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PREFACE

This volume contains the Proceedings of the 2nd Biennial Meeting of the American Society of Tropical Veterinary Medicine (ASTVM-93) held in Guadeloupe, February 2-6, 1993. The Society of Tropical Veterinary Medicine was founded in 1978 to focus scientific interest on modern strategies designed to deal with established and changing patterns of tropical diseases affecting humans and animals. The challenge of future research is to develop better diagnosis, prevention and control methods for tropical diseases of humans and animals. The Society officially became an international organization at ASTVM-93, as signified by changing the name to The Society for Tropical Veterinary Medicine.

The focus of ASTVM-93 was a Symposium on three related topics : (1) heartwater disease caused by the rickettsia, Cowdria ruminantium, *(2) its tick vector,* Amblyomma variegatum *and (3) dermatophilosis, a skin disease of cattle associated with feeding* A. variegatum *ticks. Heartwater is one of four major diseases of cattle vectored by ticks that was formerly found only in Africa. The tick and associated diseases have subsequently spread throughout the Caribbean islands and pose a threat to North and South America. Because birds have been shown to carry* A. variegatum *and to move for long distances, introduction of* A. variegatum *into the United States is quite possible. Other* Amblyomma *ticks found naturally in the United States have been shown experimentally to vector* C. ruminantium.

The Scientific Program, chaired by Gerrit Uilenberg, contained two General Sessions with papers and posters on a wide variety of topics related to tropical veterinary medicine. Ninety-four scientists participated in the five-day conference, representing 30 countries throughout the world. The Symposium included six sessions: three on Cowdria ruminantium, *two on dermatophilosis and one on* Amblyomma variegatum. *Research advances on heartwater were updated since the Heartwater Symposium that was held in South Africa in 1986. At the 1986 Heartwater Conference, the South Africans described* in vitro *cultivation of* Cowdria, *a technology that they have freely shared with other laboratories.* In vitro *cultivation has greatly enhanced many areas of research, along with application of more recently described molecular technologies. Difficulty in development of diagnostics for heartwater appears to be due, in part, to the close relationship of* Cowdria *and* Ehrlichia. *A priority of current research is to determine the cause of false positive reactions in serological tests for heartwater on Caribbean islands where the disease appears to be absent. In the* Amblyomma variegatum *session, plans to eradicate the African tick from the Caribbean were discussed. An outbreak of* A. variegatum *in Puerto Rico, where the tick was thought to be eradicated, was described. Many aspects of transmission, pathology and immune response of dermatophilosis were presented and discussed. Some progress has been made in defining the cells that play a role in the immune response, in mouse and sheep models. Effective immunization still remains elusive. Progress has also been made on the association between* Amblyomma *and severe dermatophilosis, which appears to be limited to the adult tick, but the mechanism of this association is not yet understood. Attempts are underway to identify genetic markers for susceptibility or resistance to both heartwater and dermatophilosis, for use in selection procedures. The keynote address was presented by Albert Ilemobade, Vice Chancellor, Federal University of Technology, Akure, Ondo, Nigeria entitled "Can we successfully control ticks and tick-borne diseases in Africa ?"*

The Society of Tropical Veterinary Medicine and Conference organizers are grateful for the support of those who contributed to the success of ASTVM-93 : American Cyanamid, Agricultural Division, Princeton, New Jersey ; Miles Animal Health, Leverkusen Bayerwerk, Germany ; Canon, Guadeloupe ; Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), France ; Commission of European Communities, Science and Technology for Development ; Fonds de coopération régionale Caraïbes-Guyane (FIC), France; LEICA, France ; Institut national de la recherche agronomique (INRA), Guadeloupe ; Merck and Company, Rahway, New Jersey; Perié-Médical, Antilles-Guyane ; Protatek International, Inc., St. Paul, Minnesota ; Rhône-Mérieux, Lyon, France ; Société de Distribution et de Promotion de l'Elevage (SDPE), Guadeloupe ; SPOROCHIM, Guadeloupe and the United States Department of Agriculture, Cooperative State Research Service, National Research Initiative Competitive Grants Program, Grant No. 93-00388. We are also grateful to the President of the General Council of Guadeloupe and to Mr. Jean-Pierre Poly, Departmental Director of Agriculture and Forestry, who bid welcome to the participants.

A highlight of the ASTVM-93 Banquet was acknowledgement of the contributions of Jim C. Williams to the American Society of Tropical Veterinary Medicine. Dr. Williams was presented a plaque in honor of his two terms as President of ASTVM (1987-1988, 1990-1993). During his presidency the bylaws of the Society were established and STVM became an international organization. Dr. Williams chaired the first Biennial Meeting of ASTVM that was held in Puerto Rico, February 1991. It is upon the foundation that Dr. Williams established that STVM will successfully grow and meet the needs of current and future members.

I am grateful to those who contributed to the organization of ASTVM-93 : Gerrit Uilenberg, Chair of the Scientific Program and Editor of the Proceedings ; Emmanuel Camus and Nicolas Barré, Local Arrangements and Scientific Program ; Frans Jongejan, Cowdria ruminantium *Sessions ; David Lloyd, Dermatophilosis Sessions and Glenn Garris,* Amblyomma variegatum *session. I thank the ASTVM officers for their support and participation : Jim Williams, Past President ; Alwynelle Ahl, Secretary ; Thomas Walton, Treasurer. I thank Jean-Charles and Nicole Maillard for their support of various aspects of the conference.*

The 3rd Biennial Meeting of the Society of Tropical Veterinary Medicine (STVM-95) will be held in Berg-en-Dal, Kruger Park, Republic of South Africa, August 28-September 1, 1995, in combination with the 2nd International Conference on Tick-borne Pathogens. The Conference will be chaired by Jim House, USDA Foreign Animal Disease Diagnostic Laboratory, Greenport, New York and President-elect of STVM, and Dürr Bezuidenhout, Onderstepoort Veterinary Institute, Republic of South Africa. I thank the South Africans for their warm hospitality and their willingness to host STVM-95. We invite all interested scientists to join us in beautiful Kruger Park in 1995.

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Session générale

A.S. Ahl [1]

J.A. Acree [1]

Implications of regionalization and risk assessment for developing countries

AHL (A.S.), ACREE (J.A.). Les implications de la régionalisation et l'évaluation de risque pour les pays en voie de développement. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 19-22

L'importation d'animaux et de produits animaux a été basée sur le statut d'un pays entier en ce qui concerne un agent pathogène donné ou une maladie déterminée. Cette doctrine, qui veut que le pays doit être libre, a servi à empêcher le mouvement d'agents indésirables dans une grande partie des pays plus développés. Néanmoins, les producteurs dans les pays qui n'ont pas un statut en santé animale favorable n'ont pas pu participer au commerce international et ces pays sont en général les pays tropicaux de développement. Les traités internationaux actuels sur le commerce (GATT et NAFTA) demandent que les pays soient considérés par régions en ce qui concerne les régulations d'importations pour la santé animale. La régionalisation, combinée à une évaluation consciencieuse de risque, fournit des incitations potentielles pour l'amélioration de la santé et la gestion du bétail dans une zone locale et forme également la base d'autres avantages pour les nations de développement.

Mots clés : Animal domestique - Produit animal - Transmission des maladies - Commerce international - Restriction à l'importation - Pays en développement.

INTRODUCTION

International trade policies in agriculture are ostensibly designed to maintain plant and animal health among trading partners. However, agricultural commodities have also been subject to tariffs and trade barriers in addition to measures enacted primarily for agricultural health. It has been increasingly difficult to sort out which policies were legitimately necessary for biological reasons.

Historically, the approach to imports is similar around the world and can be illustrated by briefly reviewing the U.S. situation for animals and/or products (2). The U.S. considers the animal health situation of a country for a particular disease agent, and that country is declared "free" or "not free" of that agent. After the designation is assigned, protocols and other restrictions on animal or product imports from that country are prepared to assure that imports from that country, if permitted, pose "zero biological risk". With some exceptions, this policy treats countries as single homogeneous and indivisible units. As a result, trade policies have been extremely restrictive. These "zero risk" trade policies have also been subject to political manipulation which have not always had adequate scientific review.

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Restrictive import policies have been successful in keeping exotic disease agents out of the U.S. and these concepts are reflected in trading practices of many other countries. When these policies were first developing, veterinary diagnostic capabilities were less advanced than at present, so these historic policies have served their purpose. One effect, however, has been to restrict trade of animals and animal products to those countries with advanced animal health infrastructures. Individual producer initiatives to improve husbandry practices are not encouraged by the "country doctrine". Producers in countries without favored health status have not had the incentive of potential export markets for improving the health of their animals.

Further consideration of these historic policies raises several important issues. First, there is no "zero risk" in biological systems. Biological systems and manipulation of them inherently involve risk. In addition, extremely restrictive import policies with no legal recourse invite smuggling, thus introducing unknown hazards that may not be traceable should the hazardous event occur. Thus, unknown hazards may lead to risks that are less easily contained than are known ones. Second, agents and disease are never homogeneously distributed. Geography, climatology, host range boundaries, and husbandry practices are important determinants of agent and disease distribution. For example, U.S. records show that brucellosis in cattle is restricted to a few states in the Southeastern and Southwestern parts of the country and even there, affected herds are non-uniformly distributed. We regionalize for animal health purposes within the U.S., but have not consistently done so for international trade.

In an increasingly open world trade market, demands are made for clear and defensible explanations when commodities are prohibited from moving from one country into another. These new demands require a country to justify exclusions on a biological basis and to recognize the non-uniform geographic distribution of agents. The outcome is major emphasis on regionalization and risk assessment to make management decisions about movement of commodities between countries.

NEW PARADIGMS : REGIONALIZATION AND RISK ASSESSMENT

In contrast to the country boundary, yes-no approach to animal and product importation, regionalization and risk assessment bring a different perspective to international

animal health. Regionalization recognizes that disease agents are not uniformly distributed within a country. It focuses on that part of a country or adjacent parts of countries from which an import will be generated. A region may be as small as a single premise or as large as a group of countries which choose to associate as a region for a particular exportation event or a general trading consortium. The collection of countries that make the European Economic Community (EEC) is an example of this sort of association.

Once country borders are no longer used to delineate bounds of animal health, new methods must be in place to maintain healthy national herds in importing countries. Risk assessment is the tool to assure national and international herd safety. Risk assessment is an activity which defines the hazard(s) to the importing country, describes quantitatively the likelihood of that event occurring and the magnitude of effects of the event if it did occur (1). From information such as this, the risk manager can make decisions about the import and what mitigation measures, if any, must be applied to allow the importation to occur with safety.

The hallmark of risk assessment is its scientific approach. Given the same information and assumptions, risk assessors will come to similar conclusions about the measure of risk. It is an objective measure. The risk assessment must be consistent, transparent, flexible, and documentable.

If regionalization and risk assessment are to work toward free international trade with protection for international animal health, all nations and regions must be subjected to the same scrutiny, using the same standards. The challenge is to develop systems which are accepted by all participants and which evaluate all by the same criteria, and accurately pinpoint risk.

Risk management, a part of risk analysis which concentrates on things such as policy, politics, economics, and diplomacy, also focusses on the acceptability or safety of given risk levels. There may be trade offs among the biologically assessed risks and other factors in risk management. However, the risk assessment and the risk management process should remain separate items.

HOW REGIONALIZATION AND RISK ASSESSMENT BECAME IMPORTANT

The world has become smaller with faster and more accessible travel, and instantaneous world-wide communications. Philosophical recognition of the one-environment/spaceship earth concept has played a part in changed perceptions. These perceptions may lead to making all the world as one. Continued efforts to make international trade free of tariffs and other unnecessarily restrictive regulations have led to acceptance of the General Agreement on Tariff and Trade (GATT) and the related North American Free Trade Agreement (NAFTA).

Even if these events had not occurred, the falling of the Berlin Wall, the break-up of the USSR, and the union of Europe into the EEC would have required some adjustments in the business-as-usual philosophy. For example, the EEC when fully implemented, will no longer recognize country boundaries for trade within the EEC. Ignoring the new functional geographic boundaries will result in biological risks since the new boundaries have a different meaning than in existing protocols and regulations. In addition, refusing to recognize the new EEC boundaries introduces political risks which pose economic hazards for those desiring future trade.

The former USSR poses other problems. The monolith has been broken into many subunits with political boundaries still shifting. Discipline has broken down, and war and other problems seem to inhibit the establishment of new stability in these areas. In these times of rapid change, it is far more reasonable to rely on particular and specific risk assessments than on the old "country freedom" doctrines.

ADVANTAGES TO REGIONALIZATION AND RISK ASSESSMENT

Overcoming concerns

In this major paradigm shift, international standards for regionalization and risk assessment will eventually be developed and applied equally to all participants. The advantages of regionalization and risk assessment have only cautiously been addressed. Generally the topic has been focussed on the disadvantages to a country if it fails to change, that is, the exclusion from continued participation in international trade. Though this may be true, there are advantages to regionalization and risk assessment that transcend this negative vision. Overcoming concerns about the new paradigm can open markets and opportunities the world around. Indeed, there are advantages for developing countries, for developed countries and for the entire world.

No zero risk

Regionalization and risk assessment would respect scientific facts. There is no "zero biological risk". A careful scientific review of import requests would provide a more realistic view on risk and lead to a better understanding of the actual risks involved.

Respecting geographic facts

Since diseases and agents do not respect political boundaries but rather other geographical, climatological and host range boundaries and husbandry practices, formal recognition of that in our trading practices is based on reality. All parties must realize that diseases may be restricted to one small part of a country and not be widespread in livestock all over that country. Scientific examination of the hazard and a good risk assessment allow for fewer political restrictions on trade, allowing free movement of trade while safeguarding animal health.

Strengthening animal health infrastructures

Accurate and up-to-date information on which to perform regionalized risk assessment places a premium on animal health monitoring and information capabilities. This, in turn, may encourage good record keeping which ultimately can give producers information useful to improve their individual operations.

Most tropical countries are classified as developing nations. They generally have underdeveloped animal health infrastructures, and virtually have been excluded from participation in international agricultural animal trade. Many of the diseases on the OIE List A and B diseases are associated with developing countries and/or the tropics. Regionalization and risk assessment could provide strong incentives for producers inside developing countries to make specific local improvements for their herds. A new impetus for investment in agriculture in developing countries may result. This could lead to localized animal health improvements and thus for incremental improvement of animal health within a country.

Taken together, economic incentives and success in participating in international trade could stimulate the developing country to further spread animal health improvements. This could lead to gradual and substantive improvement of veterinary infrastructure in a country, resulting in healthier national herds. This, in turn, may lead a developing country to improvement of veterinary infrastructure for dealing with other animal health problems.

Improved national herd safety

Extremely cautious import policies invite smuggling of highly desired products, thus introducing a hazard, but without adequate knowledge of that hazard and what risks it might convey. With fair consideration of a request to import smuggling could become more onerous than the legal alternatives.

Encouraging genetic diversity

More open trade can aid in the preservation of genetic diversity by helping preserve valuable genes for resistance to disease, adaptation to harsh climates, good production in adverse environments, and others. Easier movement would facilitate the improvement of animal breeds and production for the varied agricultural climates, especially tropical ones, around the world, and encourage the development of new breeds.

The freer flow of genetic material would permit more rapid development of gene banks to save the biological heritage of rare breeds and unusual disease resistance genes. This could in turn yield benefits to developing countries as breeds more suited for tropical climates while sporting the high production genes of modern breeds are melded. The animal protein production capacity of developing countries would be elevated.

Healthier and better adapted animals require lower levels of therapeutic intervention thereby decreasing the likelihood of drug residues in food from animal sources. A decrease in use of antibiotics can slow the selective pressure toward bacterial resistance, an advantage to human as well as animal health.

Insuring consistency

Regionalization would legitimize in a general way what is already done in specific instances. For example, Spain was regionalized for the purpose of the equine athletic events in the Barcelona Olympics of 1992.

Regionalization would make consistent the current and common within-country regionalization for internal animal health security. An example in the U.S. is regionalization for brucellosis and bluetongue.

Last, but not least, regionalization has long been practiced successfully by our colleagues in the international plant health community. Developing regionalization strategies would bring more consistency to agricultural import policy overall.

Improving veterinary capabilities

Together with the need for better animal health monitoring and surveillance, regionalization and risk assessment will demand promotion and strengthening of veterinary education and the veterinary profession world-wide. It will give the veterinary profession and its auxiliary sciences more visibility and a larger role in international animal health. Development of better ways to accomplish monitoring and surveillance and to contain outbreaks will be a boost to veterinary epidemiology. An example of a new

approach to the problem of exotic disease outbreak is the rapid epidemiological response teams established in EEC.

Encouraging international cooperation

Improved field epidemiological approaches will argue for joint educational ventures and further international cooperation.

The required scientific review of import requests will require risk assessment. If all countries were to strictly adhere to a common approach, such systems would require and promote international cooperation. Data collection, data sharing, data analysis would gradually become an international activity, putting all countries on equal footing in terms of systems of evaluation of imports.

Improvement of human health

Improved animal health in developing countries can provide several benefits. First, an improved animal protein supply would contribute to the nutrition of the population. Improved animal health would decrease zoonotic disease thus decreasing disease prevalence in humans. Decreased use of drugs because of better adapted and healthier animals would decrease the likelihood that drug residues would remain in animal tissues. Invigorated animal health industries can provide employment, a better future for rural populations and make significant contributions to rural development and human health.

CONCLUSIONS

World trade and its political geography is changing, bringing demands for new paradigms and new responses. In turn, regionalization and risk assessment will change world trade, opening new opportunities for agriculture in developing countries as well as provide advantages for developed countries. The challenge is to implement these ideas. The goal is accurate risk assessment and responsible risk management which are keys to healthy international herds and free international trade.

ACKNOWLEDGEMENTS

Appreciation is expressed to Dr. Lynn MILLER for her reading and review of the manuscript.

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AHL (A.S.), ACREE (J.A.). Implications of regionalization and risk assessment for developing countries. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 19-22

Animal and animal product importation has been based on the status of an entire country with respect to a particular agent or disease. This doctrine of "country freedom" has served to prevent movement of unwanted agents around much of the developed world's countries. However, producers in countries without favored animal health status have not had the opportunity to participate in international trade, and these countries are largely the tropical nations of the developing world. Current international trade treaties (General Agreement on Tariffs and Trade and the North American Free Trade Agreement) require that countries regionalize for animal health importation purposes. Regionalization combined with careful risk assessment provides potential incentives for improvement of livestock health and husbandry in a local area and forms the basis of other benefits for developing nations as well.

Key words : Domestic animal - Animal product - Disease transmission - International trade - Import control - Developing country.

AHL (A.S.), ACREE (J.A.). Implicaciones de la regionalización y evaluación del riesgo en los países en desarrollo. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 19-22

Las importaciones de animales y de subproductos animales son reguladas en cada país, en base a un determinado agente patógeno o a una enfermedad en particular. Esta doctrina de "libertad de cada país", ha evitado en la mayoría de los países desarrollados, la propagación de agentes no deseados. Sin embargo, los productores de aquellos países con un estado de salud desfavorable, no han tenido la oportunidad de participar en el comercio internacional. La mayoría de estos países, son naciones tropicales en desarrollo. Actualmente, los tratados de comercio internacional (como el Acuerdo General de Tarifas y Comercio y el Acuerdo del Libre Comercio en Norte América) requieren una regionalización de los países, con el fin de comercializar en el campo de la salud animal. La regionalización, junto con la evaluación cuidadosa del riesgo, provee un potencial de incentivos para mejorar la salud y la producción animal en una región determinada e implanta al mismo tiempo, las bases para la obtención de otros beneficios por parte de las naciones en vías de desarrollo.

Palabras claves : Animal doméstico - Producto de origen animal - Transmisión de enfermedad - Comercio internacional - Control de importación - País en desarrollo.

Simulation of infestation risk of cattle by gastro-intestinal trichostrongylids in a tropical humid climate

G. Aumont [1]

AUMONT (G.). Simulation du risque d'infestation de bovins par des trichostrongylides dans un climat tropical humide. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 23-26

La dynamique des populations de larves de trichostrongylides du troisième stade (L3) autour des bouses de bovins a été ajustée avec des modèles non-linéaires (loi marginale) dans un climat tropical humide dans des situations climatiques différentes. Ces modèles marginaux ont été combinés avec la durée de survie des bouses, le poids des animaux, le nombre de bouses par vache et par jour, la charge en animaux par hectare et la masse d'herbage disponible, en tenant compte de la tendance des bovins à paître de façon inégale, afin d'estimer la probabilité d'infestation par des larves L3. Le risque d'infestation a été calculé pour différents âges de repousse de l'herbage et différents temps de pâture dans des système de rotation des pâturages. Le risque d'infestation variait entre 0 et 1400 L3 par kg de matière sèche d'herbe et par jour, dépendante de la loi marginale. Le temps de pâture, l'âge de la repousse et la disponibilité de fourrage étaient les principaux facteurs de variation du risque d'infestation.

Mots clés : Bovin - *Trichostrongylidae* - Infestation - Herbage - Pâturage en rotation - Intensité de charge - Zone tropicale.

INTRODUCTION

Cattle are mostly reared on pasture in tropical countries. Gastro-intestinal parasitism is commonly involved as a major limiting factor of performances of these pasture systems (5, 9). To our knowledge, no epidemiologic model exists to predict infestation risk by trichostrongylids for cattle reared in tropical pastures according to different pasture management systems. In Guadeloupe (French West Indies), worm populations of cattle are mostly composed of trichostrongylids (*Haemonchus* sp., *Trichostrongylus* sp., *Cooperia* sp.) as in many other tropical countries with humid climates. These parasites induce a decrease of 8 % in calf growth from birth to weaning even with small internal populations (3). The improvement of pasture systems requires a good knowledge of third stage infective larvae (L3) dynamics to fit both parasitism control methods and grazing systems. In a humid climate, even with a marked dry season, livestock management factors were shown to be decisive for the L3 population on pasture (4, 8) and worm populations in calves and cows (3). Day to day variations in relative humidity, temperature and global radiation are low in West Indies. In such conditions, in Guadeloupe, AUMONT et al. (2) showed that there are poor relationships between climatic conditions and the L3 population size on pasture. The objectives of this study were, a) to contribute to a definition of infestation risk by trichostrongylids in cattle-grazing tropical pastures and, b) to determine this infestation risk for different cattle-grazing tropical systems in a tropical humid climate of the West Indies. A numerical approach was used to simulate different livestock management systems, involving the survival duration of pats, the body weight of cows, the number of pats per cow and per day, the stocking rate, the herbage mass availability, the age of herbage regrowth and the grazing time.

MATERIAL AND METHODS

The basic data were third stage larvae population kinetics on herbage around pats after experimental pat deposition, that were described by AUMONT et al. (2). These kinetics (S) were fitted by the following model :

$$S(t) = MG(t)D(t) \quad (a)$$

where

$$G(t) = (1+ \Phi\exp((t-\alpha)/\beta)^{**}(-1/\Phi)\ G(t) \quad (b)$$

$$D(t) = \exp(-\mu t^{2}) \quad (c)$$

G(t) represents the growing population stage and D(t) represents the mortality population stage. Φ, α, β, μ are the parameters of the model determined by non linear regression procedures. t is the time in days (d). M is the potential maximal size of the L3 population.

Three different dynamics of L3 population size expressed in L3/kg dry matter of grass (DM) were chosen because they were representative of situations prevailing in tropical grazing systems for cattle i.e. either an unimodal evolution of L3 population size with a maximum ranging from 16 days after pat deposition (situation 1) to 25 days after pat deposition (situation 2), or a bimodal evolution of L3 population size (situation 3). These kinetics are shown in figure 1.

Parasitism risk was defined as the probability of contact between animal and L3. However, the chosen unit was the number of L3 per kg DM to present interpretable data

1. Station de recherches zootechniques, Institut national de la recherche agronomique, BP 1232, 97185 Pointe-à-Pitre, Guadeloupe.

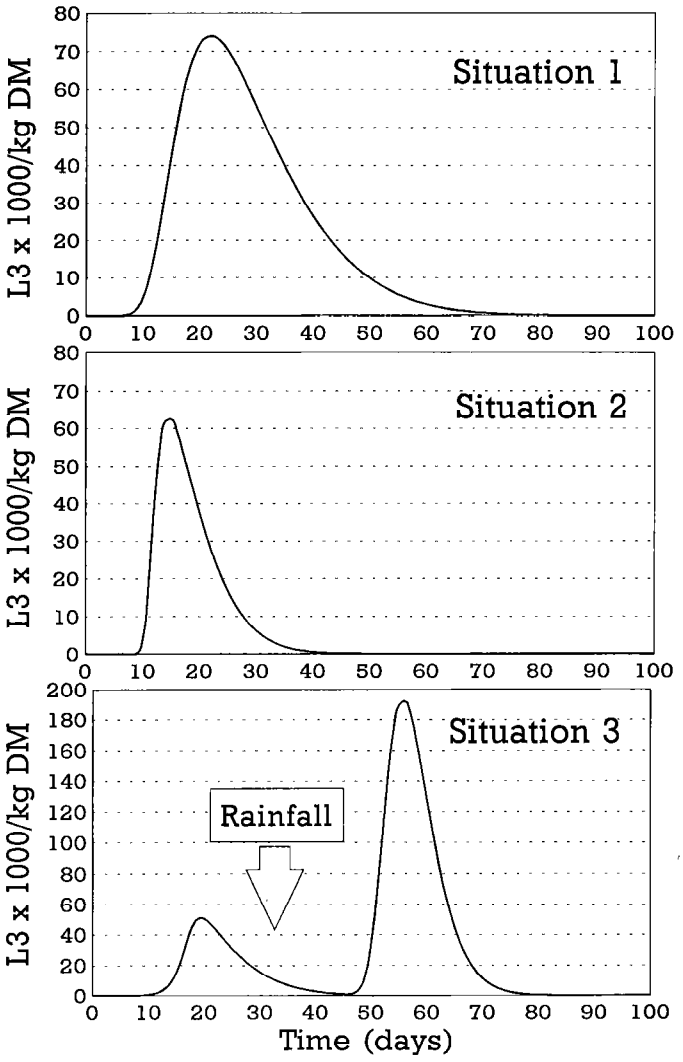


Figure 1 : Kinetics of L3 abundance (L3 x 1000/kg DM) on herbage around pats after pats deposition. Situation 1, 2 and 3 refer to the most frequent L3 kinetics that were recorded in Guadeloupe (2).

for forage scientists. The probability of grazing near pats (PG) for a fixed forage availability was defined by the following formulae :

$$PG(w) = 1-S^w \quad (d)$$

where

$$S(r,n,d) = 1-(\pi r^2 nd/10\,000) \quad (e)$$

$$w = SR/Bc \quad (f)$$

where r is the radius of pats and contaminated areas around (in meters), n is the number of pats per day and per cow, d is the survival duration time (in d), SR is the stocking rate (kg/ha), and Bc is the average bodyweight (BW) of cows (kg). S represents the part of paddock free of pats. w represents the number of cows per ha.

The PG was dependant on forage availability according to the following formula :

$$PG(\partial) = 1-\exp(\partial t) \quad (g)$$

where t was the time in d after the entrance in a paddock and ∂ was expressed in d^{-1}.

Finally the infestation risk (R) was defined as the following formula :

$$R = \sum_{i=0}^{i=n-1} \left(\int_{t=ia}^{t=s+ia} M*g(t)D(t)*PG(\partial)*PG(w)dt)/s)(s+a)/d \quad (g)$$

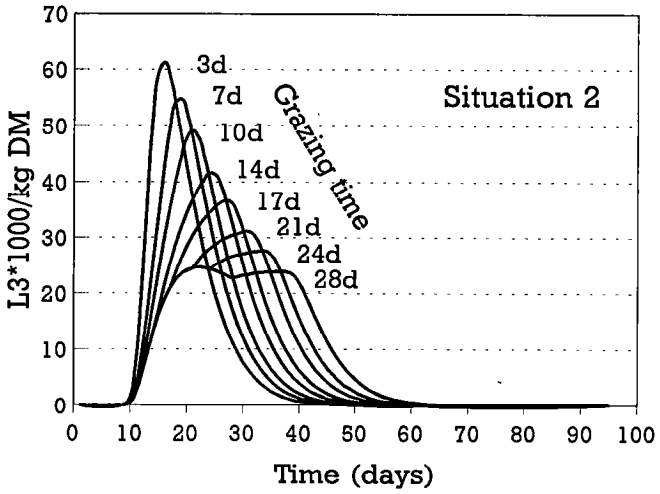
where a was the age of herbage regrowth (d), s was the grazing time (d) and n was the integer part of survival duration time divided by s+a. s+ia could never be superior to d.

In the results presented, d was fixed to 75 days (2), the Bc to 350 kg (body weight of local creole cows), the stocking rate to 1500 kg BW/ha and ∂ to 0.65 d^{-1}. The simulation was carried out for time of grazing ranging from 2 to 30 days and for age of regrowth ranging from 10 to 60 days.

RESULTS AND DISCUSSION

Our model was based on the hypothesis that infestation risk when grazing far from the pats, was of minor importance in comparison to infestation risk when grazing near pats. As a matter of fact, L3 population size further than one meter radius from the pats was shown to be 100 to 1000 fold lower than L3 populations size around pats (2, 4). The probability of grazing near pats was an important criterion for the definition of infestation risk. It integrated the patchy grazing behaviour of cattle that was clearly described by JONES and RATCLIFF (6) on tropical pastures of Queensland. As well established, cattle refuse to graze around their pats particularly when the pats are fresh or when forage availability is great. That is the reason why the forage availability (and grazing time) and stocking rate were included in the formulae for the calculation of the probability of grazing near pats. This model was similar to that built for predicting grazing time by herbage intake and/or herbage availability of ALLDEN and McD WHITTAKER (1). The parameter ∂ used in our study was determined from experimental data of MANTEAUX et al. (7) on DM intake of cows on Guadeloupean pastures.

When time of pat deposition was increased by simulation, the maximum of L3 population size around pats decreased and the time of this maximum decreased. Similar results could be recorded even with bimodal L3 kinetics such as that of situation 3 (fig. 2). This phenomenon was



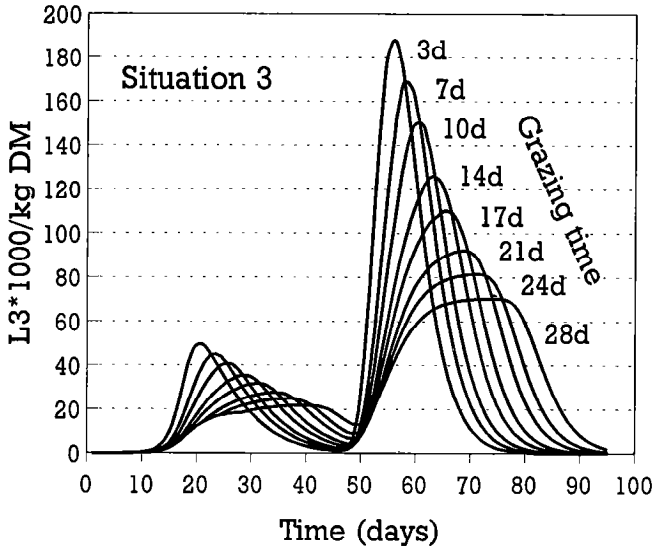


Figure 2 : Kinetics of L3 abundance on herbage around pats for different grazing time. Situations 2 and 3 refer to the most frequent L3 kinetics that were recorded in Guadeloupe (2).

due to the spacing of pat deposition and to the fact that L3 abundance around pats was defined as the means of L3 density around different pats of different ages.

The infestation risks are shown for the 3 situations by surface responses according to grazing time variations (2 to 30 days) and age of herbage regrowth variations (10 to 60 days). The infestation risks ranged from 0 to 1400 L3/kg DM (fig. 3, 4, 5). A decrease in the age of herbage regrowth and the increase in the grazing time induced a dramatic increase of the infestation risk. A minimum in the infestation risks shown as a "valley " in figures, existed for grazing time lower than 8 d and age of herba-

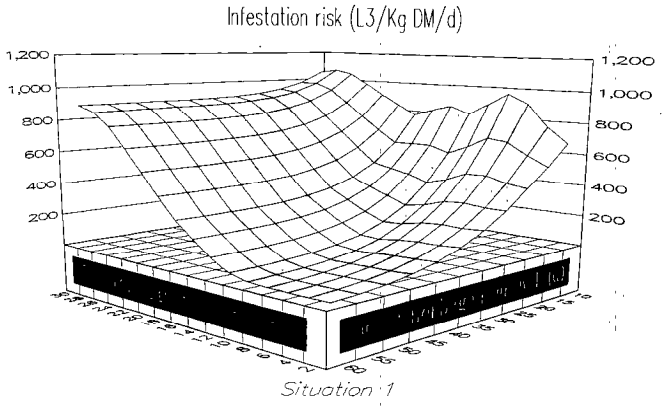


Figure 3 : Infestation risk by trichostrongylids for grazing cattle on tropical pasture according to age of herbage regrowth and grazing time. Situation 1 : unimodal L3 kinetic (maximum at the 25th day) after experimental pat deposition.

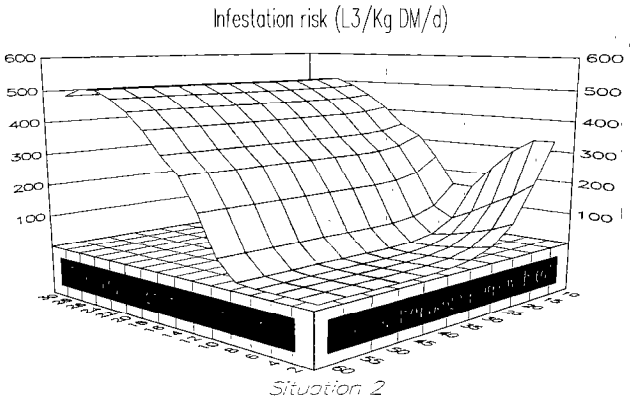


Figure 4 : Infestation risk by trichostrongylids for grazing cattle on tropical pasture according to age of herbage regrowth and grazing time. Situation 2 : unimodal L3 kinetic (maximum at the 16th day) after experimental pat deposition.

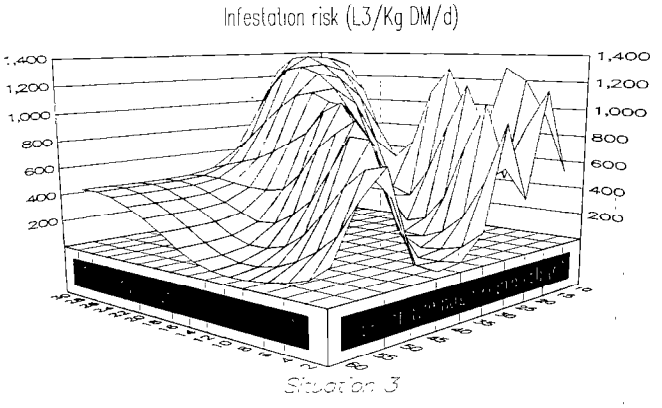


Figure 5 : Infestation risk by trichostrongylids for grazing cattle on tropical pasture according to age of herbage regrowth and grazing time. Situation 3 : bimodal L3 kinetic (maximum at the 20th and the 56th day) after experimental pat deposition.

ge regrowth of 35 d. However, when bimodal evolution of L3 population size occurred after an important rainfall for example (fig. 4), the surface of L3 infestation risk became chaotic. In such a situation, the infestation risk with a rotational system might be higher than that with continuous grazing. This study confirmed that in cattle grazing pastures of tropical humid climates, the pasture management system represents the main sources of variation in infestation risks.

These results showed that simulation approach might give consistent results in prediction of infestation risk of cattle by trichostongylids. Other hypotheses on parameters of the model could be used to set up its utilization for a great variety of situations in tropical grazing systems. However, further epidemiological studies are required to confirm simulation results. They will help to improve simulations by including the variation of eggs density in excreted pats and the frequency of bimodal or trimodal L3 kinetics in the model. Simulations in cattle appeared as relatively simple despite their particular grazing behaviour, because L3 kinetics are easy to modelize. In contrast, similar simulations for small ruminants would be more complex because in these species, a) L3 kinetics greatly vary with the climatic conditions, b) between parasite species differences exist in L3 reaction to micro-climatic conditions and c) great variations exist in egg density of faeces. Such studies are now carried out in our laboratory.

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AUMONT (G.). Simulation of infestation risk of cattle by gastro-intestinal trichonstrongylids in a tropical humid climate. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 23-26

The population dynamics of trichostrongylid third stage larvae (L3) around bovine dung were fitted with non-linear models (marginal law) in a tropical humid climate in different climatic situations. These marginal models were combined with the survival duration of pats, the weight of cows, the number of pats per cow and per day, the stocking rate and the herbage mass availability, taking into account the patchy grazing behaviour of cattle in order to estimate infestation probability of cattle by third stage larvae. The infestation risk was computed for different ages of herbage regrowth and grazing times in rotational grazing systems. The infestation risk was found to range between 0 to 1400 L3 per kg of dry matter of grass and per day depending on the marginal law. The grazing time, the age of herbage regrowth and the forage availability were the main factors of variation of the infestation risk.

Key words : Cattle - *Trichostrongylidae* - Infestation - Grassland - Rotational grazing - Stocking rate - Tropical area.

AUMONT (G.). Estimulación del riesgo de infestación por tricoestróngilos gastro-intestinales en ganado, bajo un clima tropical húmedo. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 23-26

La dinámica de las poblaciones de larvas de tercer estadio (L3) de tricoestróngilos (activas alrededor de la boñiga de bovino), en un clima tropical húmedo, se estudió con modelos no lineares (ley marginal), bajo diferentes situaciones climáticas. Los modelos marginales se combinaron con la duración de la supervivencia de los parásitos, el peso de las vacas, la cantidad de parásitos por vaca y por día, el número de animales por hectárea, la capacidad de almacenamiento y la disponibilidad de forraje. El comportamiento gregario de las vacas se tomó en cuenta con el fin de estimar la probabilidad de infestación del ganado con el tercer estado larval. El riesgo de infestación se consideró en los diferentes estadios de crecimiento forrajero y para los tiempos de pastoreo en sistemas de pastoreo rotativo. Según la ley marginal, el riesgo de infestación se situó en un rango de 0 a 1400 L3 por kg de peso de materia seca de pasto, por día. El tiempo de pastoreo, el estado de crecimiento forrajero y la disponibilidad de forrage, fueron los principales factores de variación del riesgo de infestación.

Palabras claves : Bovino - *Trichostrongylidae* - Infestación - Pasto - Pastoreo rotacional - Intensidad de carga - Zona tropical.

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A survey of goat and cattle diseases in the Artibonite Valley, Haiti, West Indies

VEIT (H.P.), McCARTHY (J.), FRIEDERICKS (J.), CASHIN (M.), ANGERT (R.). Une prospection des maladies caprines et bovines dans la Vallée d'Artibonite, Haïti. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 27-38

Une étude de 40 semaines portant sur 43 fermiers, 60 chèvres et 60 bovins a été effectuée afin d'identifier des conditions anormales ou des maladies, et les facteurs prédisposants saisonniers, liés à la gestion ou à la nutrition. Les exploitations ont été visitées 4 fois, approximativement toutes les 10 semaines, les fermiers ont été questionnés, les animaux examinés et leur sang prélevé pour l'hématocrite, le nombre total de leucocytes et le taux de certains vitamines et minéraux dans le sérum. Des poils, de la terre et du fourrage ont été prélevés pour analyse. La condition du bétail était généralement passable, la croissance et la reproduction étaient mauvaises. Une déficience calorique inattendue pendant la saison des pluies, et des déficiences graves en phosphore et moindres en vitamine A et E, ont été constatées. Une anémie due au parasitisme était fréquente chez les deux espèces, surtout chez les chèvres. Les bovins avaient des tiques, les chèvres des poux. Une diarrhée et de la mortalité néonatales étaient signalées chez les chèvres, et on a observé une dermatite exfoliative, des verrues, une dermatophytose et peut-être de l'ecthyma contagieux. Le charbon bactéridien et la babésiose étaient signalés chez les bovins, et une vaginite vésiculaire, de l'orchite et des verrues des trayons ont été observées.

Mots clés : Bovin - Caprin - Enquête pathologique - Carence minérale - Parasitisme - Méthode d'élevage - Croissance - Performance de reproduction - Alimentation des animaux - Influence de la saison - Haïti.

INTRODUCTION

Poor reproductive and growth performance in Haitian goats and cattle has been consistently observed by two of the authors (ANGERT, VEIT) for as long as 28 years and by others. The cause had been presumed to relate to nutritional deficiencies but had not been confirmed by much objective data (5). This study was initiated to identify obvious abnormal conditions or diseases that existed in a defined group of goats and cattle over a period of 40 weeks and to identify related seasonal, nutritional, and management factors. Livestock were examined up to four times in their local environment, forages and related soils were examined and analyzed, and animal handlers were interviewed at each visit. This report reviews the diseases or abnormal conditions reported or noted in the livestock, along with the major predisposing seasonal and nutritional factors. An associated report will focus on reviewing the relationship of Haitian ruminant production management to these diseases.

MATERIALS AND METHODS

Selection of farms and livestock

The region of study was within a 12-mile radius of Deschapelles, Haiti, within the Artibonite Valley, and was selected because of a long-term (about 37 years), community development program provided by Hospital Albert Schweitzer (HAS) for the study region, which included veterinary medical, animal husbandry, and horticultural extension programs, and technical support. This long-standing relationship between HAS and the participating farmers enhanced the cooperation and reliability of questionnaire responses of the farmers with our investigative team. Therefore, the farmers selected were not a random sampling of small Haitian farmers or even of those within the Artibonite Valley. Also, this region had long-term irrigated soils as well as non-irrigated highlands, allowing us to examine the effects of irrigation and/or topography on soil and forage conditions and indirectly on animal health. Finally, the Artibonite Valley region has been considered a highly productive agricultural region in the country. Hence, agricultural research in this region is potentially useful to any future agricultural production improvement in Haiti. The specific criteria for selection of farmer participants in this study were :

- They must have been participants in a new voluntary program for preventive deworming of goats offered by the HAS veterinarian just prior to this study. Participation by these farmers in this deworming program showed above average interest in their animals' health.

- They had to be regularly responsible for the care of study goats and/or cattle during the time of the study.

- They must live near the nearby communities of Desarmes, Halaire, Marin, or Hatte Bellanger for logistical and topographical reasons.

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- They must be small farm managers. They were grouped into one of three economic farming classes based on their responses to two questions "Have you ever bought any land?" and "Do you ever sell your daily labour?" Those answering "yes" to the first question and "no" to the second were classified in the highest economic group, those answering oppositely to these two questions were considered lowest on the economic scale, and those answering either "yes" or "no" to both questions were grouped between the first two groups. Farmers from all three classes were included.

- They must have a small-to medium-sized goat and/or cattle herd (range 1-31 head, mean = 5.6 head/farmer). From a pool of 121 farmers who were given initial questionnaires, 43 farmers with 56 cattle and 56 goats (about half of their stock) were selected for this study. The HAS veterinarian, four of his goats, four of the HAS cattle, and some HAS farm soils and legumes were also included for comparison with the Haitian farms, providing a starting total of 60 goats and 60 cattle and a finishing total of 45 cattle and 34 goats.

Farm visitations

The investigation team consisted of three Haitian animal technicians, a Haitian interviewer, an expatriate veterinarian, and an expatriate coordinator fluent in Haitian Creole. Visits were conducted January 27 - February 6, March 31 - April 5, June 9 - 14, and August 18 - 22, 1986. At the initial visit, all participating animals were ear tagged. At all visits, a physical examination was performed on each animal, age estimated by dentition and by farmer's estimation, and weight estimated by girth tape measure for goats and cattle and also by scale weight for goats. Any missing animals were accounted for on subsequent visits. The haircoat, skin, eyes, conjunctiva, oral cavity, mammary glands, external reproductive organs, legs, tail, and feet were examined. Rectal examination of cattle was usually performed. Respiration rates were recorded, and lungs, hearts and rumens were ausculted. Blood was collected by jugular venipuncture into EDTA and clotting tubes. All blood was stored in an ice-chilled, lightfree environment until freezing or processing two to eight hours later. Blood was analyzed for hematocrit, total and differential white blood cell counts, serum Ca, P, Mg, Fe, Se, Zn, and vitamins A and E. Bovine switch or caprine tail hair was collected in plastic bags, iced, and frozen two to eight hours later, for analysis for Ca, P, Mg, Fe, Se, and Zn. A total of 38 soil samples were taken, based on observed use for grazing or feeding of goats or cattle, crop types, topography, and irrigation availability. Standard soil samples and soil sampling boxes were used ; samples were double bagged and frozen at -2°C two to eight hours after collection until analyzed. Sixty-two forage samples in the process of consumption or harvested for consumption were collected in cotton bags, oven dried at 60°C for 24 hours, sealed in plastic bags, and stored at room temperature until analysis. On each visit, a questionnaire was administered to the person responsible for daily management of each animal. Topics covered, respectively, were reproduction, feeding and nutrition, health and disease, labour and marketing. The questionnaires were written in both English and Haitian Creole with all dialogue done in Creole. Questionnaires and physical exam data were summarized in table or graph forms. Where possible, comparison to known normal values was made.

Laboratory analyses

Hematology

Blood was collected for serum mineral and vitamin analysis, hematocrit, and total and differential white blood cell counts. Hematocrits were determined by standard micropipette procedures and total white blood cell counts done with pipette and hemocytometer procedures. Differential white blood cell counts were done after Wright staining of blood smear slides, using standard laboratory procedure (200 cell counts).

Vitamin and mineral assays of serum, hair and forages

Serum samples were iced in a light-free environment for two to eight hours after collection, centrifuged, then frozen at -2°C and analyzed for Ca, P, Mg, Fe, Se, Zn, vitamin A, and vitamin E at Virginia Polytechnic Institute and State University. Ca, Mg, Zn, and Fe were determined with a Perkin-Elmer atomic absorption spectrophotometer (model 403) using standard operation manual procedures. Serum Se was determined after digestion with nitric acid and perchloric acid in a model 460 Perkin-Elmer atomic absorption spectrophotometer at 196 nm (9). Serum P was determined by a colorimetric procedure (7) and absorbance read on a spectrophotometer at 660 nm. Serum vitamin E was assayed by a microprocedure of FABRANEK *et al.* (6), and serum vitamin A by the KIMBLE procedure (10) with modifications described by DUGAN *et al.* (4). Hair and feed samples were analyzed as described above with the additional steps of washing the hair using a previously described procedure (3), and wet ashing of hair and feed by nitric acid/perchloric acid digestion (9).

Soil analysis

Standard procedures were used for soil samples including air drying, grinding and sieve passage, pH determination, and analysis of extracts for P, K, Ca, Mg, Zn, Mn, Fe, and Cu, by inductively-coupled plasma optical emission spectrometry and chromic acid oxidation determination of organic matter.

RESULTS

Rainfall

In the region of this study, there is a cyclic wet (March-Oct.) and dry (Nov.-Feb.) season. Through 1986, the Deschapelles rainfall measured by HAS staff rain gauge was 7.2, 43.2, 75.1 cm, and 24.2 cm per quarter, respectively, with December having the least rain (less than 1 cm), July the most (29.6 cm), and the other months showing intermediate levels between those of December and July.

Questionnaire data

The questionnaires were usually answered by the person most responsible for the daily care of a given animal. Usually, the caregiver was the owner or in the owner's immediate family but rarely was unrelated and hired to do the work. We received excellent cooperation and reliability of responses, due to the selection for participants with a previous history of cooperation with HAS. Even so, the responses often lacked preciseness or objectivity to many questions. Given the lack of quantitation, subtle relationships could have been missed ; however, variations (of management, animal health or disease, and body condition) generally were noted between herds but not noted between economic groups, location, or herd size.

Feed availability for goats was reported as highest in December (early dry season) and lowest in February and March (late dry season), while feed availability for cattle was reported highest in July (mid rainy season), and lowest in February and March. The reported unavailability of feed for both goats and cattle during February and March coincides with the late dry season general shortage of feed. The difference in feed availability between goats and cattle was thought to be due to the fact that goats are fed more consistently by browsing or scavenging harvested fields or human crop residuals, while cattle are more consistently tethered and fed whatever is available from cultivated or perennial plants. This makes the rainy season more likely to provide cattle feed while the early dry season tends to provide more feed for goats.

None of the 43 farmers in this study reported planting any crop dedicated to feeding goats or cattle, and none provided any concentrate or mineral supplement, beyond salt which was used on dry corn husks to stimulate their consumption or as a treatment for presumptive parasitic diarrhea. Water was reported to be carried to goats daily by 93 % of the handlers, and all cattle were reported to be walked to water sources at least once daily. Observations led to a suspicion by our study team that the watering practices were not as fastidiously practiced as reported, especially during the rainy season. Such times left farmers busy planting, and there was a tendency to neglect livestock. Also, because of increasing rainfall, there was a presumption by some handlers that if the stock were rained on, they likely consumed sufficient water directly or via plant material.

A summary of reproductive activity is in table I. The status of goat reproduction was poorly observed by the Haitians. Only 50 % knew the age of puberty for their bucks, but it appeared to range from two to six months with three to four months most commonly reported. Inbreeding was suspected to be common by us but rarely acknowledged by the farmers. Was suspected such because of the general ease of contact between most sexually active male and female goats year round but particularly during the dry season. No defined sire selection or breeding management was identified. Does appeared to reach puberty at 10 to 12 months of age with about 50 % of farmers recognizing œstrus behavior or signs with highest sexual activity in March and April, and greatest kidding incidence in August and September confirming that breeding activity was highest when reported. Apparently, there is also a smaller breeding season in September and October with February to March kiddings. The kidding interval was estimated to be about 11 months with 1.9 kids per parturition based on composite calculations using questionnaire data and direct observations. Two abortions and one stillbirth were reported within the 47 female goats (28 pregnancies) within the 40 weeks of the study. Some owners claimed a plant called "lian pwagrate" (*Mucuna pruriens* or cow itch) caused abortion in goats. We could not verify this. Interestingly, this plant is used as an anthelmintic in other parts of the West Indies.

Cattle reproduction also was difficult to assess due to sketchy observations of farmers. Most (2/3) did not know the age that their bull reached puberty while the others estimated 15-18 months. Of those observed, all bulls over 18 months were determined to be sexually active. Higher

TABLE I Summary of reproductive activity in Haitian goats and cattle.

	Goats	Cattle
age of puberty - males	3-4 m	15-18 m
- females	10-12 m	20-24 m
breeding seasons	Mar.-Apr.	Mar.-Apr.
	Sept.-Oct.	Déc.-Feb.
gestation interval	11 m	24 m
services per gestation	ND[a]	ND
progeny per gestation	1.9	1.0
abortion rate[b]	7.2 %	0.0 %[c]
stillbirth rate[b]	3.6 %	0.0 %[c]
dystocia rate[b]	0.0 %[c]	0.0 %[c]

a - ND = Not determined due to insufficient data.
b - rate = event per pregnancy x 100 %.
c - Covered in the questionnaire but not reported by farmers or observed by investigators in study animals.

sexual activity was reported in March and April, as with goats and to a lesser degree, in December through February. There was no knowledge of breeding rates or conception rates in females of this study. Slightly less than half of the owners remembered first œstrus of their heifers or cows, but it appeared to average 24 months. Signs of œstrus recalled in order of frequency were vocalization, swollen vulvas, mounting behavior, and tail lifting. Œstrus activity was reported as highest in February to April with calvings highest November to February. Nearly all cow handlers said their cows were bred by someone else's bull, but they could not recall the number of services per conception. Most cow handlers knew approximately when their cows were bred if pregnant and when they would calve. Pregnant cattle were occasionally (12 % of the time) given extra care or feeding. Dystocia and abortions in cattle were not reported or observed in any cattle of this study. Age at first calving was known by only one-third of the farmers (N = 8) and was calculated to a mean of 29 months ; calving interval thereafter averaged 22 months (N = 11). Based on the age of first calving, it has to be presumed first œstrus must be occurring prior to 24 months, at least by 20 months, but is not recognized. Based on observed calvings, the calving interval was estimated to be 24 months. Part of the discrepancy in data also may be due to the fact that those cattle handlers who recalled breeding or gestational events were better managers of their livestock and had slightly better performance statistics. Postparturient œstrus activity was poorly recognized when it occurred ; it was reported from three to one hundred four weeks with a mean of nineteen weeks. If the true calving interval was about 24 months, there was an average of a 15-month interval between parturition and next conception.

The general perception and reported incidence of disease by these Haitian farmers is that 74 % of their goats and 82 % of their cattle were never sick. Supporting this perception, the attending HAS veterinarian reported a low incidence of severe clinical illness or deaths in goats or cattle other than persistent thinness often with internal parasitism. The observations, physical examinations, and questionnaires confirm that while there was little catastrophic disease among the study animals, there were a number of important health problems. A summary of the more commonly reported and observed diseases or conditions of goats and cattle is in table II.

Goat handlers considered "diarrhea" as their most serious sickness (91 % response*), the most frequently seen illness (53 %), and associated with the early rainy season (April and May). Most goat handlers (93 %) did not know any cause of diarrhea, a few reported sweet potato vine ingestion could cause diarrhea. HAS veterinarians see a high correlation of such diarrhea with internal parasite loads which are highest in the early rainy season. About one-third of goat handlers gave salt to their diarrhea-producing goats. Seven percent took them to the veterinarian for deworming. About 60 % did not know of, or use any treatment, 40 % of these people reporting goat deaths, 47 % recovering, the rest maintaining chronic diarrhea. Those goats in the group which were provided salt or taken to the veterinarian for deworming did not die and usually recovered. Lice were reported as the second most common problem (47 % frequency). Death rate of affected animals due to lice was reported to be 17 %, and recovery 65 %, especially with either of two treatments : one-fourth of the farmers visited the veterinarian, while one-fourth bathed the goats in the leaves of the "ti labé" tree (*Alveradoa hautiersis*). Most Haitians had no idea about cause or predisposing factors for lice infestation. Some cited rainy weather or "bad" food as the cause.

"Malcharbon" or anthrax was named as being the most frequently seen bovine disease (58 % response) and the most serious (71 % response). The disease is reported to affect both young and old cattle, but it was never mentioned to occur in goats, horses or swine. No reported treatments were named. No cases of anthrax were noted or diagnosed by the investigative group of the authors or the HAS veterinarians. Internal parasites, diarrhea, and colic attributed to parasites were named by 18 % of the handlers as the second most frequent problem. The only treatment mentioned for diarrhea was going to the veterinarian, and no deaths were reported to be associated with internal parasitism with or without treatment. Ticks were mentioned as the next most frequently noted problem (16 % response). Animals were rarely treated, and all handlers said the animals recover with no treatment.

Physical examination

Physical examinations of the goats and cattle were highly enlightening. Average weight of mature nonpregnant goats was 62 pounds. Examination of body weights over the four periods of this study indicated that goats lost weight from the early to late rainy season, while cattle gained during the rainy season (table III). Even so, only 3 of 60 goats examined were classified as emaciated. No ticks were ever found, and only one female goat had a heavy louse infestation while one other had a few lice. Many goats (26.6 %), nearly all females, had a dull hair coat with distinctively dry, flaky skin or dandruff which we called a superficial exfoliative dermatitis. A few kids had ringworm-like lesions, three goats had fibroma-like solitary skin nodules, one female had teat ulcers and her kid had mouth ulcers compatible with contagious ecthyma. Another goat had oral lesions which the owner ascribed to the ingestion of a cactus plant, "kandelab" (*Euphorbia*

* Reported response is the perception of the farmer to the incidence and seriousness of a disease in his area. It is not the actual incidence of the disease in his herd. The latter incidence and mortality figures are present in table II.

TABLE II Estimates of reported and observed diseases or conditions in goats and cattle from selected farms in the Artibonite Valley (Dechapelles region), Haiti, West Indies.

A. Goats

Goat diseases or conditions reported via questionnaires		
Condition name	Incidence (%)	Overall mortality rate (%)
1. Internal parasitism, presumptive	16.0	6.4
2. Pediculosis (lice)	12.0	2.0
3. Abortion	7.2[a]	7.2[a]
4. Neonatal death, cause unknown	7.2[a]	7.2[a]
5. Stillbirth	3.6[a]	3.6[a]
6. Neonatal death, with diarrhea	3.6[a]	3.6[a]
7. Neonatal death, with maternal mastitis	3.6[a]	3.6[a]
Goat diseases, conditions or lesions observed during study		
1. Low serum phosphorus	80.0	0.0[b]
2. Low serum vitamin E	76.0	0.0
3. Parasitic anemia, presumptive	42.0	0.0
4. Superficial exfoliative dermatitis	26.6	0.0
5. Low serum vitamin A	23.0	0.0
6. Teat lesions	7.0	0.0
7. Fibroma(s)	5.0	0.0
8. Vaginal discharge, mucoid	5.0	0.0
9. Contagious ecthyma, presumptive	4.0	0.0
10. Ringworm, presumptive	3.3	0.0
11. Pediculosis (lice)	1.9	0.0
12. Mastitis	1.7	0.0
13. Solar dermatitis	1.7	0.0

a - This is the incidence based on 28 reported pregnancies.
b - No mortalities were reported for any of the study animals within the duration of this study

B. Cattle

Cattle diseases or conditions reported during questionnaires		
Condition name	Incidence (%)	Overall mortality rate (%)
1. Internal parasitism, presumptive	18.0	0.0
2. Ticks	2.8	0.0
3. Anthrax, possible	2.0	2.0
Cattle diseases, conditions or lesions observed during study		
1. Low serum phosphorus	85.0	0.0
2. Tick infestation, few to light	55.0	0.0
3. Vesicular vaginitis	32.0[a]	0.0
4. Low serum vitamin A	31.0	0.0
5. Low serum vitamin E	27.0	0.0
6. Parasitic anemia, presumptive	19.0	0.0
7. Testicular hypoplasia	17.6[a]	0.0
8. Orchitis/epididymitis	11.8[a]	0.0
9. Papillomas	11.6	0.0
10. Focal dermatitis, presumptive	10.0	0.0
11. Teat lesions, including warts	8.1[a]	0.0
12. Parasitic diarrhea	6.7	0.0
13. Mastitis	5.4[a]	0.0
14. Oral ulcers, mild	5.0	0.0
15. Vulvar dermatitis	3.3[a]	0.0
16. Babesiosis	1.0[b]	0.0

a - This incidence is of the eligible members of the appropriate gender, not all the animal species of the study.
b - This animal was outside the study, but within a group of HAS cattle in the study. Diagnosis was made by the attending HAS veterinarian.

Table III Mean animal weights (lbs.) by sampling period.

	1st Period (1/1-2/6)[b]	2nd Period (3/3-4/5)	3rd Period (6/9-6/14)	4th Period (8/18-8/22)
Point in rainy season	(Dry)	(Early Rainy)	(Rainy)	(Rainy)
Mature Female Goats[a]	73	68	57	57
Mature Female Cattle[a]	612	639	641	663

[a] No animals who kidded or calved during the course of the study were included in these figures.
[b] Month/date.

lactes). Males appeared free of any reproductive tract lesions while three females (5 %) were noted to have a mucoid (nonpurulent) vaginal discharge without other signs of œstrus. Of 25 births, four neonatal deaths were reported, one associated with maternal mastitis, and one with neonatal diarrhea. Severe mastitis was noted in one mature lactating female, and a variety of teat wounds in four others. One male was noted to be coprophagic, while two adult females were observed with diarrhea. Conjunctival grading (pink, pale, or white) was successful in detecting the severe anemias in 62 % of the goats having hematocrit values of 20 or below but tended to miss the less anemic states (hct of 20-24 %). Blood analysis showed low packed cell volumes in 33-73 % of the goats, depending on sampling period, and high total white cell counts in 5-87 % (mean = 43 %) of the goats, compared to normal values of U.S. goats (tables IV and V). Random differential WBC showed 19 % of goats had an eosinophilia and 25 % had monocytosis.

The average weight for mature, nonpregnant cattle was 639 pounds as estimated by girth measurements (table III). Body condition was fair or better in 15/17 males and

Table IV Normal values for hematocrits (PCV) and wite blood cell counts.

	WBC (x10³/cu mm)	PCV (%)	Basophils (%)	Eosinophils (%)	Band Cells (%)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)
Goats	4-13	24-38	0-2	3-8	rare	30-48	50-70	1-4
Cattle	4-12	26-46	0-2	2-20	0-2	15-45	45-75	2-7

Table V. Hematology by sampling period.

	1st Period			2nd Period			3rd Period			4th Period		
	LOW PCV[a]	HIGH WBC[b]	LOW WBC[c]	LOW PCV	HIGH WBC	LOW WBC	LOW PCV	HIGH WBC	LOW WBC	LOW PCV	HIGH WBC	LOW WBC
Goats	42 %	27 %	2 %	35 %	5 %	5 %	33 %	87 %	0 %	73 %	57 %	0 %
Cattle	22 %	5 %	2 %	18 %	0 %	18 %	19 %	38 %	0 %	17 %	22 %	0 %

[a]*Low PCV = 24 % or less for goats, 26 % or less for cattle.*
[b]*High WBC = above 13,000 for goats, above 12,000 for cattle.*
[c]*Low WBC = below 4,000 for goats and for cattle.*

38/43 females. Examination of cattle showed ticks present 55 % of the time with 35 % of the incidents having only 1-12 ticks. Few cattle had enough ticks to cause large blood loss, but many had enough to transmit blood-borne diseases. No lice were observed. Skin lesions included seven females with warts, six of which were on the udder, one of which involved the teats so severely as to prevent milking or nursing. There were six cases of "gal", a Creole term for raised, plaque-like epidermal lesions listed as focal dermatitis in table I. The etiology of these nodules was unclear but may have been flattened papillomas, fibromas, or more likely solitary focal epidermal infections, possibly fungal or bacterial. These lesions did not appear troublesome for the affected animals. Two of the 17 males (11.8 %) had signs of orchitis and/or epididymitis, indicated by swelling and sensitivity of the testes and/or epididymal tissues. Three of five young males (under one year of age) had abnormally small or undescended testes. Twelve of the 37 sexually active females (32 %) had a vesicular vaginitis at one or more physical examinations. There were a few cases of vulvar dermatitis, a few teat wounds, and two cases of mastitis. Three cattle had a few oral ulcers, three had diarrhea or bloody feces with no other clinical signs, and one had tapeworm segments in the feces. Total white blood cell counts of cattle showed abnormally high counts in up to 38 % of the cattle at each period (mean – 16 %), and the hematocrit was low from 17-22 % of the time, per period, or a mean of 19% of the time (tables IV and V). Random cattle differential white blood cell counts showed an eosinophilia 3 % of the time and a monocytosis 10 % of the time.

Serum mineral and vitamin analysis

The caprine serum values obtained for the six minerals and two vitamins over the four periods of this study are summarized in table VI. Ca, Zn, Mg, Fe, and Se were normal to high normal in all four time periods. P values were low for two (third and fourth period) of the four time periods, and in 21 % of the animals in the other two time periods. Mean serum vitamin E was low in two of the four time periods and pervasive in that 76 % of the goats had at least one low serum vitamin E value. There was an expected increase in serum P in younger stock, presumed proportional to milk intake from nursing, and a converse reduction in nursing does versus nonlactating does. Mean vitamin A values were all normal (>20.0 µg/dl) for each period. However, 23 % of the goats had at least one subnormal serum value for vitamin A. Low serum vitamin A values are potentially more serious in goats than cattle because of a lack of plasma betacarotene in goats.

Mean bovine serum P values were subnormal in the third and fourth periods (table VII) with 23 % of individual animals also having subnormal serum P in the first two periods. Serum P tended to be higher in nursing calves. Mean serum vitamin A values were in low normal range for all four time periods and mean serum vitamin E values low normal in two of four periods ; however, 27 % of the cattle had at least one subnormal serum vitamin E value, and 31 % of the cattle had one subnormal serum vitamin A value during the study. Mean serum Fe levels were at the upper end of normal values in all four periods, while serum Ca, Mg, Se, and Zn were normal to high normal.

TABLE VI Mean and minimal acceptable mineral and vitamin serum levels of goats.

Nutrient	Overall mean serum level	Mean serum levels by period				MAC for Cattle
		1	2	3	4	
Ca (mg/dl)	11.7	13.7	11.8	10.3	10.0	8.0[a]
P (mg/dl)	4.78	6.08	6.42	2.46	2.37	4.5[a]
Mg (mg/dl)	3.90	3.67	3.79	4.18	4.17	1.0[a]
Fe (µg/dl)	261	276	274	249	229	90.0[a]
Se (µg/ml)	0.35	0.36	0.32	0.38	0.34	0.03[a]
Zn (µg/dl)	119	122	112	130	117	60.0[a]
Vit. A (µg/dl)	28.5	26.4	27.3	29.5	33.0	20.0[b]
Vit. E (µg/dl)	68	105	94	36	37	70.0[c]

[a] *McDOWELL et al., 1983. (Nomenclature « Critical Serum Levels » changed to « Minimal Acceptable Concentrations (MAC) ».)*
[b] *FRASER, CM, Ed., Merck Veterinary Manual, 6th ed., 1986.*
[c] *NORTON & McCARTHY, 1986. (All of above in bibliographical references).*

TABLE VII Mean and minimal acceptable mineral and vitamin serum levels of cattle.

Nutrient	Overall mean serum level	Mean serum levels by period				MAC for Cattle
		1	2	3	4	
Ca (mg/dl)	13.0	15.8	12.0	11.7	11.2	8.0[a]
P (mg/dl)	4.47	5.04	5.66	2.72	3.08	4.5[a]
Mg (mg/dl)	3.96	3.93	3.81	4.06	4.16	1.0[a]
Fe (µg/dl)	264	245	292	266	258	90.0[a]
Se (µg/ml)	0.36	0.35	0.36	0.41	0.36	0.03[a]
Zn (µg/dl)	145	147	137	145	152	60.0[a]
Vit. A (µg/dl)	27.7	26.0	26.2	31.5	30.7	20.0[b]
Vit. E (µg/dl)	132	161	158	84	81	70.0[c]

[a] *McDOWELL et al., 1983. (Nomenclature « Critical Serum Levels » changed to « Minimal Acceptable Concentrations (MAC) ».)*
[b] *FRASER, CM, Ed., Merck Veterinary Manual, 6th ed., 1986.*
[c] *NORTON & McCARTHY, 1986. (All of above in bibliographical references).*

Hair mineral analysis

Only Zn levels were lower in Haitian goats than in other goat hair analyses (table VIII). Other minerals (Ca, P, Fe), were higher than in reported normals, while Mg was slightly lower. We could not find normal goat hair values for Se (1.18 ppm in this study), but by using cattle hair Se values (table IX), the caprine hair Se value was low normal. More studies have been done with cattle hair, allowing greater comparison (table IX). Based on the data of the authors, P was very low, Mg and Fe were lower than normal, Ca and Se were in low normal range, while Zn was in normal range.

TABLE VIII Hair mineral concentrations in Haitian goats and concentrations reported in other studies.

Mineral	Mean Haitian goat values (ppm)	Previously reported values[a] (ppm)
Ca	2395	1605
P	343	279
Mg	205	264
Fe	36	20
Se	1.18	–
Zn	82	128

[a] *COMBS (D.K.) Dept. of Dairy Sci., University of Wisconsin, Madison. (Personal communication).*

TABLE IX Hair mineral concentrations in Haitian cattle and concentrations reported in other studies.

Mineral	Mean Haitian cattle values (ppm)	Previously reported values[a] (ppm)
Ca	590	265 – 3,208
P	38	190 – 516
Mg	60	114 – 270
Fe	11	29 – 70
Se	0.31	0.06 – 10.00
Zn	219	122 – 342

[a] *COMBS (D.K.) Dept. of Dairy Sci., University of Wisconsin, Madison. (Personal communication).*

TABLE X Comparison of hight- and medium-access Haitian goat forages with recommended sheep mineral requirements.

Mineral	Mean forage levels by period				Dietary requirements[a]
	1	2	3	4	
Ca (%)	1.00	1.11	0.54	0.50	0.21 – 0.52
P (%)	0.07	0.09	0.06	0.06	0.16 – 0.37
Mg (%)	0.15	0.15	0.21	0.14	0.04 – 0.08
Fe (ppm)	252	383	252	262	30 – 50
Se (ppm)	0.15	0.11	0.20	0.25	0.1
Zn (ppm)	79	61	49	49	35 – 50

[a] *National Research Council (NRC), Washington, D.C. (1975). All values are expressed as concentrations in dry matter.*

TABLE XI Comparison of hight- and medium-access Haitian cattle forages with recommended cattle mineral requirements.

Mineral	Mean forage levels by period				Dietary requirements[a]
	1	2	3	4	
Ca (%)	0.81	0.85	0.53	0.56	0.22
P (%)	0.07	0.07	0.06	0.08	0.20
Mg (%)	0.13	0.14	0.20	0.17	0.10
Fe (ppm)	271	427	215	311	20
Se (ppm)	0.19	0.13	0.27	0.20	0.1
Zn (ppm)	87	58	34	71	40

[a] *Requirements for Ca and P from NRC (1976). Requirements for other minerals from NRC (1983). All values are expressed as concentrations in dry matter.*

Forage analysis

Forage samples were analyzed for mineral content and accessibility at the four different sampling time points (tables X and XI). These tables show a high forage Ca level and persistently low P levels, creating an absolute P deficiency for goats (16-56 % of requirement) and cattle (30-40 % of requirement). Forage values below 0.10 % in dry matter usually are compatible with clinical signs of P deficiency (15) ; in all but two cases, all forage analyses were below 0.10 % P. In this study, the Ca to P ratio ranged from 7.0:1 to 14.3:1, well above the recommended 1:1 at low P dietary intake or 2:1 at higher P levels (11). Forage Se and Zn were generally adequate with a few forages below minimal values. Forage Fe was very high (17-21 times cattle requirements and 8-13 times the goat requirements), but still below 1000 ppm, considered the maximum tolerable limit. The high serum Fe levels and low caprine hair Zn noted raise the possibility of interference with Zn metabolism even though serum Zn levels were adequate for both cattle and goats.

Soil analysis

The soils taken for analysis showed they were classified as Eutric Flurisols by FAO/UNESCO Soil Map of the World (1972), which is equivalent to Eutric Fluvents by

the USDA Soil Taxonomy System (2). The soils were relatively fertile, had a basic reaction, contained free carbonates, and were relatively high in organic matter. The soils were low in P and Zn, with 71 % of samples expected to respond to P fertilization and 55% expected to respond to Zn supplementation when cropped with corn, sorghum, grasses, or beans. A few soils (under 10 %) had marginal magnesium levels, and the aforementioned plants could be expected to respond to magnesium supplementation in these soils.

DISCUSSION

The Haitian farmers in this study were not representative of all Haitian farmers in the Artibonite Valley or elsewhere in Haiti. As a group, these farmers appeared to have above average interest in goat and cattle production. They had variable management skills but had had access to veterinary care and animal science programs for approximately 37 years at Hospital Albert Schweitzer. Further, the Artibonite Valley is considered a "bread basket" for Haiti, in that it has been endowed with excellent soils, reliable rainfall, and fairly consistent irrigation capabilities since French colonial times. Therefore, the data collected and presented in this report are presumed to overestimate general ruminant health and underestimate the incidence or severity of goat and cattle diseases in both the Artibonite Valley itself and elsewhere in Haiti. Given this sampling bias, what was found still was not encouraging and suggests there is much potential for improvement of reproduction and production efficiency.

The major focus of this report is goat and cattle disease assessment. However, consideration needs to begin with the soils of this region. Clearly, the soils are reasonably fertile with one major exception, that is, a pervasive P deficiency. There is also a lesser Zn deficiency, relative to optimal plant growth, particularly with the interference of Zn availability due to the high pH, Ca, and Fe levels. Evaluation of the available forages indicates remarkably high Ca/P ratios and nearly complete plant deficiency of P. Only two plants (okra leaves, *Hibiscus esculentus*, at 0.2 ppm and a plant termed "balé" in Creole, *Sida* spp., at 0.1 ppm) exceeded the minimal level of P (0.10 ppm of dry matter) necessary to avoid clinical phosphorus deficiency (15).

The plants absorb P poorly, not only because it is in low absolute amounts in these soils, but also because of the high organic and carbonate levels, and the basic pH, all of which reduce plant availability. Given that little or no concentrates or mineral supplements were fed to the goats and cattle, it can be appreciated that nearly all of the animals in this study were consuming a diet very likely to cause an absolute P deficiency. In addition to the absolute P deficiency, its combination with high soil Ca creates Ca/P ratios (7.0:1 to 14.3:1) which are also acknowledged to affect growth rate, reproduction, and lactation of ruminants (17). It was noted that the mean serum P levels for both goats and cattle were low in two of four periods, and 23 % of cattle and 21 % of goats had at least one subnormal serum P value in the other two periods. The goat and cattle serum P and bovine hair P values support the diagnosis of P deficiency in both goats and cattle which was particularly bad during the middle to late rainy season (third and fourth sample periods). This points to seasonal or climatic effects which affect P availability and/or solubility in the soil and ultimately in the plants and livestock. Lactation and pregnancy provided particularly strong metabolic stress on the mature females, while lactation helped the neonates receive P. These variable effects were confirmed by trends in differences of serum P in the respective subgroups of goats and cattle.

Phosphorus deficiency is reported to produce a variety of effects including reduced growth rate, impaired reproduction, pica, and bone and joint abnormalities (15). All of these signs except noticeable bone or joint diseases were commonly observed or reported in the animals of this study. Poor haircoats or skin condition are sporadically associated with P deficiency, and such was noted with the female goats, and in other animals to a lesser degree. In cattle, another sporadic disease associated with P deficiency is called post-parturient haemoglobinuria, which appears about three to eight weeks postparturition in lactating cows. Whether this disease occurs in Haiti is unknown, but if it does, it could be mistaken for a variety of clostridial diseases (including anthrax), babesiosis, anaplasmosis, or leptospirosis. Elsewhere, anthrax is reported to occur not only in cattle but also in goats, sheep, swine, and horses (8). In this study, anthrax was reported only in cattle, but never observed. Hence, we suspect the common but unconfirmed diagnosis of anthrax may be incorrect at least some of the time. This needs to be resolved.

Infertility associated with P deficiency is often reported but not well understood (1, 11,15). It is best known in cattle but can also occur in goats (13). Cattle fed a P deficient diet show a delayed onset of puberty and postpartum œstrus, increased services per conception, and possibly an increased incidence of cystic follicles. Anœstrus is the most often reported affection in goats with cystic follicles, which is occasionally noted in goat herds with P deficiency. There is a high probability that the slow growth, longer time to puberty, and the long kidding and calving interval seen in the cattle and goats of this study are related to the obvious P deficiency as reported by others (11,12, 13).

Zinc was marginally low or suboptimal in some of the soils, plants, and animals of this study. Gonadal hypoplasia noted in male calves also is compatible with Zn deficiency. The low serum vitamin A and vitamin E, and low goat hair Zn values also raise the possibility that these nutrients were occasionally deficient and could also have produced or contributed to the infertility and poor growth

of the animals in this study in a synergistic effect with the known P deficiency. Although not measured, the authors also suspect protein and caloric deficiencies contributed to the poor reproductive performance of the livestock in this study, in addition to the P and other mineral or vitamin deficiencies noted.

The orchitis/epididymitis noted in two bulls were potentially serious lesions, possibly due to unidentified infections or traumatic cause. From a public health standpoint, infection by *Brucella abortus*, *Mycobacterium bovis*, or *Leptospira* species would be serious for both cattle and humans. Certainly, there are other bacteria which are pathogenic for the reproductive tract of bulls. The absence of reports or observations of bovine abortions or stillbirths was encouraging. Fetal deaths, if present, were occurring in early pregnancy in order to escape gross detection. The vesicular vaginitis noted in 12 of 37 (32 %) mature female cattle was pervasive enough to warrant further investigation as to its etiology. Infectious agents, such as ureaplasmas or viruses could be potential causes, but there was a lack of other clinical signs such as signs of systemic disease, fever, abortions, or stillbirths, to support such causes. Nutritional deficiencies (e.g., protein, vitamin A, vitamin E, or Zn) may also be causing fragility of vaginal epithelium, which could produce the vesicle-like erosive or ulcerative vaginal lesions.

The anaemia (low hct) noted in the goats about 43 % of the time and in cattle 19 % of the time most certainly reduced reproductive efficiency as well. Anemia in these cattle and goats was suspected to be caused by internal parasitism. There was a high incidence of eosinophilia in goats (19 % of random blood smears) and a low incidence in cattle (3 % of random blood smears). Also, there were variable episodes of occasionally bloody diarrhea which, based on the HAS veterinarian's testimony, corresponded with high faecal egg counts and were clinically responsive to deworming treatment. Finally, the mean hct dropped 11% in the late rainy season ; this parallels the time of expected high worm infestation. Anemia caused by *Babesia* and *Anaplasma* species was reported and confirmed in only a few random Haitian animals (personal communication, Rod Frank, DVM, HAS, Deschapelles, Haiti). When seen, these infectious agents caused clinical disease in larger herds, such as those owned by HAS.

Beyond reproductive diseases, there were a variety of integumentary system diseases or lesions. There were two goats with lice. At least one doe and its kids had lesions compatible with contagious ecthyma. This disease can be highly detrimental by predisposing the udder to mastitis due to teat lesions with soreness and resultant reduced nursing. The kids can starve due to the dam's teat sensitivity and resultant reluctance or refusal to nurse and/or the kid's oral nursing pain. Therefore, even though it was a singular observation, the possible presence of contagious ecthyma in this area of Haiti needs diagnostic confirmation because of its significant biological effects. The presence of the superficial exfoliative dermatitis in 16 of 60 goats (26.6 %) was of concern mainly because of its high incidence rather than any observed adverse effect. The authors are unsure of its etiology but suspect it may be due to vitamin E deficiency which produces a dermatosis very similar to what was seen (16) and because the goats in general had a high incidence of low serum vitamin E levels (76 % of goats had one or more subnormal serum vitamin E levels). The fact that only females had this skin lesion is interesting. If the condition is due to vitamin E or some combination of nutrient deficiencies (e.g. Zn, P, as well) one could rationalize that females might have higher requirements for such nutrients, and are more predisposed to the clinical dermatitis observed.

The common presence of small numbers of ticks on cattle suggested that they might be important vectors for blood-borne disease without producing significant blood losses. Integumentary lesions in cattle were generally minor with the one exceptional case of udder and teat warts (papillomatosis) which obliterated normal milking or nursing of this cow. The common (6/37 females or 16 %) occurrence of udder and/or teat warts suggests milking or nursing is involved in its spread. Digestive system lesions or signs were also minor, with the exception of three cattle with diarrhea, sometimes bloody. These diarrhea cases appeared to be secondary to internal parasitism, the latter of which is likely involving 19 % or more of the cattle based on hematologic abnormalities.

CONCLUSION

This study included examination of a selected group of nutrients (Ca, P, Mg, Fe, Se, Zn, Vit A, Vit E) through parts of the food chain (soil, forages, serum, and hair). There was an absolute and relative P deficiency which was noted in the soil, forages, and animals. Correction of the above would require soil and animal supplementation with products high in P and low in or free of Ca. There were less obvious vitamin E and vitamin A deficiencies in both goats and cattle. There was a Zn deficiency in some soils, which appeared in some forages and some low goat hair Zn values. Soil Zn supplementation is likely to be beneficial for most of the soils tested.

External parasitism was uncommon (3 % incidence) in goats with only one goat heavily infested with lice and one other having just a few lice. External parasitism (ticks only) was seen in 55 % of cattle but only in low numbers. Therefore, the ticks may be a vector for blood-borne diseases but were not directly contributing to sufficient blood-letting to produce the anaemias noted in the animals of this study.

Anaemia, eosinophilia, high white blood cell counts, and diarrhea responsive to deworming and relating to the

rainy season were noted to be worse in goats but common to both species. Weight loss of goats during the rainy season was also noted. This combination of clinical signs was compatible with internal parasitism. Not all anaemias noted were necessarily due to internal parasitism; conversely, numerous animals with normal hematocrits could have been internally parasitized but were not so identified. However, if abnormally low hematocrits are used as a screening marker for internal parasitism goats had serious parasitism about 43 % of the time and cattle 19 % of the time.

There was circumstantial evidence (by gross examination) for contagious ecthyma in goats. This needs diagnostic confirmation.

A superficial exfoliative dermatitis or dermatosis was often seen in female goats. Its cause awaits further evaluation, but vitamin E deficiency and/or zinc deficiency dermatoses have been reported in goats and may be occurring here.

There was evidence of vaginal and testicular lesions in cattle but not goats, without evidence for widespread stillbirths or abortions in cattle, hence, the cause(s) for, or the significance of, the bovine lesions are unclear.

There was a relatively high incidence of bovine udder and teat papillomas (infectious warts) suggesting milking or nursing transmission.

One disease issue of cattle identified by this study but not resolved was the Haitian farmers' perception that anthrax was the "most frequently seen" bovine disease and "most serious." This investigation did not confirm this diagnosis since no cattle in the study were seen or reported with a disease similar to anthrax. In considering the reported signs, the "charbon" or anthrax may be some other clostridial disease, postparturient hemoglobinuria, acute babesiosis, acute anaplasmosis, acute leptospirosis, or an acute toxicosis, unique to cattle. This issue needs clarification.

A group of low incidence diseases was noted and includes possible dermatophylosis, mastitis, and neonatal diarrhea in goats, and mastitis, focal cutaneous masses, and babesiosis (one case in HAS bull) in cattle. These diseases or lesions were uncommon and/or mild, but with major management changes such as formation of large herd units, these diseases could occur more frequently or more seriously.

The disease incidence noted in goats and cattle was not related to the economic status of the owner, the location of the stock, or size of the total herd. Most variability occurred from herd to herd, suggesting owner management differences were significant.

There was only slight evidence of widespread catastrophic disease in the goats and cattle of this study but plenty of evidence for a variety of disease problems which collectively produce major production and reproductive inefficiency. Estimates of production and reproduction efficiency based on the data of this study suggest that goat and cattle raising on small Haitian farms is well below 50 % of what is theoretically possible.

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VEIT (H.P.), McCARTHY (F.), FRIEDERICKS (J.), CASHIN (M.), ANGERT (R.). A survey of goat and cattle diseases in the Artibonite Valley, Haiti, West Indies. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 27-38

A 40 week study of 43 farmers, 60 goats and 60 cattle was conducted in order to identify abnormal conditions or diseases and predisposing seasonal, managemental or nutritional factors. Farms were visited, farmers interviewed and animals examined up to 4 times, about every 10 weeks, and bled for Ht, total WBC, selected serum vitamins and minerals, hair collected for mineral analysis. Soil and forages were collected for analysis. Animals were generally in fair condition, with poor growth and reproduction. Unexpected wet season caloric deficiency, severe P deficiency and lesser vit. A and E deficiencies were noted. Anaemia, secondary to parasitism, was common to both species, worse in goats. Cattle had ticks, while goats had lice. Goats had reported neonatal diarrhea and mortality; observed exfoliative dermatitis, warts, dermatophytosis and possible contagious ecthyma. Cattle had reported anthrax and babesiosis; observed vesicular vaginitis, orchitis and teat warts.

Key words : Cattle - Sheep - Disease survey - Mineral deficiency - Parasitism -Farming system - Growth rate - Reproductive performance - Animal feeding -Seasonal effect - Haiti.

VEIT (H.P.), McCARTHY (F.), FRIEDERICKS (J.), CASHIN (M.), ANGERT (R.). Estudio de las enfermedades en caprinos y bovinos en el Valle Artibonita, Haití, Antillas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 27-38

Se llevó a cabo un estudio en 43 establecimientos , en 60 caprinos y 60 bovinos, con el fin de identificar los estados anormales o enfermedades, así como las predisposiciones estacionarias, de manejo y/o nutricionales. Los establecimientos fueron visitados, los finqueros interrogados y los animales examinados hasta 4 veces, a intervalos de 10 semanas. Se tomaron muestras de sangre para hematocrito, recuenta de leucocitos, selección de vitaminas y minerales séricos. El análisis de minerales se hizo gracias a la colecta de pelo de los animales. Se recolectaron también suelo y forrajes, para los análisis respectivos. Los animales se encontraron a menudo en mala condición, con baja tasa de crecimiento y reproducción. Se observaron deficiencias calóricas durante la época lluviosa, deficiencia de P severa y en menor escala, deficiencias de vitamina A y E. La anemia, como resultado de parasitosis, fue comun en ambas especies, aunque peor en las cabras. En el ganado bovino se observaron garrapatas, mientras que en el caprino se encontraron piojos. En cabras se reportó diarrea neonatal y mortalidad, se observaron dermatitis exfoliativas, estrías, dermatofitosis y un posible ectima contagioso. En bovinos se reportó antrax y babesiosis, se observaron vaginitis vesiculares, orquitis y estrias en los pezones.

Palabras claves : Bovino - Caprino - Encuesta patológica - Carencia mineral - Parasitismo - Sistema ganadero - Crecimiento - Reproductividad - Alimentacíon animal - Efecto estacional - Haití.

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The relationship of Haitian small farm management to goat and cattle diseases

VEIT (H.P.), McCARTHY (F.), FRIEDERICKS (J.), CASHIN (M.), ANGERT (R.). La relation entre la gestion des petites exploitations en Haïti et les maladies caprines et bovines. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 39-45

Une étude de 40 semaines a porté sur 43 fermiers, 60 chèvres et 60 bovins, afin d'identifier des conditions anormales ou des maladies, et les facteurs prédisposants saisonniers, liés à la gestion ou nutritionnels. Les exploitations ont été visitées 4 fois, approximativement toutes les 10 semaines, les fermiers questionnés, les animaux examinés et leur sang prélevé pour l'hématocrite, le nombre total de leucocytes et le taux de certains vitamines et minéraux dans le sérum. Des poils, de la terre et du fourrage ont été prélevés pour analyse. Des déficiences sérieuses en phosphore ont été mises en évidence dans le sol, dans le fourrage et chez les animaux, et des déficiences moindres en vitamine E et A, dues à un manque de fertilisation du sol et/ou des insuffisances alimentaires. Une anémie, présumée d'origine parasitaire, était un signe clinique fréquent chez les chèvres (incidence 43 p. 100) et les bovins (incidence 19 p. 100). Les maladies infectieuses et le parasitisme externe et interne étaient partiellement limités par l'isolement des animaux, leurs mouvements et leur maintien à l'attache, rarement par des médicaments. Généralement, la condition du bétail était passable, les performances reproductives et pondérales mauvaises, mais des maladies graves étaient rares. La nourriture des ruminants provenait entièrement de résidus de cultures vivrières ou de plantes spontanées. Etant donné la pénurie et/ou le coût élevé du fourrage, de l'engrais, des compléments en vitamines et en minéraux, des médicaments et des vaccins, le système actuel de gestion des petites exploitations en Haïti réussit à prévenir des maladies graves, mais n'est pas efficace pour des maladies mineures, la reproduction et la croissance.

Mots clés : Bovin - Caprin - Résistance aux maladies - Conduite du troupeau - Alimentation des animaux - Carence minérale - Performance de reproduction - Gain de poids - Croissance - Influence de la saison - Méthode d'élevage - Haïti.

INTRODUCTION

It has been observed that there was relatively poor growth and reproductive performance of Haitian goats and cattle in the Artibonite Valley of Haiti, West Indies, with minimal gross catastrophic illness (4). The majority of such stock appeared to be in fair or better physical condition, even though there was widespread phosphorus deficiency, lesser vitamin A and E deficiencies, and suspected parasitic anaemia (4). Very little information regarding basic management of such animals was available. Accordingly, a study was done to physically examine a selected group of Haitian goats and cattle, to analyze their serum or hair for selected minerals and vitamins, to analyze related soil and forage minerals and vitamins, and to interview their caregivers. This report focuses on the relationship between the management of these animals, and the reported or observed diseases, conditions and lesions found.

MATERIALS AND METHODS

Selection of farms and livestock

The materials and methods are reported in greater detail elsewhere (4). Briefly, the region of study was in the Artibonite Valley region, within a 12-mile radius of Deschapelles, Haiti. The selected Haitian farmers had received long-standing agricultural support from Hospital Albert Schweitzer (HAS) veterinarians, which enhanced their cooperation and reliability with our investigative team. The specific criteria for participation of farmers in this study included prior participation in a goat deworming program, direct responsibility for animals in this study, local residence, ownership or use of a small farm, and small herd status.

Farm visitations

Details of 4 farm visits were described elsewhere (4). Briefly, all participating animals were ear tagged at the initial visit and at all visits, physical examinations were performed on each available animal. Blood was collected by jugular venipuncture for hematology and serum mineral and vitamin assays. Bovine switch or caprine tail hair was collected and stored for mineral assays. A total of 38 soil samples were taken and frozen at -2°C for analysis. Sixty-two forage samples were collected, oven dried, sealed in plastic bags, and stored at room temperature until analysis. On each visit, a questionnaire was administered to the person responsible for daily management of each animal by a Haitian interviewer. Topics covered, respectively, were reproduction, feeding and nutrition,

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health and disease, labour and marketing. The questionnaires were written in both English and Haitian Creole with all dialogue done in Creole. Questionnaires and physical examination data were tabulated and summarized in table or graph forms. Where possible, comparison to known normal values was made.

Laboratory analyses

Hematology

Blood was collected for hematocrit, total white blood cell count, and differential white blood cell counts. Hematocrits were determined by standard micropipette procedures, and total white blood cell counts were done with pipette and hemocytometer procedures. Differential white blood cell counts were done after Wright staining of blood smear slides, using standard laboratory procedure (200 cell counts).

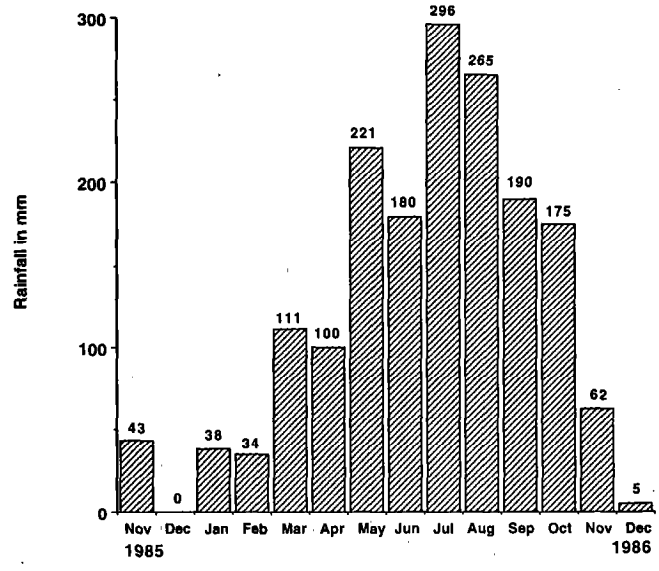
Vitamin and mineral assays for serum, hair, forages and soil

Samples were collected and stored for analysis at Virginia Polytechnic Institute and State University as previously described (4).

RESULTS

This region of Haiti (Deschapelles) had a typical rainfall pattern for 1986 as shown on figure 1. Essentially, the winter months (Nov.-Feb.) were relatively dry (mean = 3.5 cm/m), while the spring through fall months (Mar.-Oct.) were relatively wet (mean = 19.2 cm/m).

Feed availability for goats and cattle was reported least plentiful in February and March, and most available for goats around December, and for cattle around July. Body weights of mature female goats and cattle (non-pregnant) over the 4 sampling periods are shown in table I. Goat weights were obtained by both scale weighing and chest taping, while cattle weights were obtained by tape measure only. Based on questionnaire responses and observations, most goats were loose during the day in the dry season (Nov.-Feb.), and allowed to browse freely. Evenings, most goats were taken into the owner's yard. From March to November, or while there were crops growing for human consumption, goats were restricted in movement by tethering or by yoke collar (to prevent entrance to fenced yards and gardens). Again, these animals were usually kept in yards in the evenings. Food and water was provided to about 1/2 the goats in the evenings, while in the yards. Feed for goats consisted of various crop residues, tree leaves, volunteer grasses, and



Ronald Bluntschli. Mennonite Central Committee, Deschapelles. Personal communication.

Figure 1 : Rainfall in Deschapelles, Haiti (1985-1986).

***TABLE I** Mean animal weights (lbs.) by sampling period.*

	1st Period (1/1-2/6)	2nd Period (3/3-4/5)	3rd Period (6/9-6/14)	4th Period (8/18-8/22)*
Point in rainy season	Dry	Early rainy	Rainy	Rainy
Mature female goats[a]	73	68	57	57
Mature female cattle[a]	612	639	641	663

[a] *No animals who kidded or calved during the course of the study were included in these figures.*
* *Month/date.*

a few legumes. No reported or observed plantings for goats or cattle were noted. Most goat handlers moved their tethered goats twice daily, and most said they daily carried feed to their goats. The most commonly reported cut and carried goat feeds were leaves of the West Indian Birch (*Bursera simaruba*), West Indian Elm (*Guazuma ulmifolia*), and Hog plum (*Spondias mombin*) trees, and Sorghum (*Sorghum vulgare*) crop residue. Ninety-two percent of the handlers reported carrying water to the goats at least once daily, but this was not confirmed by our study team.

About 43 % of the cattle were reported to be tethered year round, with the remainder tethered only during the rainy season (Mar.-Oct.). The tethering during the rainy season was done to protect crops, while tethering during

the dry season was done to prevent cattle from being injured, either accidentally or by irate landowners protecting property or crops.

Cattle were usually left tethered in the same area day and night. They rarely were reported to be given extra feed in the evenings, but about 1/3 had feed carried to them in the day time. The most common cut and carried feeds for cattle were residues of corn, plantain, sorghum, sugar cane or leaves of the West Indian Elm (*Guazuma ulmifolia*). All cattle were reported to be walked to a water source at least once daily, and moved about twice daily for better feed access.

Haitian goat or cattle handlers in this study relied entirely on memory. No written records were made even though there was general access to literate individuals. Therefore, precise information regarding signs of sexual activity or reproduction for bucks or does was sketchy, as was previously reported (4). A summary of caprine and bovine reproductive activity is given here (table II). All buck kids (N = 8) in this study were sold before they were 1 year of age. This encouraged outbreeding, although inbreeding was suspected to occur. Older bucks were usually more restricted and were also frequently sold, while female kids were often kept. Normal œstrus behaviors in does were noted, along with vulvar swelling. Very few owners knew the gestation period for does. About 25 % of goat handlers fed extra feed and/or water to presumed pregnant does, and about 1/3 gave extra food and/or water to a nursing doe. No special care was given to newborn kids. First parturitions, when recalled, occurred at about a mean of 14 months. Only 2 handlers recalled a second parturition, at 18 and 12 months later. Based on kiddings observed, kidding interval is estimated to be 11 months, with 1.9 kids/parturition. No dystocias were reported or observed; two abortions and one stillbirth were reported, but none were seen. Handlers claimed to see the first caprine postparturient œstrus at a mean of 6 weeks, when observed. Based on kidding intervals observed, conception must average about 6 months postparturition. Other than some mastitis, and teat lesions possibly associated with contagious ecthyma, the does had minimal lesions relating to reproduction. Three does had an excessive mucoid vaginal discharge, without œstrus. Nothing abnormal was seen or reported in the bucks or their male reproductive tracts. A summary of caprine diseases or conditions is in table IIIA.

Cattle reproduction was slightly better understood or appreciated by the Haitian handlers, but also was based on no apparent record keeping. About two-thirds of owners of bulls did not know the age of puberty for their bulls. Infertility in bulls was either not recognized or considered. Only 1 farmer expressed an awareness of bull infertility ; he presumed it to be due to thinness. There was no knowledge of frequency of breeding or female conception rates. About half of cow handlers remembered first heat, with a mean of 24 months. Alrnost all cow handlers said their cows were bred by someone else's bull, but they did not know the number of services per conception. About 60 % of the handlers recognized normal œstrus signs. About 1/3 of handlers reported seasonal sexual activity to be highest February to April (table II). This was confirmed by the highest reported and observed calvings being November to February. A majority of handlers of pregnant cattle knew when they were bred and expected to calve. A few (12 %) owners gave extra feed to pregnant cows. No dystocia, abortions or stillbirths were reported or observed. Age at first parturition was reported 1/3 of the time (mean = 29 months), with a mean calving interval of 22 m reported and 26 m observed. The likely actual interval is about 24 m. First postparturient œstrus, when remembered, ranged from 3-104 weeks with a mean of 19 weeks. Based on a 24 month calving interval, conception occurred about 15 months postparturition. Observed reproductive abnormalities in cattle included orchitis and/or epididymitis in 2 mature bulls, testicular hypoplasia in 3 young bulls, and a vesicular vaginitis in 12 of 37 sexually mature females. Also, six females had udder warts, one so severely that nursing or milking would have been difficult or impossible. These and other lesions of cattle are summarized in table IIIB.

HANDLERS reported 74 % of the goats and 82 % of the cattle to never have been sick. The HAS veterinarian confirmed that few goats or cattle were noted to suffer severe or fatal clinical illness in this region, and these observations also failed to reveal any major severely debilitating or fatal disease. On the other hand, there was considerable evidence of nutritional diseases, specifically deficiencies in P, vitamin A and E, reported elsewhere (4). Further, there was a high incidence of anaemia, associated with other hematologic abnormalities and with the middle to late rainy season, all suggestive of internal parasitism. There was also a variety of foetal and neonatal goat losses, previously reported (4).

TABLE II Summary of reproductive activity in Haitian goats and cattle.

	Goats	Cattle
age at puberty – males	3-4 m	15-18 m
– females	10-12 m	20-24 m
breeding seasons	March-April Sept.-Oct.	March-April Dec.-Feb.
gestation interval	11 m	24 m
services per gestation	ND[a]	ND
progeny per gestation	1.9	1.0
abortion rate[b]	7.2%	0.0%[c]
stillbirth rate[b]	3.6%	0.0%[c]
dystocia rate[b]	0.0%[c]	0.0%[c]

a : ND = Not determined due to insufficient data.
b : rate = event per pregnancy x 100.
c : Covered in the questionnaire but not reported by farmers or observed by investigators in study animals.

TABLE III Estimates of reported and observed diseases or conditions in goats and cattle from selected farms in the Artibonite Valley (Deschapelles region), Haiti, West Indies.

A – Goats

I. Goat diseases or conditions reported via questionnaires		
Condition name	Incidence (%)	Overall mortality rate (%)
1. Internal parasitism, presumptive	16.0	0.4
2. Pediculosis (lice)	12.0	2.0
3. Abortion	7.2[a]	7.2[a]
4. Neonatal death, cause unknown	7.2[a]	7.2[a]
5. Stillbirth	3.6[a]	3.6[a]
6. Neonatal death with diarrhea	3.6[a]	3.6[a]
7. Neonatal death with maternal mastitis	3.6[a]	3.6[a]
II. Goat diseases, conditions or lesions observed during study		
1. Low serum phosphorus	80.0	0.0[b]
2. Low serum vitamin E	76.0	0.0
3. Parasitic anaemia, presumptive	42.0	0.0
4. Superficial exfoliative dermatitis	26.6	0.0
5. Low serum vitamin A	23.0	0.0
6. Teat lesions	7.0	0.0
7. Fibroma(s)	5.0	0.0
8. Vaginal discharge, mucoid, chronic	5.0	0.0
9. Contagious ecthyma, presumptive	3.3	0.0
10. Ringworm, presumptive	3.3	0.0
11. Pediculosis (lice)	3.3	0.0
12. Mastitis	1.7	0.0
13. Solar dermatitis	1.7	0.0

a : This is the incidence based on 28 pregnancies.
b : No mortalities were reported for any of the study animals within the duration of this study.

B – Cattle

I. Cattle diseases or conditions reported via questionnaires		
Condition name	Incidence (%)	Overall mortality rate (%)
1. Internal parasitism, presumptive	18.0	0.0
2. Ticks	2.8	0.0
3. Anthrax, possible	2.0	2.0
II. Cattle diseases, conditions or lesions observed via study		
1. Low serum phosphorus	85.0	0.0[c]
2. Tick infestation, few to light	55.0	0.0
3. Vesicular vaginitis	32.0[a]	0.0
4. Low serum vitamin A	31.0	0.0
5. Low serum vitamin E	27.0	0.0
6. Parasitic anaemia, presumptive	19.0	0.0
7. Testicular hypoplasia	17.6[a]	0.0
8. Orchitis/epididymitis	11.8[a]	0.0
9. Papillomas	11.6	0.0
10. Focal dermatitis, presumptive	10.0	0.0
11. Teat lesions, including warts	8.1[a]	0.0
12. Parasitic diarrhoea	6.7	0.0
13. Mastitis	5.4[a]	0.0
14. Oral ulcers, mild	5.0	0.0
15. Vulvar dermatitis	3.3[a]	0.0
16. Babesiosis	1.0[b]	0.0

a : This incidence is of the eligible members of the appropriate gender, not all the animal species of the study.
b : This animal was outside the study, but with a group of HAS cattle in the study. Diagnosis was made by the attending HAS veterinarian.
c : No mortalities were reported for any of the study animals during this study.

All diseases or abnormal conditions are listed in table III, which includes both reported and observed disease. The reported diseases are calculated on the basis of the memory, given earnestly and willingly, but not presumed to be highly accurate. The observed conditions or diseases are reasonably accurate within this select group of Haitian goats and cattle. Comparing the reported diseases with those observed, it is clear that the Haitian farmers appreciated the caprine and bovine conditions involving external parasites and deaths, and internal parasitism to a lesser degree. They tended to either ignore or miss the less biologically severe conditions and could not appreciate the vitamin and mineral deficiencies revealed by analysis. In short, they underestimated the disease problems of their stock.

Labour management of the Haitian stock showed that the animal handlers are usually the owners (50 % of goats, 59 % of cattle) or some other family member, often one of the children. Men and women, in equal incidence, cared for goats. Adult males more frequently cared for cattle. Among children, boys more commonly cared for the stock. Regardless of age, gender, or ruminant species, the same person usually tethered, moved, and carried food or water for a given animal. When recognizable illness occurred, the local Haitian veterinary technicians or the HAS veterinarian were used by all the cattle handlers, and three-fourths of the goat handlers. The remaining goats were cared for by the owner or a relative. About half of these animals were not treated, while the others were given some kind of home treatment or extra care.

Milking of cows was fairly universal, although recollections of the amounts of milk collected were vague, and the milking schedule was rather loosely arranged. Lactating goats were rarely milked, although children

often against parental approval were known to sneak milk from goats. Use of cattle for traction was not practiced in this region, although cattle are used for plowing 18 miles away in Lachapelle, and elsewhere in southern Haiti.

Questionnaire information regarding marketing only extended to the point of sale of such animals, not beyond. Nearly all of the 43 farmers in this study raised their goats and cattle for sale, not for home consumption. Most owners (58 %) thought their animals were sold for short-term breeding and eventual slaughter, 39 % said for long-term breeding (with eventual slaughter presumed), and only 3 % said animals were sold strictly for slaughter. About one-third of the owners expected to eat meat from their animals. In most cases of local sales, they likely bought back some of the meat from local butchers.

Preferences in purchasing goats and cattle gravitated towards large size as a primary desirable attribute. Some cattle owners were attracted to excellent lactators. Distinct small size in either species was discriminated against. Does and bucks were sold for a mean of $20 each, female kids for $ 11, male kids for $ 9, with a considerable price range for each category.

Mean prices for cows were $ 251, bulls $ 262, heifer calves $124 and bull calves $ 98. In this study, males were sold readily, hence, there was an 80 % sales attrition rate (12/15) of caprine males from start to finish and 41 % sales attrition rate for bovine males (7/17). Examination of mean body weights versus mean sale price shows goats selling for $ 0.32 per lb. live weight, and cattle selling for about $ 0.38 per lb live weight.

DISCUSSION

The Haitian farmers' perception of disease problems was less than the real incidence. Part of this perception occurred because of a true difference in opinion or concept, as to what is disease. Part of the difference is due to an inability to appreciate or understand the disease. Finally, part of the underestimation of disease was due to a lack of record-keeping and objective analysis by the farmers. There appeared to be a total absence of written records for livestock management. Part of this is simply an inability of some farmers to read or write; however, many farmers do have access to literate family members, neighbours or friends to assist in reading or writing when desired. There simply is no tradition to keep records. Further, no one has demonstrated sufficient benefit of such written records in the evaluation of reproductive or growth efficiency on small farms and improved management and/or increased profits. The lack of written records also retards or prevents data acquisition relative to nutrition, either from the perspective of feedstuff efficiency or individual animal performance to a given ration. The Haitian management style for feeding goats and cattle is simple: all feed is essentially scavenged from crop residues, annuals, or perennial plants indigenous to the area. There were no reported or observed plantings specifically for goat or cattle use. Therefore, there is little to no direct stimulus for concern about feed efficiency since there is no out-of-pocket feed expense for goats and cattle and no apparent desire to spend money to purchase feedstuffs. Further, due to the general surplus of available labour, animal feeding and care is assigned little to no value. Presumably, if there were significant labor costs, labour efficiency might take on relevance or value for consideration, but it was not important to the subjects of this study. In summary, reproductive, growth, feed, and labour efficiency and the record keeping necessary to measure them had little or vague perceived value to the studied Haitian farmers. Demonstration projects that show important material benefits for the Haitian farmer by use of written records might motivate some of them to acquire and use record keeping skills.

As previously mentioned, goat and cattle feeding is primarily a scavenging process involving free grazing or browsing within limited areas during the dry season, and tethering or yoke use for the wet season ; some stock, especially cattle, are tethered year-round. What is particularly important is that tethered, yoked, or free, roaming stock are rarely kept together, even on farms with larger (up to 30 head) herds. Rather, they are usually isolated as individuals or in small groups. This isolation of stock is the single best feature of Haitian small farm livestock management because it has tended to inhibit spread of external and internal parasites, and infectious diseases. Those who have tampered with the above isolation by placement and aggregation of goats and cattle into larger groups for pasturing, supplemental feeding, reproductive or other management purposes, usually have had significantly increased incidence and/or severity of infectious and parasitic diseases. The above isolationism needs to be maintained in any future attempts at "improving" Haitian small farm management. Interestingly, the one place where animal isolation is commonly broken, is in the evening when most goats and a few cattle are brought together in the yard, near the house. During this time, stock are often close enough to each other to transmit infectious agents and parasites directly, or via faecal ingestion. This evening aggregation should be further examined for ways to minimize infectious and parasitic disease transmission. One way to reduce parasite egg ingestion in yards would be to create off-the-ground feeders and to promote rapid and total cleaning of the yards of faecal material. This manner of internal parasite transmission is likely a primary mode of vertical and horizontal transmission for goats. The mean caprine body weights declined from the early to late rainy season and there were concurrent anaemia and white blood cell abnormalities (4) ; these findings are suggestive of internal parasitism. Using more specific rotational or placement procedures for day time feeding of goats and cattle could also reduce parasitic egg or larvae ingestion from fecal contamination. Improvements of daytime feeding would enhan-

ce what is already partially effective management for infectious or parasitic disease control. Based on questionnaire responses regarding etiology of both external and internal parasitic diseases, Haitian farmers have only a vague understanding of how these parasites spread. They likely have learned their present management procedures by empirical experience and evolved traditions. In any case, the present livestock feeding management works fairly well but could be much better. The use of antiparasitic veterinary drugs has excellent efficacy but their costs, and difficulty in delivery, storage and administration, will likely continue to retard wide scale use for small farms in Haiti. Education and use of optimal management for infectious, and particularly parasitic, disease control could be very cost effective, given that free or low cost labour is currently so freely available.

This study also noted a couple of indigenous herbal antiparasitic treatments (4). Objective evaluation for efficacy of such reported treatments would seem to be worthwhile.

Due to the fact that goats and cattle did not feed exclusively on crop residues, the severe soil and plant phosphorus (P) deficiency was not as critical as it could be. Crop residues from long-term irrigated soils tended to be the most deficient in P, presumably because of long-term cropping depletion without P replacement. There was a tendency (not statistically validated) for the perennials and annuals growing at field boundaries and on non-irrigated soils to have higher, but still deficient P levels. Therefore, the dietary blending of scavenged materials produced at least one positive nutritional benefit by slightly increasing mean dietary P. Such random mixed feeding might have an impact on other nutrients such as Vitamins A, E, and Zn intake, but our data were not sufficient to allow for interpretation of this. The ratio of Ca to P in soils and plants of the study region was so severe so as to require dietary supplementation of animal feeds with a P-containing supplement containing little or no Ca, in order to correct not only the absolute P deficiency, but the incredibly high Ca/P ratio. Both the absolute P deficiency and the high Ca/P ratios (above 7/1) can greatly impair reproduction and growth (1,2,3). Buying and feeding such a mineral supplement, or a more comprehensive vitamin-mineral-concentrate supplement would be a relatively unique management practice for the Haitian farmers in this study. Likely, they would need to be thoroughly convinced that such an effort was worthwhile. Given that the Haitian farmers lack ability or experience in evaluating livestock reproduction, production or feeding efficiency, evaluation of the cost-benefit ratio of a feed supplement would be a new mental exercise for the Haitian farmers of this study. A final drawback is that in the past, a variety of agricultural programs have been introduced to Haitian farmers which required non-indigenous supplies or equipment, only to be eventually cut-off, curtailed, or manipulated by increased pricing and/or taxation. Haitian farmers have seen this occur often enough to realize these circumstances create a fragile dependency for the users of such supplies or equipment. Therefore, there is a strong tendency for Haitian small farmers to be "minimalists," that is, to avoid use of anything, including feed supplements,which would make them dependent on anyone else. This is a survivalist attitude, not easily overcome by strictly mathematical arguments of greater profitability. Such minimalist attitudes are part of the Haitian small farmers attitude towards life in general, not only livestock production. Therefore, part of the strategy to change goat and cattle management goes beyond the technology ; it requires emotional reassurances, as well. An ideal solution would be to find one or more local sources of phosphorus which Haitian farmers could easily acquire and use, at low cost, in order to bypass importation, distribution, taxation and other hidden costs or control procedures. Unfortunately, such has yet to be identified in Haiti.

CONCLUSIONS

The Haitian farmers of this study tended to underestimate, or not appreciate some of their goat and cattle disease problems, particularly those with a nutritional etiology, or those having subtle lesions or effects.

The Haitian farmers of this study keep little objective data regarding their livestock reproductive or production activities. No written records were noted to be kept or used. This lack of objectivity prevents understanding and appreciation of improved efficiency of many aspects of livestock production, as well as true incidence and effect of disease problems. Any future enhancement of livestock production efficiencies will need to include basic record keeping and use of such for increased profitability.

The Haitian farmers of this study have a slight bias towards favoring cattle over goats. This is seen in the tendency for more young and adult males to be put in charge of the cattle, while goats are more often left to the care of women or younger girls. Also, cattle were usually given better attention, in regards to feeding and watering. Finally, they sold at a mean 16 % higher price per pound of body weight ($ 0.38/lb. *vs* $ 0.32/lb. live weight for goats). There is also a distinctive size bias, in the sense that Haitian farmers will pay a premium for perceived largeness, within species, beyond body weight differences.

Haitian farmers in this study did not use cattle for traction, even though knowledge of such is well-known, nor did they use goat milk routinely for human consumption. The reasons for this are unclear, but deserve further evaluation, given there are some positive reasons to do so.

The Haitian farmers of this study rely almost totally on a scavenger system for feeding their ruminants. This system has 2 major advantages :

- low cost : there are minimal expenses associated with this system, and low dependency on others for feed, supplies or equipment ;

- fair to excellent infectious and parasitic disease control, with excellent individual animal observation and care, within the environmental limits of each farm.

The main disadvantages of this system are :

- high labour needs : labour is given little to no value, due to the current lack of paid employment opportunities ;

- nutrition is highly variable, and subject to large problems with quality and quantity of feedstuffs ;

- limited water availability ;

- œtrus detection and breeding activities are potentially more difficult to control and manage.

The Haitians manage their goats and cattle reasonably well, given their knowledge and resource limitations. Some low cost, large improvements in production efficiency could occur with appropriate management improvements in record-keeping and use, and in disease and parasitic control measures.

The deficiency of phosphorus in the soil, plants and animals of this study appears to be causing the largest single production limitation. A feasible phosphorus supplement for the livestock and/or soils is badly needed, and should be incorporated into the nutritional management of the farms in this study as soon as possible.

ACKNOWLEDGEMENTS

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VEIT (H.P.), McCARTHY (F.), FRIEDERICKS (J.), CASHIN (M.), ANGERT (R.). The relationship of Haitian small farm management to goat and cattle diseases. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 39-45

A 40 week study of 43 farmers, 60 goats and 60 cattle was conducted in order to identify abnormal conditions or diseases and predisposing seasonal, managemental or nutritional factors. Farms were visited, farmers interviewed and animals examined up to 4 times, about every 10 weeks and bled for Ht, total WBC, selected serum vitamins and minerals hair collected for mineral analysis. Soil and forages were collected for analysis. There were serious soil, forage and animal phosphorus and lesser vitamin E and A deficiencies due to a lack of appropriate soil fertilization, and/or dietary insufficiency. Presumptive parasitic anaemia was a common clinical sign in goats (43 % incidence) and in cattle (19 % incidence). Infectious diseases, external and internal parasitism were partially controlled by animal isolation, movement and tethering, rarely by therapeutics. Overall, livestock condition was fair, reproductive and growth performance poor, but catastrophic disease rare. Ruminant feeding was entirely from scavenging of crop residues for human consumption, or voluntary plants. Given the scarcity and/or high cost of forages, fertilizer, vitamin-mineral supplements, drugs and vaccines, the present system of Haitian small farm management is successful in catastrophic disease prevention, but is inefficient for minor diseases, reproduction and growth.

Key words : Cattle - Goat - Disease resistance - Livestock management - Animal feeding - Mineral deficiency - Reproductive performance - Live weight gain - Growth rate - Seasonal effect - Farming system - Haiti.

VEIT (H.P.), McCARTHY (F.), FRIEDERICKS (J.), CASHIN (M.), ANGERT (R.). Relación entre los sistemas de manejo en pequeños establecimientos haitianos y las enfermedades en ganado caprino y bovino. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 39-45

Se llevó a cabo un estudio en 43 establecimientos , en 60 caprinos y 60 bovinos, con el fin de identificar los estados anormales o enfermedades, así como las predisposiciones estacionarias, de manejo y/o nutricionales. Los establecimientos fueron visitados, los finqueros interrogados y los animales examinados hasta 4 veces, a intervalos de 10 semanas. Se tomaron muestras de sangre para hematocrito, recuento de leucocitos, selección de vitaminas y minerales séricos. El análisis de minerales se hizo gracias a la colecta de pelo de los animales. Se recolectaron también suelo y forrajes, para los análisis respectivos. Se observaron serias deficiencias de P y en menor grado de vitaminas E y A, tanto en suelos, como en pastos y animales, ya sea debido a la falta de fertilización apropiada de los suelos y/o a insuficiencias nutricionales. Se observaron frecuentemente síntomas clínicos de anemia parasítica en cabras (con una incidencia de 43 p. 100) y bovinos (con una incidencia de 19 p. 100). Las enfermedades infecciosas externas y el parasitismo interno fueron parcialmente controlados mediante el aislamiento, fijación y la redistribución de los animales, pero raramente mediante tratamientos terapeúticos. La condición general del hato fue pobre, con bajas tasas de crecimiento y reproducción, sin embargo, las enfermedades graves fueron raras. La alimentación de los rumiantes se compone principalmente de restos de cosechas para consumo humano o de plantaciones voluntarias. Dada la escacez y/o el alto costo de los forrajes, los fertilizantes, los suplementos minerales y vitamínicos, los medicamentos y las vacunas, el sistema actual de manejo en las pequeñas explotaciones haitianas es exitoso en lo concerniente a la prevención de catástrofes sanitarias, pero insuficiente en lo referente a enfermedades menores, reproducción y crecimiento.

Palabras claves : Bovino - Caprino - Resistencia a las enfermedades - Manejo del ganado - Alimentación animal - Carencia mineral - Reproductividad - Aumento de peso - Crecimiento - Efecto estacional - Sistema ganadero - Haití.

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Development of a computer simulation model for anaplasmosis with emphasis on the Caribbean*

TOUSSAINT (J.), HABTEMARIAM (T.), ORYANG (D.), WILSON (S.). Développement d'un modèle de simulation informatique pour l'anaplasmose, notamment dans les Antilles. *Revue Élev. Méd. vét. Pays trop., 1993,* **46** (1-2) : 47-48

L'anaplasmose, qui continue à être une énigme dans la région des Caraïbes, est responsable de pertes économiques élevées dans la production animale. L'épidémiologie de cette maladie est complexe et la voie efficace et rationnelle à suivre pour son contrôle demeure encore incertaine. On a donc pensé que l'élaboration d'un modèle épidémiologique utilisant des modèles de simulation sur ordinateur serait utile pour une meilleure compréhension de cette maladie. Un modèle de simulation sur ordinateur qui donne les tendances réalistes de la dynamique de la maladie a donc été développé. De plus, ce cadre fournit aux décideurs un outil d'évaluation de différentes alternatives de lutte, pour un planning rationnel et pour l'allocation de fonds. L'élaboration du modèle a été basé sur la mise en œuvre : d'une base de connaissances épidémiologiques pour l'anaplasmose ; d'un modèle conceptuel pour les sous-populations de bovins et de tiques ; d'un modèle d'analyse de systèmes pour les sous-populations de bovins et de tiques ; d'un modèle mathématique ; d'un modèle de simulation informatique ; sur l'évaluation du modèle de simulation et l'utilisation de ce dernier pour l'évaluation des alternatives de lutte contre *Anaplasma*. La base de connaissances a été développée en utilisant la "Epidemiologic Problem Oriented Approach" (EPOA) pour la collecte et la compilation de l'information dans une base condensée de connaissances épidémiologiques sur l'anaplasmose. L'information sur l'anaplasmose a été extraite de manuels sélectionnés de médecine vétérinaire, de revues contemporaines, de documents divers, et de questionnaires remplis par des vétérinaires antillais. L'information épidémiologique a été présentée en diagrammes afin de conceptualiser l'épidémiologie détaillée de la maladie. En même temps, elle montre les parties fondamentales du système anaplasmose pour mieux décrire et analyser la maladie. Des diagrammes d'analyse de systèmes ont également été utilisés pour établir une corrélation entre le niveau pathologique et un niveau particulier qui était décrit et défini par des équations différentielles classiques. Toutes les équations étaient approchées en utilisant la méthode d'intégration de Euler. Ainsi, la dynamique de la maladie a été révélée. Ces diagrammes ont fourni le cadre sur lequel le modèle a été construit. L'évaluation du modèle a montré qu'il est stable. Des tendances biologiquement solides et raisonnables ont été affichées. Ce cadre a ensuite été utilisé pour évaluer les présentations diverses de la maladie et les alternatives différentes pour la lutte. Les manifestations de la maladie observées comprenaient la présentation de populations de bovins et de tiques avec et sans maladie, la dynamique de la maladie quant elle fut introduite par des bovins et des tiques infectés. Les alternatives de lutte testées sont : les effets de niveaux différents de lutte acaricide sur la population de tiques et l'évolution de la maladie ; l'influence de la génétique sur l'incidence de la maladie ; les effets de niveaux différents d'application d'antibiotiques sur la dynamique de la maladie, quant elle fut introduite par des bovins et des tiques infectieux. Le modèle de simulation informatique doit être testé et validé systématiquement pour être sensible aux conditions du terrain.

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**Seuls les résumés de cette communication sont présentés dans ce volume.*

TOUSSAINT (J.), HABTEMARIAM (T.), ORYANG (D.), WILSON (S.). Development of a computer simulation model for anaplasmosis with emphasis on the Caribbean. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 47-48

Anaplasmosis continues to be an enigma in the Caribbean region causing high economic losses in animal production. Since the epidemiology of this vector-borne disease is quite complex, and since an effective and rationally designed approach to its control is still unclear, it was felt that developing an epidemiologic model using computer simulation models will be useful to shed more light in this area. To represent the epidemiology of anaplasmosis, an epidemiologic simulation model which portrays realistic trends of disease dynamics has been developed. Due to the high economic losses in animal production and the complexity of controlling the disease, the model serves as tool to better understand the complex epidemiology of anaplasmosis. Additionally, the model provides decision makers with a tool to be used to evaluate various anaplasmosis control alternative for rational planning and allocation of funds. The development of the model was based on developing : an epidemiologic knowledge-base for anaplasmosis ; a conceptual model for the cattle and tick subpopulations ; a systems analysis model for the cattle and tick subpopulations ; a mathematical model ; a computer simulation model ; testing the computer simulation model and using the model to evaluate *Anaplasma* control alternatives. The knowledge-base was developed using the Epidemiologic Problem Oriented Approach(EPOA) to collect and compile the information into a condensed, but systematic epidemiologic knowledge-base of anaplasmosis. The information on anaplasmosis was retrieved from : selected textbooks of veterinary medicine ; current journals ; papers and questionnaires filled by Caribbean veterinarians. The epidemiologic information was presented using flowchart diagrams to conceptualize the detailed epidemiology of the disease. At the same time, it displays the fundamental parts of the anaplasmosis system to better describe and analyze the disease. Systems analysis diagrams were also used to relate the health states to specific rates which were described and defined by differential equations based on classical mass action theory. All states equations are approximated using Euler's integration method. In this way, the dynamics of the disease was revealed. These diagrams provided the framework on which the model was built. Testing of the model shows that it is stable. Trends which were biologically sound and reasonable were displayed. The model was then used to evaluate disease patterns and various control alternatives of anaplasmosis. Disease patterns observed included : cattle and tick populations patterns with and without disease ; disease dynamics when disease was introduced via infective cattle and infectious ticks. The control alternative tested were : effects of various levels of acaricides on tick population and disease dynamics ; the influence of genetics on the incidence of the disease ; the effects of various levels of antibiotics on disease dynamics when disease was introduced via infective cattle and infectious ticks. The computer simulation model needs systematic testing and validation to observe its sensitivity to filed conditions.

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TOUSSAINT (J.), HABTEMARIAM (T.), ORYANG (D.), WILSON (S.). Desarrollo de un modelo de simulación para la anaplasmosis con énfasis en el Caribe. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 47-48

La anaplasmosis sigue siendo un enigma en la región del Caribe, causando de pérdidas económicas en la producción animal. En vista de que la epidemiología de esta enfermedad vectorial es relativamente compleja y debido a que aún no existe un enfoque efectivo y razonable para el control, se pensó que un modelo epidemiológico permitiría aclarar un poco el campo de trabajo. Para representar la epidemiología de la anaplasmosis se desarrolló un modelo de simulación epidemiológica representativo de los patrones dinámicos de la enfermedad. Dada la importancia de las pérdidas económicas en la producción animal y la complexidad del control de la enfermedad, el modelo sirve como herramienta para una mejor comprension del complejo epidemiológico de la anaplasmosis. Además, el modelo permite el establecimiento de "marcadores de decision", a ser utilizados para la evaluación de varias alternativas de control de la anaplasmosis, para lograr una planificación racional y para la distribución de fondos. El desarrollo del modelo se basó en desarrollo : de una base de conocimientos de la anaplasmosis ; de un modelo conceptual para las sub-poblaciones de ganado y de garrapatas ; de un modelo de análisis de sistemas para las sub-poblaciones de ganado y de garrapatas ; de un modelo matemático ; de un modelo de simulación informática ; evaluación del sistema de simulación informática y uso del modelo para la evaluación las alternativas de control de *Anaplasma*. La base de conocimientos se desarrolló mediante el uso de EPOA (Epidemiologic Problem Oriented Approach), tanto para la recolección de datos, como para la complilación de la información en una base densa, pero epidemiológicamente sistemática de la anaplasmosis. La información se obtuvo de : libros de texto de medicina veterinaria seleccionados, publicaciones periódicas, artículos y cuestionarios completados por los veterinarios del Caribe. Con el fin de mostrar la epidemiología detallada de la enfermedad, se utilizaron diagramas para la representación de la información epidemiológica. Se muestran las partes fundamentales del sistema de la anaplasmosis, para proveer una mejor descripción y un mejor análisis de la enfermedad. También se utilizaron diagramas de análisis de sistemas para correlacionar los niveles de salud con tasas específicas, descritas y definidas mediante ecuaciones diferenciales basadas en la teoría clásica de acción de masas. Todos los estadios de las ecuaciones son aproximativos, gracias al método de integración de Euler. De esta manera se reveló la dinámica de la enfermedad. Estos diagramas proveen el marco utilizado para la construcción del modelo. La evaluacion del modelo demostró su estabilidad. Se utilizaron parámetros factibles y razonables desde el punto de vista biológico. Seguidamente el modelo se utilizó para la evaluación de los patrones de la enfermedad y de varias alternativas de control de la anaplasmosis. Los patrones de la enfermedad observados comprenden : patrones de la población de ganado y de garrapatas, con y sin presencia de la enfermedad ; dinámica de la enfermedad cuando ésta se introdujo vía ganado infectado o garrapatas infectadas. Las alternativas de control evaluadas fueron : efecto de los diversos niveles de acaricidas sobre la población de garrapatas y sobre la dinámica de la enfermedad ; influencia de los factores genéticos sobre la incidencia de la enfermedad ; efecto de los diferentes niveles de antibióticos sobre la dinámica de la enfermedad cuando ésta fue introducida vía ganado infectado o garrapatas infectadas. El modelo de simulación informática debe ser evaluado y una validado sistematicamente, con el fin de determinar la sensibilidad bajo condiciones de campo.

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Persistence of tick-derived *Anaplasma marginale* in cultured bovine turbinate and endothelial cells

BLOUIN (E.F.), KOCAN (K.M.), MURPHY (G.L), GE (N.). Persistance d'*Anaplasma marginale* isolé de tiques dans des cellules bovines des cornets nasaux et endothéliales. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 49-56

Des monocouches de cellules bovines des cornets nasaux (cellules CN) et de cellules bovines endothéliales ont été inoculées avec des *Anaplasma marginale* dérivés de glandes salivaires de *Dermacentor andersoni*. Des passages des couches ont été faits à des intervalles de 2 ou 4 semaines et examinés aux microscopes classique et électronique, ainsi que par une sonde d'ADN spécifique pour *A. marginale*. Des inclusions intracellulaires ont été observées dans les cellules CN après 2-4 semaines. Le nombre de cellules avec des inclusions augmentait au cours de 1-2 semaines, ensuite il y avait une disparition graduelle. Un fragment du gène *msp1ß* du stade érythrocytaire d'*A. marginale*, marqué par isotope radioactif, a hybridé avec de l'ADN extrait de cultures de cellules CN jusqu'à 7 semaines après inoculation (passage 4). Des rickettsies individuelles ont été observées au microscope électronique dans des prélèvements faits à ce moment. Des veaux sensibles inoculés avec des cultures suspectes n'ont pas montré d'anaplasmose clinique, mais ont développé des titres significatifs d'anticorps détectés par ELISA. De l'ADN de cultures de cellules endothéliales 9 semaines après inoculation s'est également lié à la sonde spécifique pour *A. marginale*. Il semble qu'*Anaplasma marginale* des glandes salivaires de *D. andersoni* persiste en culture dans des cellules bovines CN et endothéliales, mais qu'il n'y a pas de développement normal ni infectiosité pour les bovins.

Mots-clés : Anaplasma marginale - Culture - Sonde à ADN.

INTRODUCTION

Anaplasma marginale is a tick-borne rickettsial organism (Rickettsiales : Anaplasmataceae) that infects erythrocytes of cattle and causes significant mortality and production losses in many parts of the world (11). The life cycle of *A. marginale* also involves developmental stages in the tick vector where a complex developmental sequence occurs in gut, gut muscle and salivary gland cells (5). One of the major constraints in anaplasmosis research has been lack of a continuous *in vitro* culture system. Most attempts to cultivate the organism have involved the erythrocytic stage of *A. marginale*. Organisms were found to be viable and retain infectivity in whole erythrocyte cultures but replication or further development did not occur after approximately 48 hours (8). When established mammalian and insect cell lines were incubated with infected erythrocytes, cells and organisms were taken up by some cell types and survived for extended periods of time ; further development was not apparent (8, 12). The tick gut stage of *A. marginale* was used to infect an embryonic tick cell line (3). This stage was reported to infect and grow within these cells but cultures were not infective for cattle, and host cells eventually destroyed the intracellular parasites through lysosomal digestion (3, 4). In this study salivary glands infected with *A. marginale* were used as inoculum for cultured cells. This stage is most likely transmitted to the vertebrate host during tick feeding. Manipulation of the feeding schedule of *D. andersoni* males resulted in large numbers of *A. marginale* colonies in salivary glands (7). We have recently shown that the salivary gland stage will infect bovine erythrocytes *in vitro*, although further development does not occur (2). This report describes results of attempts to establish infections of *A. marginale* using the salivary gland stage to inoculate bovine turbinate and endothelial cell cultures.

MATERIAL AND METHODS

Agent

The Virginia isolate of *A. marginale* (VAM) was used to infect a donor calf by transfusion of whole blood from a carrier calf.

Infection of ticks

Dermacentor andersoni males, reared at Oklahoma State University, were placed in orthopedic stockinettes attached to donor calves when parasitemia reached 3-5 %. Ticks were allowed to feed for seven days, after which they were removed and placed in a humidity chamber (90-98 % RH) at 25° C for 5 days. Ticks were then allowed to feed on a second, susceptible calf for 12 days and removed. Uninfected male *D. andersoni* were fed on a separate, susceptible calf for 12 days in similar fashion to provide uninfected control ticks. Samples from all batches of ticks were collected and salivary glands were examined by light and electron microscopy (LM and EM) for presence of *A. marginale*.

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Collection and preparation of inoculum

Immediately after removal from animals, infected and uninfected ticks were surface decontaminated under a laminar flow cabinet by washing in sequential solutions of H_20, 3 % H_20_2, 70 % ETOH, H_20, 0.5 % bleach (hypochlorite), 1 % detergent (roccal), H_20 and several washes in sterile H_20 with penicillin/streptomycin. Salivary glands were dissected from individual ticks and placed in sterile Minimum Essential Medium (MEM) on ice. Glands were transferred to a sterile glass tissue grinder and suspended in complete medium to a volume of 1 ml medium/ten pairs of glands and homogenized. Crude homogenate was transferred to centrifuge tubes and spun at 1000 x g for 10 min at 4 °C. The supernatant was removed and immediately inoculated onto cell monolayers. Samples of supernatants and pellets were fixed for EM to confirm presence and morphology of A. *marginale* .

Inoculation of cell lines

Established monolayers of bovine turbinate cells obtained from Washington State University (T. Crawford) and bovine endothelium (aorta) from ATCC (# CPAE CCL209) were passaged three days prior to inoculation into 25 cm^2 flasks and maintained in Glasgow MEM supplemented with 15 % calf serum, 5 % tryptose phosphate broth and penicillin/streptomycin (100 IU/100 µg/ml) at pH 6.8-7.0. The volume of medium in each flask was reduced to 2 ml to which 1 ml supernatant from infected or uninfected salivary gland centrifuged homogenate was added. Flasks were placed on a rotator in a 37°C incubator and shaken slowly (80 rpm) for 30 min to disseminate the inoculum. After 2 h medium was removed from cultures and monolayers were washed and then replenished to 5 ml with fresh medium.

Maintenance of cultures

Culture medium was replenished in cultures every 3 days by replacing 2/3 of the old medium. Cell layers were passaged at 2 or 4 week intervals by trypsinizing and splitting monolayers 1:3.

Light microscopy

Culture flasks were examined daily for the presence of inclusion bodies. Smears were made from samples taken from monolayers with a sterile needle and stained with a modified Wright stain prior to being passaged.

Electron microscopy

Portions of monolayers were removed from flasks with a cell scraper and fixed in cold 2 % glutaraldehyde in 0.1M sodium cacodylate buffer and processed according to procedures of KOCAN et al. (6). Ultrathin (silver-reflective) sections were cut on a Sorval MT 5000 ultramicrotome with a Diatome diamond knife and stained with uranyl acetate and lead citrate (13). Sections were observed on a JEOL 100 CX electron microscope.

DNA hybridization

The 965 bp *Hind* III/*Xho*I fragment (1) from within the *msp1ß* gene of the erythrocytic stage of A. *marginale* (Virginia isolate) was amplified by the polymerase chain reaction and cloned into the vector pBluescriptII SK (Stratagene). For nucleic acid hybridizations, the 965 bp fragment was purified from low melting temperature agarose and radiolabeled with ^{32}P-dCTP. Culture samples (approximately 1000 cells) were centrifuged (2000 x g, 10 min), and cell pellets were washed three times with sterile phosphate buffered saline. Cells were lysed by incubating with 0.35 ml digestion buffer (10 mM Tris-HCL (pH 8), 1 mM EDTA, 0.5 % SDS, 0.2 mg/ml proteinase K) at 56 °C for 3 h. Protein and cell debris were removed by phenol/chloroform extraction. DNA was precipitated in 100 µl TE (10 mM Tris-HCL, 1 mM EDTA, pH8).

Serial dilutions of each sample were prepared, so that three dilutions were analyzed by DNA hybridization (1:1, 1:10, 1:100). DNA samples were treated and bound to nitrocellulose using a slot blot manifold (Schleicher & Schuell Inc.), according to manufacturer's instructions. Blots were hybridized with the radiolabeled *msp1ß* fragment and washed under stringent conditions (at 72°C), as described by MURPHY and DALLAS (9), then exposed to X-Ray film overnight.

Animal inoculation

Cultures of turbinate cells from which samples hybridized to the *A. marginale* DNA probe or that were found to harbor rickettsial organisms were used to inoculate susceptible calves to determine infectivity of culture material. Cells in 2 ml of MEM medium from 2 suspect flasks were inoculated IV into calves. Five animals were inoculated with cultures collected at different times post inoculation (PI) (table I). Calves were monitored for presence of intraerythrocytic parasites in blood smears and for clinical signs of anaplasmosis. Serum samples were collected before and after inoculation and evaluated by the anaplasmosis complement fixation test (Oklahoma Animal Disease Diagnostic Lab.). An ELISA test developed previously to detect antibody to erythrocyte, tick gut and tick salivary gland stages of *A.marginale* was used to screen serum samples collected before and after inoculation (10). Fifty male *D. andersoni* were fed on one of the inoculated calves (PA 145) at 10 weeks PI, to attempt tick infection, and subsequently refed on a susceptible calf (PA 163) to test for the transmission

TABLE I Inoculation of calves with bovine turbinate cell cultures infected with the salivary gland stage of **anaplasma marginale.**

Animal	Inoculum Day/Pass	Clinical Reaction	Organisms/Days Post Inoculation	% Decrease PCV	CF*	Susceptible To Challenge
PA 83	Day 14	Neg	Pos/Day 13	27	Neg	yes
PA 106	Day 28	Neg	Pos/Day 12	18	Neg	yes
PA 78	Day 54/P3	Neg	Pos/Day 7	14	Neg	yes
PA 145	Day 57/P6	Neg	Pos/Day 20	20	Neg	yes
PA 76	Day 66/P4	Neg	Pos/Day 4	27	Neg	yes

* *Complement Fixation Test.*

of *A. marginale.* All animals were challenged-exposed with either infected *A. marginale* blood or ticks to prove their susceptibility.

RESULTS

Infection of ticks

Salivary glands from all groups of infected ticks used were found to be infected with colonies of *A. marginale* by LM and EM. Calves on which these ticks fed developed clinical anaplasmosis. Colonies were not observed in uninfected control ticks nor did calves they fed on develop anaplasmosis. Symbiotic rickettsiae were not observed in either infected or uninfected tick salivary glands.

Inoculum

Supernatant from infected glands collected after centrifugation contained many individual *A. marginale* as well as mitochondria, secretory granules and other tick cell components (photo 1a, b). A few intact *A. marginale* colonies, as well as smaller groups of rickettsiae were also seen in each inoculum sample. *Anaplasma marginale* in the inoculum appeared to be morphologically intact and binary fission was apparent. Colonies of *A. marginale* were seen in pellets recovered after centrifugation, along with tick tissue and organelles. *Anaplasma* were not seen in inocula from any uninfected gland preparations.

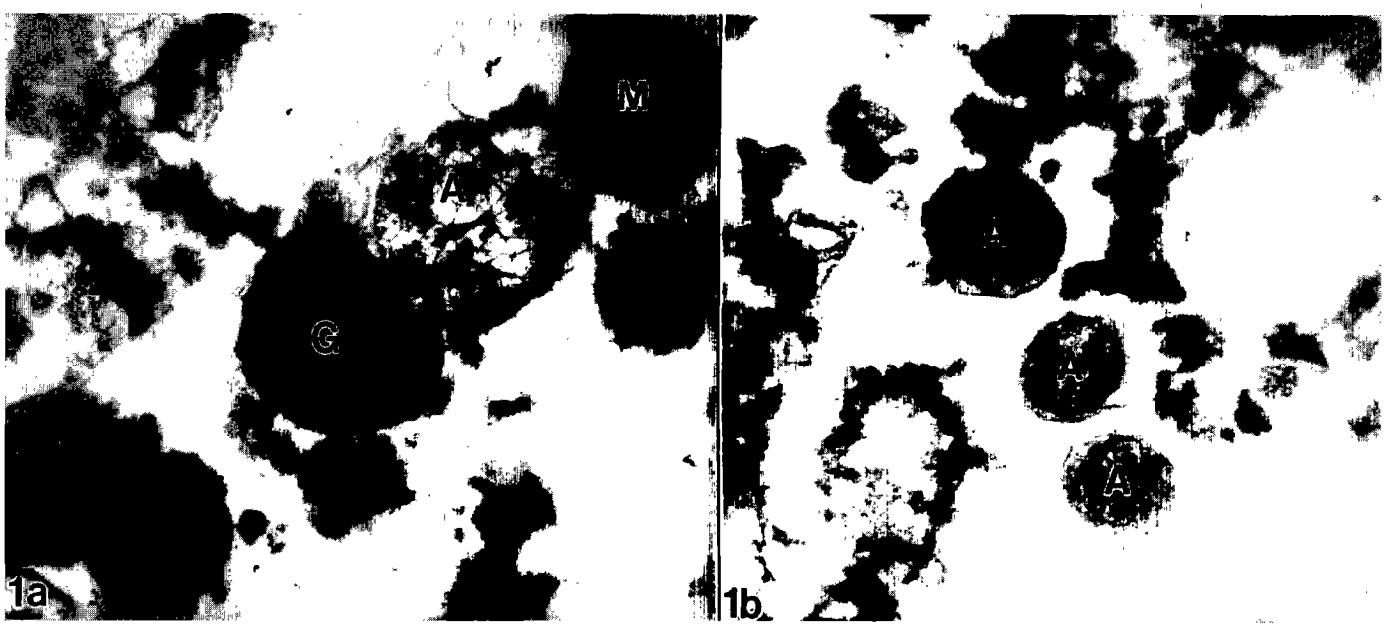

Photo 1 : Electron micrographs of an inoculum prepared from salivary glands of Dermacentor andersoni *infected with* Anaplasma marginale. *a) An individual* Anaplasma *organism (A) in a host cell mitochondria (M) and a granule (G). (x 36,720). b) Three individual* Anaplasma *organisms (A) free from the colony. (x 36,720)*

Light microscopy and electron microscopy

In samples collected at 60 min PI rickettsiae were seen attached to host cell membranes (photo 2a). Colonies were also observed in association with host cells with individual rickettsiae adhered to the host cell membrane (photo 2b). Changes in rickettsial morphology were observed after 18 hours PI. Rickettsiae became polymorphic and changed from reticulated forms to denser forms. At two weeks PI round intracytoplasmic inclusions were observed in turbinate cells (photo 3a). These inclusions increased in number for 1-2 weeks, contained several subunits and were often observed in close association with the nucleus (photo 3a, b). With EM, inclusions were found to contain a variable number of subunits with dense

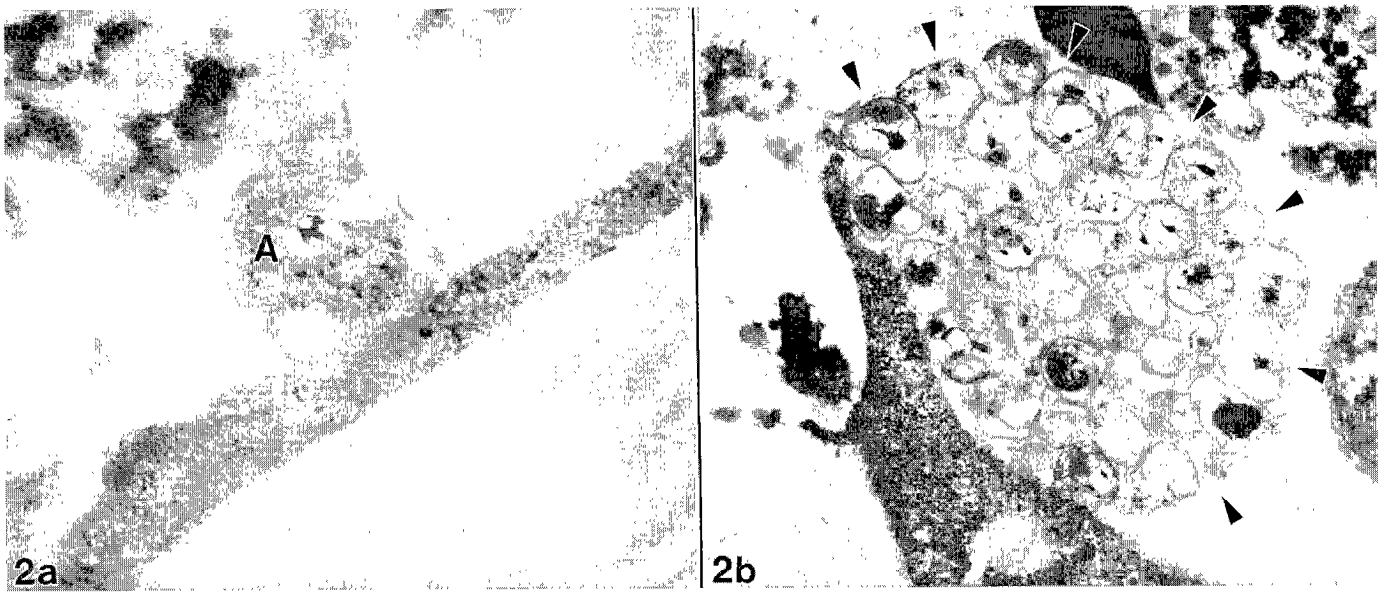

Photo 2 : Electron micrographs of bovine turbinate cell cultures at 60 min PI with tick salivary gland material infected with A. marginale. *a) An individual* Anaplasma *organism (A) in contact with a host cell membrane (x 60,780). b) A colony (arrows) of* A. marginale *in association with a bovine turbinate cell membrane (x 17, 720).*

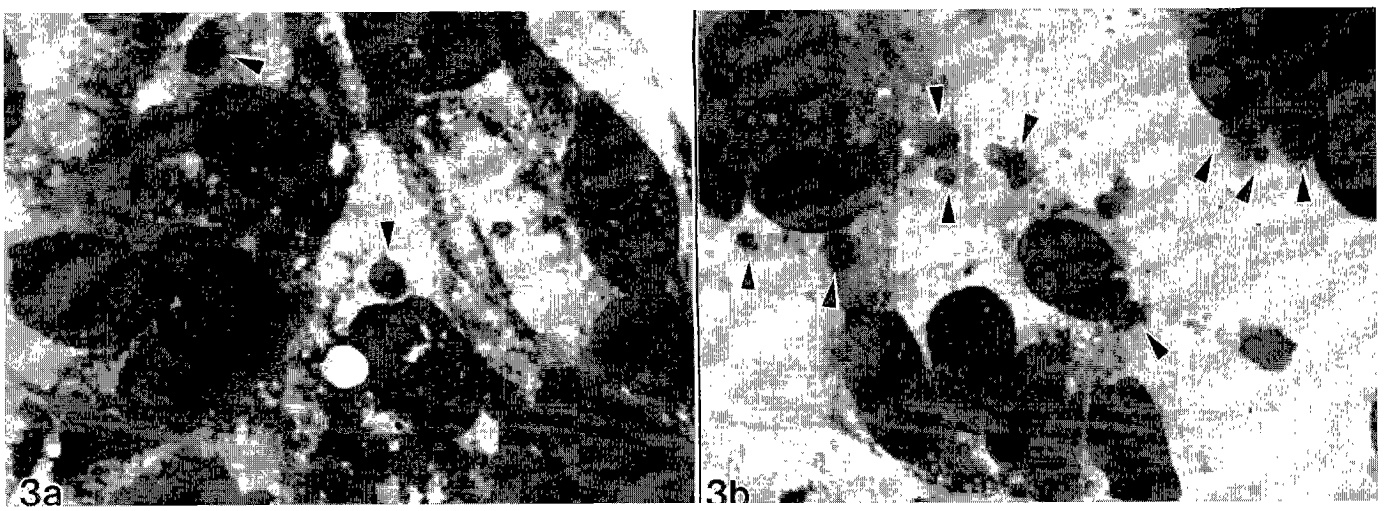

Photo 3 : Bovine turbinate cell culture inoculated with the salivary gland stage of A. marginale. *a) Intracellular inclusions (arrows) at 2 weeks PI. Note inclusions appear to contain multiple subunits. b) Intracellular inclusions (arrows) at 3 weeks PI (passage 1).*

internal material (photo 4). In some cultures these inclusions were not seen until 6 weeks PI. After the initial increase, the number of inclusions decreased as cultures were passaged and eventually could be found only occasionally. In older cultures inclusions and subunits appeared to be devoid of internal structure but rickettsial organisms were occcasionally seen within the inclusion (photo 5). Phagocytic vacuoles were abundant in endothelial cultures and obscured any other material in the host cell cytoplasm during the first 1-2 weeks. After approximately 10 days inclusions were found but did not increase in numbers following passage as was observed in turbinate cells. Similar inclusions were not observed in control cultures. Colony formation or rickettsial replication were not observed during culture passage. Individual rickettsiae were identified in some turbinate cultures with EM ; these organisms had a reticulated core with a denser peripheral area and were variable in size (photo 6). The plasma membrane and cell wall were separated and a single large vacuole was often present within rickettsiae (photo 6). The number of organisms identified in each sample was small but persisted after passage and organisms were identified after 6 weeks PI. Organisms were not seen in some samples that hybridized to the *A. marginale*-specific DNA probe, however amorphous inclusions with reticulated areas resembling rickettsial DNA were seen in several of these samples (photo 7).

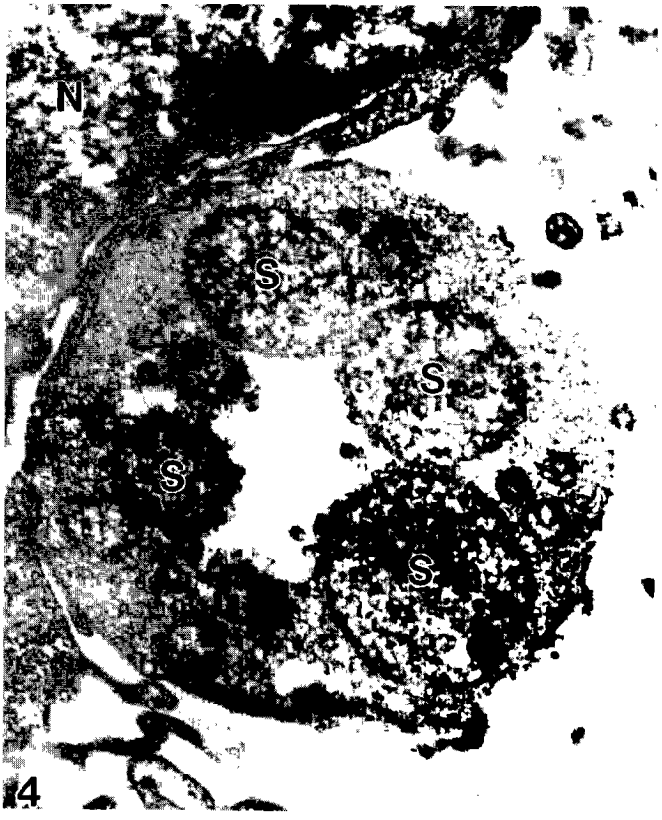

Photo 4 : Electron micrograph of an inclusion body in association with a bovine turbinate cell nucleus (N) at 2 weeks PI. Note dense subunits (S). (x 18,465)

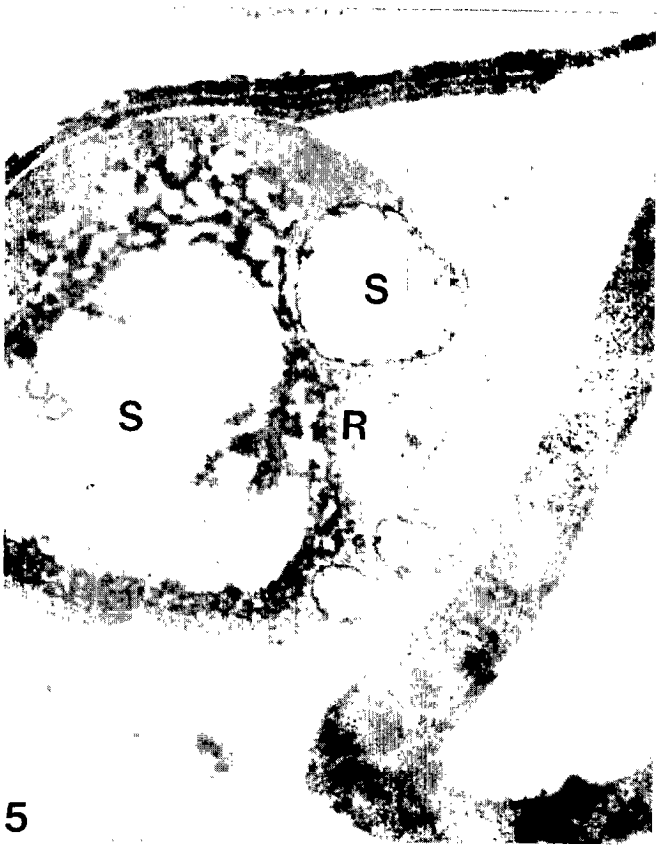

Photo 5 : Electron micrographs of an inclusion body from turbinate cell culture at 41 days PI. Subunits (S) appear to be devoid of substance but 1 distinct rickettsial organism (R) is present. (x 18,900).

DNA hybridization

Culture samples with inclusion bodies, as well as those with distinct rickettsial organisms contained DNA that hybridized to the *A. marginale*-specific DNA probe. Probe-positive DNA was present in endothelial cultures after four passages at 6 weeks PI (photo 8) and beyond 9 weeks PI in unpassaged cultures. In turbinate cultures probe-positive DNA was present after four passages at seven weeks PI (photo 8). Approximately 10 % of turbinate culture flasks sampled and 15 % of endothelial cultures hybridized to the *A. marginale* DNA probe. Control samples of bovine erythrocytes and tick salivary glands infected with *A. marginale* were DNA probe positive while uninfected erythrocytes and tick salivary glands, and turbinate and endothelial cell cultures inoculated with uninfected material were negative.

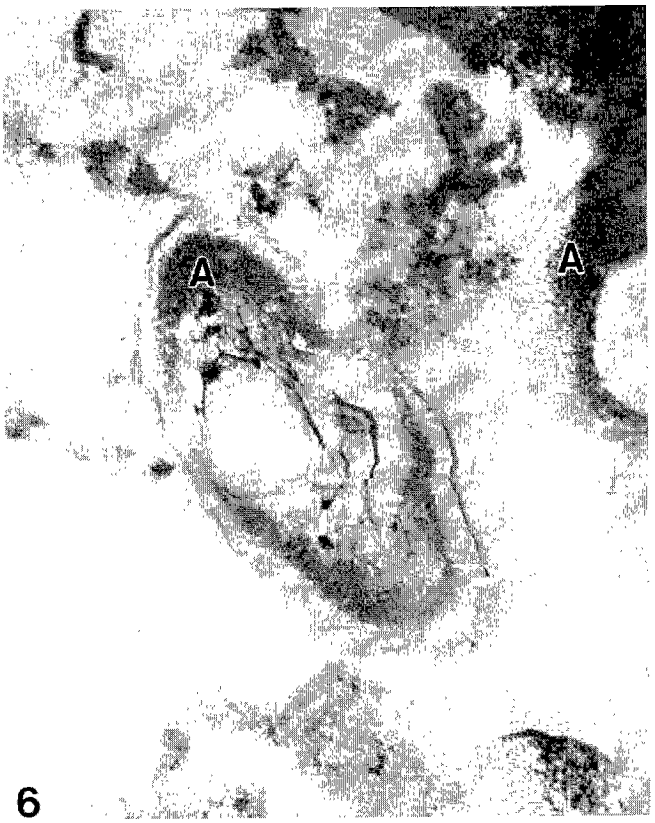

Photo 6 : Electron micrograph of an individual Anaplasma *organism (A) from bovine turbinate cell culture at day 43 PI. (x 64,800)*

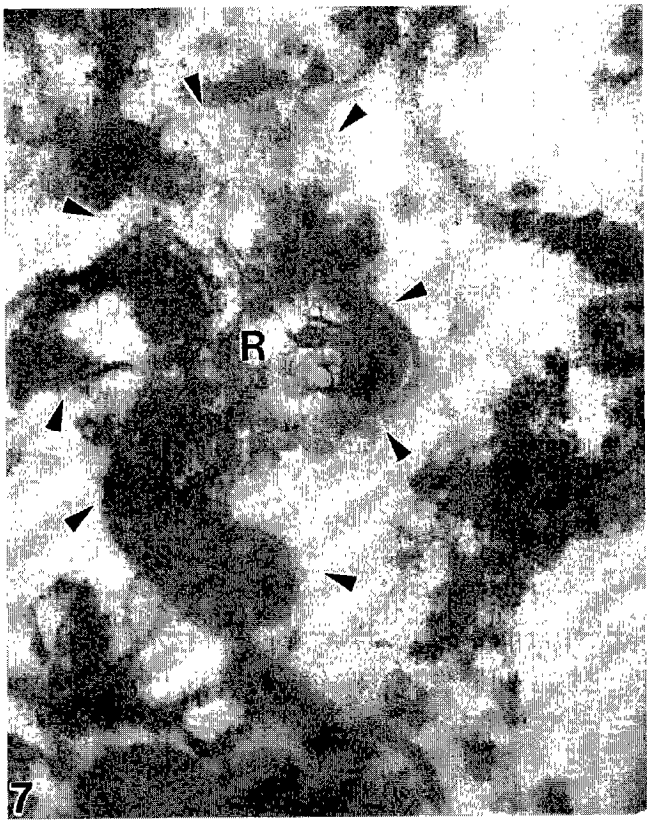

Photo 7 : Electron micrograph of an amorphous inclusion (arrows) from bovine turbinate cell culture at day 43 PI. Note the central area of reticulation (R). (x 64,800).

Calf inoculation

Calves that were inoculated with bovine turbinate culture material did not develop clinical anaplasmosis, their sera were negative by the complement fixation test and they proved to be fully susceptible to challenge-exposure (table I). Small numbers of intraerythrocytic organisms were seen in each calf as early as 7 days PI and calves had a decreased percentage in packed cell volume (PCV) of 4-27 % after inoculation (table I). When sera from these animals were evaluated with ELISA, 3 of 5 animals had significant antibody titers to different stages of *A. marginale* (table II). *Anaplasma marginale* was not seen in ticks which had fed on PA 145 for attempted tick infection and they did not cause clinical anaplasmosis in calf (PA 163) used for transmission feeding of these ticks although small numbers of intraerythrocytic inclusions were seen for 4 weeks. PA 163 proved to be susceptible upon challenge ; however peak parasitemia was low (17 %) and percent reduction in PCV was only 17 %.

DISCUSSION

Monitoring salivary glands of *D. andersoni* with LM and DNA probe for presence of *A. marginale* colonies after feeding and during isolation assured that all cultures received organisms in inoculum. Isolation of individual rickettsiae from colonies and other host cell material initially involved longer preparation time, differential and density gradient centrifugations, and sonication. Although more of the salivary gland material was extracted, the number of *A. marginale* recovered after each step decreased. Host cell fragments and organelles were present in semi-purified preparations. With extended preparation time, rickettsiae appeared to change morphologically ; the central chromatin became more diffuse and disappeared in some cases. Prolonged manipulation of released parasites may have had a detrimental effect on the rickettsiae by reducing their infectivity. Mechanical disruption salivary glands was sufficient to release many individual rickettsiae, leaving some larger groups, a few intact colonies, and host cell material. Rickettsiae recovered using minimal prepa-

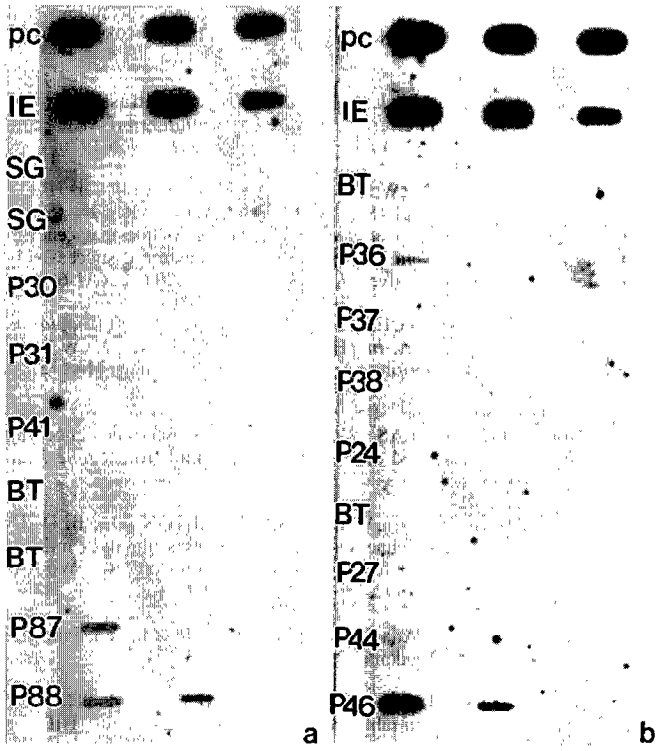

Photo 8 : Hybridization of a ^{32}P-labeled A. marginale msp1ß *gene to cell culture samples. pc = plasmid control, IE =* A. marginale*-infected rbcs, SG = uninfected salivary gland control, BT = bovine turbinate cell inoculated with uninfected salivary gland inoculum. a) Two positive culture samples : P87 = bovine turbinate cells at day 41 PI (passage 4), P88 = endothelial cells at day 41 PI (passage 4). b) Two positive culture samples : P36 = bovine turbinate cells at day 50 PI (passage 4), P46 = bovine turbinate cells at day 18 PI.*

ration were found to be morphologically intact and, within colonies, evidence of replication by binary fission was apparent.

It is likely that only organisms which had entered host cells persisted in culture since the inoculum was removed after 2 hours and monolayers were washed and later trypsinized. The appearance and subsequent proliferation of intracellular inclusion bodies in turbinate cells suggested a developmental stage of the organism. The extended period before appearance (2-6 weeks PI) may be necessary for transition and accommodation of the parasite to the host cell. Although inclusions and their subunits were not typical of *Anaplasma,* samples containing these forms hybridized with the *A. marginale*-specific DNA probe. Some of these inclusions did contain distinct rickettsiae which may have originated from inclusions. Because distinct transitional stages were not identified it is also possible that rickettsia from the inoculum survived in host cells without further development. Parasites may also change form in culture. Amorphous inclusions, represented by photo 7, contained areas resembling reticulated chromatin and were the only suspicious bodies found in some culture samples that hybridized with the *A. marginale*-specific DNA probe.

Inoculation of susceptible calves with infected cultures did not result in clinical anaplasmosis or serologic conversion as determined by the anaplasmosis CF test but intraerythrocytic *A. marginale* were observed in all 5 animals. However, 3 calves had significant levels of specific antibodies to different stages of the parasite, suggesting that they were exposed to *A. marginale* antigens. Since we know that infected salivary gland material will produce cli-

TABLE II Serum ELISA values (optical densities) for 3 calves inoculated with culture material using 3 stages of* A. marginale *as antigen.

Animal	Dilution	Preinoculation	7 DPI*	14 DPI	28 DPI	60 DPI
Erythrocyte stage						
PA 145	1 : 1280	1.07	1.18	1.18	1.9	1.03
PA 83	1 : 1280	1.18	1.01	–	1.03	1.03
PA 76	1 : 1280	0.99	1.13	1.23	1.26	1.33
Salivary gland stage						
PA 145	1 : 2560	0.6	0.56	0.49	1.5	0.6
PA 83	1 : 1280	0.85	0.87	–	1.1	0.94
PA 76	1 : 320	0.55	0.28	0.57	0.97	0.23
Gut stage						
PA 145	1 : 5120	0.4	0.44	0.37	1.55	0.46
PA 83	1 : 1280	0.73	0.84	–	1.06	0.82
PA 76	1 : 320	0.73	0.86	0.70	0.92	0.73

* *Days post inoculation.*

nical anaplasmosis in calves, numbers of infective organisms in the cultures may have been too low for initiation of clinical disease.

The results of this study suggest that the salivary gland stage of *A. marginale* may survive and persist for extended periods in bovine turbinate and endothelial cells. Atypical forms occur which do not produce disease or result in protective immunity when inoculated into susceptible calves.

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BLOUIN (E.F.), KOCAN (K.M.), MURPHY (G.L), GE (N.). Persistence of tick-derived *Anaplasma marginale* in cultured bovine turbinate and endothelial cells. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 49-56

***Anaplasma marginale* from salivary glands of *Dermacentor andersoni* was used to inoculate monolayers of bovine turbinate and endothelial cells. Monolayers were passaged at 2 or 4 week intervals and monitored with light and electron microscopy and with an *A. marginale*-specific DNA probe. Intracellular inclusions were observed in turbinate cells after 2-4 weeks. The number of inclusion-bearing cells increased over 1-2 weeks and gradually disappeared. A radiolabeled fragment from within the *msp1ß* gene of the erythrocytic stage of *A. marginale* hybridized to DNA extracted from bovine turbinate cell cultures as late as 7 weeks post inoculation (passage 4). Individual rickettsiae were observed with electron microscopy in samples taken at this time. Susceptible calves inoculated with suspect cultures did not develop clinical anaplasmosis but did develop significant antibody titers as detected with ELISA. DNA from endothelial cell cultures at 9 weeks post inoculation also bound the *Anaplasma*-specific DNA probe. Anaplasma marginale from salivary glands of *D. andersoni* appears to persist in cultured bovine turbinate and endothelial cells but typical development and infectivity for bovines do not occur.**

Key words : *Anaplasma marginale* - Culture - DNA probe.

BLOUIN (E.F.), KOCAN (K.M.), MURPHY (G.L.), GE (N). Persistencia de *Anaplasma marginale* en cultivos de células endoteliales y turbinales bovinas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 49-56

Se utilizó *Anaplasma marginale*, proveniente de glándulas salivales de *Dermacentor andersoni*, para la inoculación de monocapas de células endoteliales y de turbinas (cornetes) de bovinos. Se hicieron pasajes a 2 y 4 semanas de intervalo en las monocapas celulares, así como pruebas con microscopio de luz y electrónico y con ADN específico para *A. marginale*. Dos a cuatro semanas después se observaron inclusiones intracelulares en las células de turbinas. El número de células portadoras de inclusiones aumentó durante 1 a 2 semanas, para luego desaparecer en forma gradual. Siete semanas post-inoculación, se obtuvo un híbrido entre un fragmento marcado del gen *msp1ß* del estado eritrocítico de *A. marginale* y ADN extraído de los cultivos de células turbinales de bovino (cuarto pasaje). Gracias al microscopio electrónico, se observaron formas individuales de rickettsias en las muestras extraídas. La inoculación de terneros susceptibles no provocó el cuadro clínico de anaplasmosis, pero indujo un título significativo de anticuerpos detectables mediante el ELISA. De la misma manera, nueve semanas post-inoculación, el ADN proveniente de cultivos de células endoteliales, se unió al segmento de ADN específico para *Anaplasma*. El *Anaplasma marginale*, extraído de glándulas salivales de *Dermacentor andersoni* parece persistir en cultivos de células endoteliales y de cornetes bovinos, pero a pesar de esto no se produce el tipico estado de desarrollo e infectividad en los bovinos.

Palabras claves : *Anaplasma marginale* - Cultivo - Sonda de ADN.

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Ovine trypanosomosis : a seroepidemiological survey in coastal Guyana

VOKATY (S.), McPHERSON (V.O.M.), CAMUS (E.), APPLEWHAITE (L). La trypanosomose ovine : une prospection séro-épidémiologique dans la zone côtière du Guyana. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 57-59

Le but de cette étude était de déterminer les taux de séroprévalence de *Trypanosoma vivax* et de *Trypanosoma evansi* chez des moutons de la zone côtière du Guyana. Des prélèvements de sang ont été faits sur 193 moutons, pris au hasard, dans 22 fermes de la Région 5, Mahaica/Berbice, une région côtière du Guyana. L'âge, la race, le sexe et la ferme d'origine ont été enregistrés pour tous les moutons prélevés. Cent soixante-seize prélèvements de sérum ont été examinés par le test d'immunofluorescence indirecte pour *T.vivax* et *T.evansi*. La fluorescence a été notée comme 0 (négative), 1+ (très faible), 2+ (faible), 3+ (forte) ou 4+ (très forte), sur des sérums dilués à 1:160. Les échantillons ont été considérés comme positifs dès qu'une fluorescence était visible. Les résultats des tests ont été reçus pour 161 prélèvements. Cent trois sérums (64 %) étaient positifs ; 38 (23,6 %) d'entre eux étaient positifs pour *T.evansi* seulement, 11 (6,8 %) pour *T.vivax* seulement, et 54 (33,5 %) pour les deux. Étant donné qu'il existe des réactions croisées entre *T.vivax* et *T.evansi*, il était difficile de déterminer l'espèce responsable pour les réactions positives pour les deux espèces. Le taux global de séroprévalence de 64 % suggère que la trypanosomose est endémique chez les ovins de la côte guyanaise. Ces résultats constituent la première preuve sérologique de *T.evansi* au Guyana. Tandis que *T.vivax* est considéré comme pathogène pour le mouton, l'importance clinique de *T.evansi* reste inconnue. Le vecteur de ces deux espèces de trypanosomes pour les moutons de la côte septentrionale de l'Amérique du Sud n'est pas connu non plus.

Mots clés : Ovin - Trypanosomose - *Trypanosoma evansi* -*Trypanosoma vivax* - Epidémiologie - Sérum - Immunofluorescence indirecte - Prévalence - Guyana.

INTRODUCTION

The trypanosomes, *Trypanosoma vivax* and *Trypanosoma evansi* , are vector-transmitted hemoparasites commonly found in livestock in Africa and Latin America. *Trypanosoma vivax* is found in cattle, sheep, goats and wild ruminants in Africa, where it is spread by the tsetse fly, *Glossina* sp. It causes Nagana in African cattle and sheep, a disease complex characterized by fever, anaemia, reduced fertility, weight loss and mortality (3, 12). In the New World, *T. vivax* infection has been recorded in cattle, buffalo, sheep and goats (15). The tsetse fly is not found in the Americas. Infection is probably mechanically transmitted by biting flies. Three species of Tabanids have been proven to be experimental vectors of *T. vivax* infection of cattle in South America, *Cryptotylus unicolor* (8), *Tabanus importunus* (14) and *Tabanus nebulosus* (13). However, the experimental transmission of trypanosomes by biting insects does not necessarily imply that they play a significant role in the field (9). Suggested reservoirs of *T. vivax* in the New World include cattle and deer (15).

Trypanosoma evansi is found in the Middle East, Asia, the Far East, Central and South America and Africa. It has clinical significance in horses, donkeys, camels, buffaloes, cattle and dogs, causing a disease called surra (12). This disease is characterized by intermittent fever, anaemia, dependent oedema, lethargy, loss of condition, nervous signs and eventually death (11). Natural infection has been found in several species of wild animals including the capybara (*Hydrochoerus capybara*), a large rodent found in South America, which has been suggested to be the reservoir (16). Cattle and buffalo in endemic areas can be subclinically infected and may act as reservoirs for other animals (15). In Africa and Asia, the incidence of surra is associated with wet seasonal conditions which increase the population of biting flies, resulting in "surra seasons" (15). The vector in Central and South America has been postulated to be biting flies (4) or the vampire bat, *Desmodus rotundus* (10).

In March 1992, a baseline survey of ovine health on small farms was conducted in Region 5, Mahaica/Berbice, a coastal area of Guyana. The objectives of this study were to evaluate the presence, significance and frequency of selected diseases in target sheep flocks in order to develop appropriate, effective and economical preventive medicine recommendations. As part of this survey, serological testing was done for *Trypanosoma evansi* and *Trypanosoma vivax*.

MATERIALS AND METHODS

In March 1992, demographic data and blood samples were collected from a systematic random sample of sheep on twenty-two farms. Sheep were categorized as ewe, nursing lamb, weaned lamb or ram. Farm of origin,

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4. Veterinary Division, Ministry of Agriculture, Regent and NewGarden Sts., Georgetown, Guyana.

sex, approximate age, breed and body condition scores were recorded. Blood samples were collected by jugular venipuncture from 163 sheep. Blood samples were centrifuged, serum was pipetted and frozen until laboratory submission.

Serum samples were subjected to Indirect Fluorescent Antibody (IFA) testing for *Trypanosoma vivax* and *Trypanosoma evansi*, using the following technique. Antigen slides were made using *T. vivax* from an experimentally infected goat and *T. evansi* from infected mice. The smears were air dried at room temperature, fixed in acetone and stored at -20°C. Smears were thawed at room temperature for 15 min, then divided into 3 rows of 7 wells with permanent marker. The test sera, diluted to 1:160 concentration, were incubated for 30 min in humid chambers at 37°C, washed for 10 min in a PBS bath, then incubated with conjugated goat anti-ovine IgG L+H and Evans blue. Slides were then covered with Indirect Fluorescent buffer mountant, air-dried and examined under a 10x eyepiece with 50x objective on a fluorescent microscope. Fluorescence was graded as 0 (negative), 1+ (very weak), 2+ (weak), 3+ (strong) or 4+ (very strong). Samples were considered to be seropositive if any fluorescence was observed (>0).

RESULTS

Age at time of sampling varied between 3 months and 7 years of age, with a mean of 2.2 years. One hundred and eleven (69 %) were female and fifty (31 %) were male. Most (90 %) sheep were Creole or mixed breed, with the rest considered to be mainly of Barbados Blackbelly type. Mean recorded body condition score was 3; however, these data were considered unreliable as the scoring method was not sufficiently standardized between scorers.

Trypanosoma serology results were received for one hundred and sixty-one (161) samples. One hundred and three (64 %) sera were sero-positive for *Trypanosoma* sp. on Indirect Fluorescent Antibody test. Of these, 38 (23.6 %) sera were positive to *T. evansi* only, eleven (6.8 %) were positive to *T. vivax* only and 54 (33.5 %) were positive for both. Of 43 samples from lambs under 1 year of age, 29 (67 %) were positive to *Trypanosoma* sp. The youngest seropositive lamb was 3 months old.

DISCUSSION

The overall seroprevalence rate of 64 % for *Trypanosoma* sp. suggests that trypanosomosis is endemic in sheep in coastal Guyana. As cross reactions occur between *T. vivax* and *T. evansi*, it was difficult to determine the true species of exposure for the sera which tested positive to both species. This seroprevalence result corroborates the finding of APPLEWHAITE, who found a seroprevalence rate of 63.4 % of *T. vivax* in sheep in Guyana using an ELISA (Enzyme Linked Immuno Sorbent Assay) procedure. The same study found trypanosome infection in 4.6 % of sampled sheep, based on examination of stained thick blood films (2). In a survey of cattle in Guyana in 1975, CRAIG found 5 samples out of 1019 (0.6 %) to be positive for *T. vivax*, using examination of stained thick blood films. All infected cattle were from coastal regions (6).

The pathogenicity of New World *T. vivax* is variable but tends to be lower than of African strains (15). Studies in cattle, sheep and goats have demonstrated that *T. vivax* infections may be acute, subacute or chronic (1). Trypanosome susceptibility varies between ruminant species, between breeds and between individuals within a breed (6). Asymptomatic infections and mixed infections with *Babesia* and *Anaplasma* are common. In symptomatic domestic ruminants, clinical signs include intermittent fever, anaemia and loss of condition (15). Bovine trypanosomosis has been associated with clinical disease, abortion and high mortality in Colombia (17) and Venezuela (5). Recent evidence in French Guiana has associated ovine *T. vivax* infection with abortion and mortality (7). In sheep in Africa, hair loss from the back, tail and scrotum and peripheral lymphadenopathy have also been associated with *T. vivax* infection (6). Control measures for *T. vivax* in Africa include the use of insecticides, trypanocides and tsetse fly trapping. Research is currently underway in Africa in the areas of vaccine development and breeding trypanosome resistant cattle and sheep (12).

This was the first serological evidence of *T. evansi* in sheep in Guyana. Strains of *T. evansi* from different geographic areas vary greatly in virulence and economic importance for domestic animals (15). The clinical significance and economic importance of *T. evansi* infection in sheep in South America are not clearly understood.

CONCLUSION

Further studies are necessary to evaluate the clinical significance and economic impact of *T. vivax* and *T. evansi* infection in sheep in Guyana. If these studies determine trypanosomosis to be an important constraint to productivity, research to identify the vectors and reservoirs in Guyana would be justified.

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VOKATY (S.), McPHERSON (V.O.M.), CAMUS (E.), APPLEWHAITE (L). Ovine trypanosomosis : a seroepidemiological survey in coastal Guyana. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 57-59

The objective of this study was to determine the seroprevalence rates of *Trypanosoma vivax* and *Trypanosoma evansi* in sheep in coastal Guyana. Blood samples were taken from a systematic random sample of one hundred and ninety-three (193) sheep on twenty-two (22) farms in Region 5, Mahaica/Berbice, a coastal area of Guyana. Age, breed, sex, and farm of origin were recorded for all sampled sheep. One hundred and seventy-six (176) serum samples were submitted for Indirect Fluorescent Antibody (IFA) testing for *T. vivax* and *T. evansi*. Fluorescence was graded as 0 (negative), 1+(very weak), 2+(weak), 3+(strong) or 4+(very strong), measured at 1:160 dilution of serum. Samples were considered to be sero-positive if any fluorescence was observed. Indirect Fluorescent Antibody results were received for one hundred and sixty-one (161) samples. One hundred and three (64 %) sera were sero-positive for *Trypanosoma* sp. Of these, 38 (23.6 %) sera were positive to *T. evansi* only, 11 (6.8 %) were positive to *T. vivax* only and 54 (33.5 %) were positive for both. As cross reactions occur between *T. vivax* and *T. evansi*, it was difficult to determine the true species of exposure for the sera which tested positive to both species. The overall sero-prevalence rate of 64 % suggests that trypanosomosis is endemic in sheep in coastal Guyana. This was the first serological evidence of *T. evansi* in Guyana. Although *T. vivax* is believed to be pathogenic in sheep, the clinical significance of *T. evansi* remains unknown. The vector of both species of trypanosomes in sheep on the north coast of South America also is not known.

Key words : Sheep - Trypanosomosis - *Trypanosoma evansi* - *Trypanosoma vivax* - Epidemiology - Sera - Indirect immunofluorescence - Prevalence - Guyana.

VOKATY (S.), McPHERSON (V.O.M.), CAMUS (E.), APPLEWHAITE (L). Tripanosomosis ovina : estudio sero-epidemiológico en la zona costera de la Guyana. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 57-59

El objetivo del presente estudio fue la determinación de las tasas de seroprevalencia de *Trypanosoma vivax* y *Trypanosoma evansi* en ovejas, en la zona costera de la Guyana. Se tomaron muestras de sangre en un grupo de ciento noventa y tres (193) ovejas, escogidas al azar en veintidós (22) fincas de la región 5, Mahaica/Berbice, zona costera de la Guyana. Se recolectaron datos concernientes a la edad, raza, sexo y establecimiento de origen de las ovejas incluídas en el estudio. Ciento setenta y seis (176) muestras de suero fueron sometidas al test de Inmunofluorescencia Indirecta de Anticuerpos (IFA) para *T. vivax* y *T. evansi*. La fluorescencia se graduó en 0 (negativa), 1+ (muy leve), 2+ (leve), 3+ (fuerte) o 4+ (muy fuerte) y fue medida en diluciones de suero de 1:160. Las muestras fueron consideradas seropositivas cuando no se observó ninguna inmunofluorescencia. Los resultados de la Inmunofluorescencia Directa de Anticuerpos fueron obtenidos para ciento sesenta y un (161) muestras. Ciento tres (64%) sueros fueron positivos para *Trypanosoma* sp. De estos, 38 (23,6 %) se mostraron positivos para *T. evansi*, once (6,8 %) para *T. vivax* y solo 54 (33,5 %) para ambos. En vista de la existencia de reacciones cruzadas entre *T. vivax* y *T. evansi*, la determinación de las especies de exposición fue difícil en aquellos sueros con resultados positivos para ambas especies. La seroprevalencia general de 64 % sugiere que la tripanosomosis en ovejas es endémica en esta zona costera de Guyana. A pesar de que se presume que *T. vivax* es patógeno en la oveja, se desconoce aún la importancia clínica de *T. evansi*. De la misma manera, se ignora cual es el vector de ambas especies de tripanosomas en ovejas, en la costa norte de Sur América.

Palabras claves : Ovino - Tripanosomosis - *Trypanosoma evansi* - *Trypanosoma vivax* - Epidemiología - Suero - Inmunofluorescencia indirecta - Prevalencia - Guyana.

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Serological evidence for the presence of *Brucella* antibodies in sheep and goats on Saint Croix, U.S. Virgin Islands

AHL (A.S.), BARTLETT (P.C.), FRERICHS (W.M.). Preuve sérologique de la présence d'anticorps contre *Brucella* chez les petits ruminants à Sainte-Croix, Iles Vierges américaines. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 61-63

Une prospection sérologique pour des anticorps contre *Brucella* chez des moutons et des chèvres a été exécutée à Sainte-Croix, Iles Vierges américaines. La séroprévalence (aux titres suspects ou positifs) d'anticorps contre *B.melitensis* était de 11,3 p. 100 pour les moutons et de 2,5 p.100 pour les chèvres. Ceci est le premier rapport, à notre connaissance, d'anticorps contre *B.melitensis* chez les petits ruminants dans les îles Caraïbes.

Mots clés : Ovin - Caprin - Technique immunologique - *Brucella melitensis* - Anticorps - Sainte-Croix.

INTRODUCTION

Brucella melitensis, a zoonotic disease, is usually carried by goats and sheep, although it is the least host-specific of the brucellosis-causing organisms. *B. melitensis* is well known in many developing and some developed nations. For example, this organism is known to occur in Egypt (4), Syria (11), Israel (6), India (2), Saudi Arabia (13), and sub-Saharan Africa (1, 5, 12). In the New World, it has been reported from Argentina, Peru, and Mexico (7). *B. melitensis* is not known to occur in the Caribbean islands. However, many sheep and goats were brought to this area from Old World countries known to have *B. melitensis*. With no specific *Brucella* surveillance efforts aimed at sheep and goats, it is possible that the bacteria were brought to the New World with their hosts and established in the region. This seroprevalence survey is an attempt to determine if further studies might be warranted.

MATERIALS AND METHODS

Saint Croix is the largest of the U.S. Virgin Islands, approximately 32 km long and 13 km at its widest dimension (8) (map 1). It is the largest of the Virgin Islands with both rolling hills and level terrain which makes it more suitable for agriculture. Food animal agriculture includes dairy and beef cattle and an even larger population of sheep and goats. Most sheep and goats are maintained as backyard animals, tethered or fenced in small pastures or paddocks, usually in groups of fewer than 30 animals. Sheep and goats are often kept in the same enclosure.

A LAGRANDE PRINCESSE
B HANNAH'S REST
C HUMBUG
D GOLDEN GROVE
E SALLY'S FANCY
F GREEN CAY
G CALQUOHOUN
H GLYNN

Map1 : Saint Croix, USVI, location of goats and sheep used in this serological survey for Brucella melitensis *antibodies.*

The goat sera for this study were collected when goats were treated with anthelmintics or otherwise attended by a veterinarian from the USVI Department of Agriculture. The sheep included in this study were all from the University of the Virgin Islands (UVI) Agricultural Experiment Station flock. This flock was formed during 1985-87 by purchasing local sheep from several flocks on Saint Croix. The sheep have been kept together in the same corrals and pastures during the 2 years prior to this study.

Sera from a total of 161 goats from 9 herds were collected. Sera were obtained from 53 sheep in the UVI flock, representing 5 original flocks plus several sheep born into the UVI flock. The sera were frozen and shipped to the National Veterinary Services Laboratories in Ames, Iowa, for serological testing. Each sample was tested for *B. ovis* antibody by the complement fixation test (9), for *B. abortus* antibody by the standard plate test, and for *B. melitensis* antibody by the standard tube test (14).

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Titers were measured and interpreted as suspicious of a previous infection (suspect) or as evidence of previous infection (reactor) as outlined in tables I and II.

***TABLE I** Serological titers for* **Brucella melitensis** *in goats on Saint Croix, USVI. The location on the island and the titer interpretation are also shown.*

Goat	Location*	Tube Aggl. Titer		Interpretation
1	A	Inc[1]	1:25	Suspicion of previous infection
2**	A	Inc	1:50	Evidence of previous infection
3	B	Pos[2]	1:25	Evidence of previous infection
4	C	Inc	1:25	Suspicion of previous infection

* *See map 1.*
** *This goat showed a titer of incomplete at 1:50 for* B. abortus, *interpreted as a borderline suspect for previous infection or possibly a cross-reaction with* B. melitensis.
[1] *Inc = Incomplete.*
[2] *Pos = Positive.*

***TABLE II** Serological titers for* **Brucella melitensis** *in sheep on Saint Croix, USVI. The location represent the site of flock of origin for each sheep tested.*

Sheep*	Location**	Titer		Interpretation
1	D	Inc[1]	1:100	Evidence of previous
2	D	Inc	1:50	infection for all reported
3	D	Pos[2]	1:25	titers in sheep
4	E	Inc	1:50	
5	F	Inc	1:50	
6	G	Pos	1:25	

**A seventh sheep, negative for* B. melitensis, *showed a positive 2+ titer at 1:10 for* B. ovis ; *this sheep came originally from a flock at location H (map 1).*
* *See map 1.*
[1] *Inc = Increase.*
[2] *Pos = Positive.*

RESULTS AND DISCUSSION

Four goats from 3 herds in 3 different areas of Saint Croix showed *B. melitensis* antibodies, with suspect or reactor titers. This gives a seroprevalence of 4/161 or 0.0248. Three of 9 herds had at least one goat with a reactor or suspect titer for a herd prevalence of 33 %. Map 1 shows locations of the herds from which animals were sampled. The location of these animals, titers, and interpretation of the titers are given in table I.

All goats were negative for *B. ovis* antibodies. One goat showed a *B. abortus* titer, incomplete at 1 :50, which could be interpreted as a borderline suspect for previous infection with *B.abortus* or could possibly represent a cross-reaction with *B. melitensis*.

Of the 53 sheep tested, 6 showed titers high enough to use as evidence of previous infection with *B. melitensis*. One showed a 2+ titer at 1 :10 for *B. ovis ;* this animal originated in a flock at location H (map 1). Antibodies for *B. abortus* were not detected in the sheep. The titers and herd of origin for individual sheep are shown in table II. Since the sheep in the UVI flock represent a recently mixed flock, flock prevalence calculations are not appropriate. However, half of the positive sheep came originally from a single source flock located at D (map 1).

CONCLUSION

From a sample of sheep and goats on Saint Croix, we have found serological evidence for previous infection with *B. melitensis* for both species. It is possible that these titers represent cross-reactivity with other antigens. More definitive methods for diagnosis of *B. melitensis* in sheep and goats must be undertaken before the question of occurrence can be settled (3, 10).

On Saint Croix, the other Virgin Islands, and most of the Caribbean islands as well, sheep and goat owners live in close proximity to their animals and often practice backyard slaughter of animals for home consumption. If *B. melitensis* does occur in these animals, it would not be surprising to find cases of human brucellosis as well. The need for a comprehensive study of this agent in sheep and goats is suggested by the results of this serological survey.

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AHL (A.S.), BARTLETT (P.C.), FRERICHS (W.M.). Serological evidence for the presence of *Brucella* antibodies in sheep and goats on Saint Croix, U.S. Virgin Islands. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 61-63

A serological survey for *Brucella* antibodies in sheep and goats was completed on Saint Croix, United States Virgin Islands (USVI). Seroprevalence (at suspect or reactor titer levels) for *B. melitensis* antibodies was 11.3 % for sheep and 2.5 % for goats. This is the first report, of which we are aware, of *B. melitensis* antibodies in sheep or goats in the Caribbean islands.

Key words : Sheep - Goat - Immunological technique - *Brucella melitensis* - Antibody - Saint Croix.

AHL (A.S.), BARTLETT (P.C.), FRERICHS (W.M.). Evidencia serológica de la presencia de anticuerpos de *Brucella* en cabras y ovejas en Santa Cruz, Islas Vírgenes de los E.U.A. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 61-63

Se llevó a cabo un estudio serológico para la determinación de anticuerpos de *Brucella* en Santa Cruz, Islas Vírgenes de los E.U.A. (USVI). La seroprevalencia (con títulos de sospecha o de reacción) para anticuerpos contra *B. melitensis* fue de 11.3 % en ovejas y de 2.5 % en cabras. De acuerdo a nuestros conocimientos, se trata del primer reporte de anticuerpos contra *B. melitensis* en ovinos o caprinos de las islas del Caribe.

Palabras claves : Ovino - Caprino - Técnica inmunológia - *Brucella melitensis* - Anticuerpo - Santa Cruz.

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Babesia bovis-specific CD4+ T cell clones from immune cattle express either the Th0 or Th1 profile of cytokines

BROWN (W.C.), ZHAO (S.), WOODS (V.M.), DOBBELAERE (D.A.E.), RICE-FICHT (A.C.). Des clones de cellules T CD4+ spécifiques pour *Babesia bovis*, de bovins immunisés, expriment le profil de cytokines des cellules Th0 ou des Th1. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 65-69

Le rôle central des cellules T dans la réponse immunitaire contre les hémoprotozooaires, aussi bien comme cellules "helper" pour la production d'anticorps sous dépendance de cellules T que comme cellules effectrices agissant directement ou indirectement sur les parasites intracellulaires par l'élaboration de cytokines, a conduit les auteurs à examiner la réponse immunitaire cellulaire chez les bovins aux antigènes de *Babesia bovis*. Des clones de cellules T produits à partir de quatre bovins immunisés contre *Babesia bovis* par stimulation *in vitro* par des antigènes solubles ou associés à la membrane des mérozoïtes, ont été caractérisés en ce qui concerne leur réactivité contre divers antigènes et des isolats de *B. bovis* et de *B. bigemina* d'origine géographique différente. Les clones ont été catégorisés dans sept groupes différents basés sur les divers types de réactivité. Ce tableau de clones de cellules T, ainsi que des clones additionnels spécifiques pour la protéine de 77 kDa associée au complexe apical des mérozoïtes (Bb-1) ou la protéine majeure de 42 kDa (MSA-1), ont été analysés pour des cytokines. Des tests biologiques pour mesurer IL-2/IL-4, IFN-γ et TNF-α/TNF-ß, et l'analyse par northern blot pour la détection d'ARN messager codant pour IL-2, IL-4, IFN-γ, TNF-ß and TNF-α bovins, ont montré la production différentielle de cytokines par des clones ayant des spécificités antigéniques différentes. Deux clones de cellules T spécifiques pour Bb-1 ont produit le profil de cytokines de Th1: IL-2, IFN-γ, TNF-ß et TNF-α, mais non IL-4. Des clones spécifiques pour la protéine 42 kDa produisaient des taux indétectables de tous les cytokines, mais ont exprimé un profil non restreint ou Th0 de cytokine ARN messager pour cytokines : IL-2, IL-4, IFN-γ, and TNF-α. Finalement, la majorité des clones Th réagissant avec des antigènes de mérozoïtes non définis exprimaient le profil Th0 de cytokines, et plusieurs clones avaient le phénotype Th1, tandis qu'aucun des clones n'a exprimé un profil de cytokines de Th2. Etant donné que dans d'autres infections à protozoaires, les cellules Th1, l'IFN-γ, le TNF-α, et le TNF-ß, mais non pas l'IL-4, sont associés au développement d'une immunité protectrice, le Bb-1 est un candidat logique pour un vaccin contre *B. bovis*.

Mots clés : Babesia bovis - Cellule T - Clone - Antigène - Immunité - Réponse immunitaire.

Babesia bovis causes a virulent form of babesiosis, characterized by fever, anaemia, anorexia, cachexia, low parasitemia and a generalized circulatory disturbance, often resulting in high mortality rates among non-immune cattle. Characteristic of this disease is the sequestration of parasitized erythrocytes in the capillary beds of the brain and lung, leading to cerebral babesiosis and respiratory distress syndrome (13). Similarity in the immunopathology caused by this and related malarial parasites has led to the hypothesis that the diseases caused by these hemoparasites share common mechanisms. Cytokines, including gamma interferon (IFN-γ) and tumor necrosis factor (TNF) released by parasite antigen-activated T cells and macrophages are implicated in anaemia, cytoadherence of parasitized erythocytes to the brain microcapillary endothelia and accumulation of infected erythrocytes and neutrophils in the pulmonary vasculature.

Protective immunity in experimental murine malaria and babesial infections is mediated by T cells and macrophages, and the same cytokines involved in immunopathology play a role in immunity. Although the T helper cell (Th) subsets that are involved in protective immunity to the intraerythrocytic stages of these parasites have not been conclusively identified, it has been suggested that both subsets of Th1 cells appear to be involved in protective immunity against this stage of malarial parasites, with Th1 cells appearing early and Th2 cells appearing late in the infection. IFN-γ and lymphotoxin, or TNF-ß, produced by both CD4+ T helper 1 (Th1) and CD8+ T cells are important for the resolution of hemoparasite infections. These cytokines inhibit parasites by direct toxic effects on the intraerythrocytic parasite, by inhibition of the intrahepatocytic development of exoerythrocytic stages, and through the activation of macrophages and neutrophils, resulting in enhanced phagocytosis of parasitized erythrocytes and the production of TNFα, which itself has antiparasitic properties *in vivo*. Th2 cells, through the elaboration of B cell growth and differentiation factors IL-4 and IL-5, probably function in maintaining an anti-parasitic antibody titer once the initial infection has been controlled. In other protozoal infections, the role of Th2 cells is not benign. In experimental leishmania infection for example, Th1 cells producing IFN-γ conferred protection, whereas Th2 cells, producing IL-4, exacerbated disease (10).

Identification of parasite antigens which evoke immunity and/or immunopathology during the course of hemopara-

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sitic infection is essential for vaccine development. Optimally, a vaccine would include proteins with both T and B cell epitopes that induce anamnestic cellular and humoral immunity upon natural exposure to the parasite. However, very little is known about the nature of either protective babesial antigens or the immune responses in cattle that they evoke. In studies with *B. bovis*, attempts to characterize protective antigens have historically relied upon the use of antibodies to identify immunodominant proteins. Although antibody may play a role in merozoite neutralization (8), it has become increasingly evident that serologically immunodominant antigens are not always protective against a challenge *B. bovis* infection; in some studies protective immunity to *B. bovis* was inversely related to antibody titer (13). These observations, together with the documented relevance of T cells and macrophages in both immunity and immunopathology associated with infection by related hemoparasites, have prompted a detailed investigation of cell-mediated immune responses in *B. bovis*-infected cattle. Because *B. bovis* has no known exoerythrocytic stage which could serve as a target for MHC-restricted cytotoxic T cells, this research has focused on the identification of babesial merozoite antigens which induce Th cell responses in immune cattle, and characterization of the cytokines produced by *Babesia*-specific Th cells. In addition, the authors were interested to determine if discreet subsets of Th cells, identified by distinct cytokine profiles, are present in ruminants as has been described for mouse and man.

Initial studies performed with cattle rendered immune to *B. bovis* either by application of *B. bovis* (Mexico)-infected ticks and treatment with Berenil, or by intravenous administration of avirulent, cultured *B. bovis* (Mexico) merozoites, revealed the preferential stimulation of $CD4^+$ T cells by unfractionated merozoite antigen (1, 2). Parasite-specific T cell lines were established from these cattle and additional cattle infected with a virulent blood stabilate prepared from the Texas isolate of *B. bovis*, and subsequently treated with Berenil. All animals were protected upon challenge infection with virulent organisms. The differential pattern of response by T cell lines derived from the immune cattle suggested the presence of multiple immunodominant epitopes (1). Several T cell lines were cloned by limiting dilution to obtain a panel of T cell clones with differing antigenic specificities (5). All of the clones were $CD4^+$ and MHC class-II restricted. To summarize these findings to date, Th clones stimulated with either soluble or membrane enriched fractions of *B. bovis* merozoites can be categorized into seven different groups (table I).

[An original description of five groups (5) has been revised as new Th clones have been characterized.] These distinctions are based on the differential patterns of proliferative responses to :

- different parasite isolates, including the Mexico, Texas and Australian isolates of *B. bovis* and a Mexico isolate of *B. bigemina* ;

- different forms of unfractionated antigen, including crude membrane (CM), soluble cytosolic (HSS), culture supernatant exoantigen (EXO) ;

- soluble cytosolic antigen fractionated by either anion exchange with a Mono Q column or gel filtration with a Superose-12 column by use of FPLC (Pharmacia).

The majority of Th clones obtained from animal C97 reacted with the membrane antigen only, and recognized all isolates of *B. bovis* tested, but not *B. bigemina* (group I). Some clones reactive with only CM did not recognise the Australian isolate (group II), and the remaining Th clones proliferated in response to soluble HSS antigen (groups III-VII). Clones in group VII, unlike the others, responded

TABLE I ***Helper T cell clones define seven antigenic epitopes in*** **Babesia bovis** ***merozoites.***

Group	Response of clones in each group to the following antigens								
	Unfractionated			Fractionated		Parasite isolate			
	CM	EXO	HSS	Mono Q (M NaCl)	Superose-12 (Size in kDa)	Tex	*B. bovis* Mex	Aust	*B. bigemina* Mex
I	+	—	—	NT[a]	NT	+	+	+	—
II	+	NT	—	NT	NT	+	+	—	—
III	+	—	+	Wash, 0.2-0.25	30-70	+	+	+	+
IV	+	—	+	0.35-0.45	25	+	+	+	—
V	+	—	+	NT	Vo	+	+	+	—
VI	+	—	+	0.35-0.45	50/NT	+	+	—	—
VII	+	+	+	0.35-0.45	NT	+	+	—	—

a. NT refers to not tested.

strongly to the soluble exoantigen. Other clones were grouped according to the soluble antigen fractionation pattern and response to Australian parasites and *B. bigemina.*

We have begun to characterize the antigens present in the fractionated soluble HSS analyzed by SDS-PAGE and silver staining (5). Three Th clones reactive with all babesial parasites tested (group III) recognized a common peak of activity in HSS fractionated by either gel filtration or anion exchange, suggesting the recognition of a common epitope. Upon gel filtration, activity eluted in a broad peak ranging from 30 to 70 kDa, and upon anion exchange, activity was present both in the unbound protein fractions and in fractions eluting at the beginning of the NaCl gradient, with 0.2-0.25 M NaCl. SDS-PAGE analysis of the fractions revealed a single band of approximately 43 kDa common to those fractions with antigenic activity (unpublished observations). However, additional experiments are needed to confirm the identity of the antigen that stimulates these T cell clones. In similar studies performed with *Theileria parva*, we found that antigenic activity in fractionated parasite extract was detected with T cell clones even when no protein bands could be visualized on silver-stained gels (3). Two additional Th clones reactive with all *B. bovis* isolates recognized distinct peaks of activity upon gel filtration (groups IV and V).

To determine whether different subsets of Th cells responsive to different *B. bovis* antigens were present, this panel of Th clones has been characterized for the expression of cell surface differentiation antigens indicative of a memory cell phenotype, and for the production of cytokines. Analysis of the cells using monoclonal antibodies (kindly supplied by C. HOWARD, Compton Laboratories, UK and N. MacHUGH, ILRAD, Nairobi, Kenya) and fluorocytometry revealed that all of the ten $CD4^+$ T cell clones examined expressed high levels of CD45RO, an isoform of the common leukocyte surface antigen, CD45, that is found on memory T cells. In contrast, expression of CD45R, an isoform of CD45 associated with naive T cells, was low. All clones also expressed high levels of L-selectin, a lymph node homing receptor. Biological assays for cytokines secreted into the supernatants of mitogen-activated T cell cultures revealed a differential production of T cell growth factor (IL-2/IL-4), IFN-γ and TNF-α/TNF-ß, suggesting functional differences among the Th clones. All clones in groups I-VI produced IFN- γ, and some clones in each group produced TNF and IL-2/IL-4 activities, whereas none of the clones in group VII produced any detectable cytokine (5, unpublished observations). Because bioassays that distinguish bovine IL-2 and IL-4 activities are currently not available, analysis of the expression by these cells of mRNA encoding IFN-γ, IL-2, IL-4, and TNFα was performed to determine whether these *B. bovis*-specific Th clones could be classified as Th0, Th1 or Th2 cells. Northern blotting revealed that the majority of Th clones in groups I-VI expressed an unrestricted, or Th0 profile of cytokine mRNA, whereas the three Th clones in group VII expressed a Th1-like profile of cytokine mRNA (unpublished observations).

Functional and phenotypic analysis of two sets of Th clones specific for two recombinant *B. bovis* proteins has similarly been performed. In the first study, the authors examined T cells from *B. bovis* immune cattle for responsiveness to the major merozoite surface antigen, MSA-1 (4). This antigen is a 42 kilodalton integral membrane glycoprotein previously shown to induce immunodominant antibody responses in cattle protectively immune to *B. bovis* and to induce neutralizing antibody (7, 8). Recent studies have also shown that MSA-1 B cell epitopes common to New World strains of *B. bovis* are not present in either Israel or Australia strains (8, 9). To understand the potential role of this protein in protective immunity, T helper cell responses specific for MSA-1 were characterized in *Babesia*-immune cattle. Peripheral blood mononuclear cells (PBMC) from immune cattle proliferated against affinity purified recombinant MSA-1 protein expressed in *E. coli.* MSA-1 preferentially stimulated the growth of $CD4^+$ T cells in cell lines cultured with antigen for 4 weeks. MSA-1-reactive cell lines responded to a membrane fraction of *B. bovis* merozoites, suggesting recognition of the native protein. However, the *B. bovis*-reactive T cell lines and Th clones established by stimulation with crude parasite membrane antigen described above failed to respond to recombinant MSA-1, indicating that this antigen is not immunodominant for T cells. The majority of MSA-1-specific Th clones reacted to unfractionated merozoite membrane antigen from New World *B. bovis* isolates, but none of the clones responded to Australia *B. bovis* or to a Mexico isolate of *B. bigemina*. Six clones tested did not secrete detectable levels of cytokines when stimulated with mitogen alone; however several Th clones produced low levels of cytokines when stimulated with mitogen and IL-2. Northern blot analysis revealed the expression of IL-2, IL-4, IFN-γ and TNF-α mRNA in mitogen-stimulated Th clones, showing that the clones examined expressed an unrestricted Th0 phenotype. These findings show that the MSA-1 protein, although serologically immunodominant and capable of inducing neutralizing antibodies as well as a T helper cell response, is not an immunodominant T cell antigen for the cattle used in this study. Furthermore, the parasite strain specificity of the Th clones supports previous findings of extensive polymorphism in the MSA-1 glycoprotein, and suggests that like B cell epitopes, T cell epitopes reside in a non conserved portion of the protein .

The authors also examined T cells from B. bovis-immune cattle for responsiveness to a recombinant form of the 77kDa merozoite protein, Bb-1 (6, 12), which was of interest for studies on T cell immunogenicity because :

- the Bb-1 gene is conserved among New World and Australian parasites ;

- the gene predominated during immunoselection with bovine sera obtained from cattle naturally infected with *B. bovis* ;

- affinity purified bovine antibody reacted with a 77 kDa merozoite protein on immunoblots, and with the apical end of the merozoites by immunofluorescence staining ;

- the Bb-1 protein is inducible by either nutritional or oxidative stress.

Together, these results suggested a functional importance for the Bb-1 protein and a logical target for immune intervention, and showed that the Bb-1 protein is immunogenic for naturally infected animals, where upon natural exposure it could presumably boost memory T cell and B cell responses in cattle vaccinated with this antigen. We first showed that recombinant Bb-1 induced proliferative responses of PBMC of two immune cattle. Antigenicity for PBMC resided in the N-terminal half of the protein. Both $CD4^+$ and $CD8^+$ Bb-1-specific T cell clones were obtained by cloning Bb-1-reactive cell lines. To map the T cell epitopes recognized by the Th clones, a nested set of truncated fusion proteins spanning the Bb-1 protein was prepared and tested for antigenicity in proliferation assays. Two Th clones recognized different T cell epitopes, which mapped to the N terminal half of the protein. Both clones expressed the Th1 profile of cytokines : IL-2, IFN-γ, TNF-β, and TNF-α, but not IL-4, suggesting the preferential induction of Th1 cells by the Bb-1 protein. The authors then characterized the antibody-reactive regions of the Bb-1 protein by Western blot analysis of the truncated recombinant fusion proteins using rabbit antiserum raised against intact Bb-1 protein. In contrast to the T cell epitopes, the antibody-reactive region mapped to the C-terminal half of Bb-1 which contains 28 tandem repeats of a tetrapeptide, PAEK or PAET.

In conclusion, the nature of the protective immune response against many hemoprotozoan parasites is not well understood, and *Babesia* is no exception. When T cells were selected in vitro with crude parasite extracts, the T cells that were subsequently cloned out of the population did not recognize the serologically immunodominant antigen, MSA-1. Furthermore, preliminary studies on fractionation of the soluble antigen of *Theileria* and *Babesia* parasites suggest that the immunodominant T cell antigens are not abundant. In agreement with these observations, WRIGHT and coworkers found that protective antigens of *B. bovis* were present in minute quanties in the organism, and did not stimulate strong antibody responses (13). These findings support the concept put forth by BYRON WAKSMAN, that the best choice of a vaccine antigen may be one which does not evoke a strong immune response during natural infection (11). In studies performed with *B. bovis*, the Th cell response generated against crude parasite extracts was comprised of Th0 and Th1 cells, but not Th2 cells. Interestingly, when a single antigen, Bb-1, was used to select T cells *in vitro*, Th1 cells were preferentially induced, providing a rationale for choosing Bb-1 as a potential vaccine antigen. The use of T cell clones as probes to identify parasite antigens and to characterize the immune response may facilitate the identification of protective parasite immunogens.

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BROWN (W.C.), ZHAO (S.), WOODS (V.M.), DOBBELAERE (D.A.E.), RICE-FICHT (A.C.). *Babesia bovis* -specific CD4+ T cell clones from immune cattle express either the Th0 or Th1 profile of cytokines. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 65-69

The central role of T cells in the immune response against hemoprotozoan parasites, both as helper cells for T-dependent antibody production, and as effector cells acting directly or indirectly on intracellular parasites through the elaboration of cytokines, has prompted us to investigate the bovine cellular immune response against *B. bovis* antigens. T cell clones generated from four *B. bovis*-immune cattle by *in vitro* stimulation with soluble or membrane associated merozoite antigen were characterized for reactivity against various forms of antigen and different geographical isolates of *B. bovis* and *B. bigemina*. The clones were categorized into seven different groups based on differential patterns of reactivity. This panel of T cell clones and additional clones specific for either the 77 kDa merozoite apical complex associated protein (Bb-1) or the 42 kDa major merozoite protein (MSA-1) were analyzed for cytokines. Biological assays to measure IL-2/IL-4, IFN-γ and TNF-α /TNF-β and Northern blot analysis to detect mRNA encoding bovine IL2, IL-4, IFN-γ, TNF-β and TNF-α revealed the differential production of cytokines by clones with different antigen specificities. Two Bb-1-specific T cell clones produced the Th1 pattern of cytokines : IL-2, IFN-γ ,TNF-β and TNF-α, but not IL-4. Clones specific for the 42 kDa protein produced undetectable levels of all cytokines, but expressed an unrestricted or Th0 pattern of cytokine mRNA : IL-2, IL-4, IFN-γ and TNF-α. Finally, the majority of Th clones reactive with undefined merozoite antigens expressed the Th0 pattern of cytokines, and several clones were of the Th1 phenotype, whereas none of the clones expressed a Th2 profile of cytokines. Because in other protozoal infections Th1 cells, IFN-γ, TNF-α, and TNF-β, but not IL-4 are associated with the development of a protective immunity, Bb-1 is a logical candidate for a *B. bovis* vaccine.

Key words : *Babesia bovis* - T cell - Clone - Antigen - Immunity - Immune response.

BROWN (W.C.), ZHAO (S.), WOODS (V.M.), DOBBELAERE (D.A.E.), RICE-FICHT (A.C.). Los clones de células T CD4+, de bovinos inmunes, específicos para *Babesia bovis*, se expresan de acuerdo a un perfil de citoquinas Th0 o de Th1. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 65-69

La razón que nos motivó a investigar la respuesta inmunológica celular en el bovino contra antígenos de *Babesia bovis*, es el papel central que juegan las células T en la respuesta inmunológica contra los hemoprotozoarios, tanto como células colaboradoras para la producción de anticuerpos T-dependientes, que como "células de efecto", actuando directa o indirectamente sobre los parásitos intracelulares, mediante la elaboración de citoquinas. Los clones de células T, provenientes de cuatro bovinos inmunizados contra *B. bovis* (por estimulación *in vitro*, con una asociación de un antigeno de merozoito soluble o de membrana), fueron caracterizados para la reactividad contra varias formas de antigeno y de aislamientos geográficos de *Babesia bovis* y *B. bigemina*. Los clones se clasificaron en siete grupos diferentes, basados en los patrones diferenciales de reactividad. El análisis de las citoquinas se hizo para esta muestra de clones de células T y otros clones específicos, ya sea para la proteína asociada al complejo 77 kDa del merozoito apical (Bb-1) o a la proteína del merozoito mayor 42kDa (MSA-1). Estos experimentos biológicos para medir el IL-2/IL-4, IFN-γ y TNF-α /TNF-β y el analisis por "Northern blot" para detectar el ARNm que codifica la IL-2, IL-4, IFN-γ, TNF-β y TNF-α, demostraron la diferencia en cuanto a la producción de citoquinas por parte de los clones con diferentes especificidades antigénicas. Dos clones celulares Bb-1 específicos para células T, produjeron citoquinas del patron Th1: IL-2, IFN-γ ,TNF-β y TNF-α, pero no IL-4. Los clones específicos para la proteína 42 kDa produjeron niveles no detectables de todas la citoquinas, pero mostraron un patrón sin restricción o patrón Th0 de citoquina de ARNm: IL-2, IL-4, IFN-γ y TNF-α. Finalmente, la mayoría de los clones Th reaccionaron con antigenos indefinidos del merozoito, expresando el patron Th0 de citoquinas. Varios clones fueron del fenotipo Th1, mientras que ninguno de ellos mostró un perfil Th2 de citoquinas. Bb-1 es el candidato lógico para una vacuna contra *B. bovis*, debido a que en otras infecciones por protozoarios las células Th1, IFN-γ, TNF-α y TNF-β, pero no IL-4, se asocian con el desarrollo de la inmunidad protectora.

Palabras claves : *Babesia bovis* - Célula T - Clon - Antígeno - Inmunidad - Respuesta inmunologica.

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Use of a multiplex polymerase chain reaction-based assay to conduct epidemiological studies on bovine hemoparasites in Mexico

FIGUEROA (J.V.), ALVAREZ (J.A.), RAMOS (J.A.), VEGA (C.A.), BUENING (G.M.). Utilisation d'un test multiple basé sur la réaction de polymérase en chaîne pour des enquêtes épidémiologiques sur les hémoparasites des bovins au Mexique. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 71-75

Une étude a été effectuée sur la possibilité d'appliquer la technique de la réaction de polymérase en chaîne (PCR) pour la détection simultanée des hémoparasites bovins *Babesia bigemina*, *B. bovis* et *Anaplasma marginale*. Des échantillons de sang de bovins ont été récoltés dans des ranches d'une zone endémique identifiée au préalable dans la péninsule de Yucatan au Mexique, et ont été préparés pour analyse par PCR. Ils ont été soumis à l'amplification de l'ADN dans un tube à réaction contenant des amorces d'oligonucléotides spécifiques pour l'ADN de chaque espèce d'hémoparasite. Les produits de la PCR ont été détectés par hybridation en Dot-Blot de l'acide nucléique utilisant des sondes d'ADN non-radioactives, spécifiques, marquées à la digoxigénine par PCR. Quatre cent vingt échantillons analysés par le test multiple PCR-sonde ADN ont montré des taux de prévalence de 66,7 p.100, 60,1 et 59,6 p.100 pour *B. bigemina*, *B. bovis* et *A. marginale*, respectivement. L'analyse multiple par PCR a montré que des animaux ayant des infections simples, doubles ou triples pouvaient être détectés par les sondes d'ADN spécifiques. La procédure est proposée comme un outil de valeur pour l'analyse épidémiologique dans les régions où ces espèces d'hémoparasites infectent les bovins simultanément.

Mots clés : Bovin - *Babesia bigemina* - *Babesia bovis* - *Anaplasma marginale* - Épidémiologie - Enquête pathologique - Dot-Blot - Sonde à ADN - Mexique.

INTRODUCTION

One of the most economically important diseases of cattle in tropical and subtropical areas of the world, is bovine babesiosis (24). In America, two species have been recognized in cattle, *B. bovis* and B. *bigemina* (21). Both *Babesia* species share the tick *Boophilus* spp. as the vector for their biological transmission to cattle (5, 24). Clinically the disease is manifested as fever, anorexia, dullness, weakness, ataxia, hemoglobinuria, icterus, anemia, and presence of intraerythrocytic parasites (5, 24). Bovine anaplasmosis is a biologically and mechanically arthropod-transmitted disease caused by the rickettsia *Anaplasma marginale* which is widely distributed throughout the world (5, 25). Clinically, bovine anaplasmosis is at times very similar to bovine babesiosis. However, anaplasmosis is manifested as a progressive anemia (usually without hemoglobinuria) associated with the presence of intraerythrocytic inclusion bodies (5, 15, 25).

Under field conditions, the three organisms may be involved in the causation of disease (7, 24, 25, 30) since they can be transmitted by a common tick vector. Traditionally, peripheral blood smears have been utilized to detect the presence of intraerythrocytic bodies (5, 15, 24, 25). However, microscopic examination of Giemsa-stained smears often does not detect low levels of parasites because the parasitemia fluctuates during the carrier state in cattle (5, 17).

The reported use of the polymerase chain reaction technique (26) with its high sensitivity for detection of infectious organisms (1, 12, 19, 28) led to the development of an assay for the simultaneous detection of the hemoparasites *B. bigemina, B. bovis* and *A. marginale* in carrier cattle (13). In this study, we test the applicability of the assay to determine the distribution of the 3 hemoparasites in cattle from the Yucatan State of Mexico.

MATERIALS AND METHODS

Hemoparasite-infected bovine erythrocytes

Babesia bigemina and *B. bovis* Mexico isolates were cultivated as previously described (18, 29). *Anaplasma marginale*-infected blood (USA isolate) was collected from an experimentally infected calf at peak of parasitemia. Percentage of parasitized erythrocytes (PPE) was estimated by light microscopy of Giemsa-stained smears (5) and dilutions of infected blood were prepared in normal blood to serve as positive controls in the PCR assay (13).

Primers

Six sets of oligonucleotide primers were utilized in the PCR assays and their nucleotide sequences have previously been reported (13). Three sets (external primers, each set species specific) were used to amplify DNA from genomic hemoparasite templates present in the blood samples. Expected size of amplified fragments was 280 bp, 350 bp and 200 bp for *B. bigemina, B. bovis* and *A.*

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marginale DNA, respectively (13). Three more sets of oligonucleotides (internal primers) were used to prepare nonradioactive DNA probes specific for each species of hemoparasite as described below.

PCR DNA amplification

For blood sample analysis, 20 µl of packed and washed bovine erythrocytes were processed as previously described (12, 28). Briefly, pelleted erythrocytes were resuspended in saponin lysis buffer (28). After one wash in reaction mixture buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin, without dNTPs and primers), pellets were directly resuspended in 99.5 µl of reaction mixture buffer containing 200 µM of each dNTP and 1µM of each external primer and incubated at 100 °C for 10 min.

After a brief centrifugation, 2.5 U of Taq polymerase (Promega Co., Madison, WI) was added to each tube and the PCR tubes were mixed and placed in a TempCycler 60 (Coy, Ann Arbor, MI) for thermocycling. The first cycle consisted of 2 min template denaturation at 95 °C, 1 min primer annealing at 60 °C, and 1.5 min primer extension at 73 °C. The second to 36th cycle were as above except that the denaturation step was decreased to 1 min. A final extension of 15 min at 73 °C was included in the amplification reaction. To avoid amplicon contamination, individual steps were performed in separate rooms and dedicated pipets were utilized as recommended (16).

Experimental animals

This study was carried out in southeast Mexico, in the state of Yucatan. Yucatan, a "dry" tropical area, with a warm, subhumid climate Aw (14), has a total cattle population of 646,371 head (23) . The sample size (n = 903) was determined according to the exact population equation (8), considering the total number of head in the region as the population "N", regardless of age. An error of 0.2 and a confidence level of 95 % were assumed, based on a previous estimation of a 60 % serological prevalence for bovine babesiosis. Collected bovine blood samples were then placed into five groups, considering the age in months of the animals : Group A, < 3 ; Group B, 3 to 9; Group C, 9 to 18; Group D, 18 to 36; and Group E, > 36 months (23). For this experiment in particular, blood samples from 421 randomly selected animals in the region were processed for multiplex PCR assay analysis.

Sample collection and preparation

Peripheral blood was aseptically obtained from cattle's jugular or tail veins into evacuated glass tubes containing EDTA (Vacutainer, Becton-Dickinson de México, S.A. de C.V.). Blood samples were processed as previously described (11). Briefly, blood samples were washed three times by centrifugation with TEN buffer (0.1 M Tris-HCl, pH 8.0 ; 0.15 M NaCl, 10 mM EDTA) ; supernate and buffy coat were discarded each time. Packed erythrocytes were stored frozen at -20 °C in 500µl aliquots. Blood was maintained at -20°C until analyzed. After two freeze-thaw steps 20 µl aliquots were removed and the PCR procedure performed as described above.

Preparation of PCR-labeled probes

Purified pBbi55 plasmid DNA (12), purified p60 DNA (27) kindly provided by T.F. McELWAIN (Washington State University, Pullman WA, USA), and purified p25 plasmid DNA (1) served as templates for the synthesis of probes via PCR using the internal set of primers. The procedure was carried out essentially as described (13) in which digoxigenin-dUPT is incorporated into the newly synthesized DNA (9). The size of DNA probes are 170 Bp, 291 Bp and 160 Bp for *B. bigemina, B. bovis* and *A. marginale*, respectively (13).

Analysis of PCR products

Aliquots from the amplification reaction were analyzed by dot-blot nucleic acid hybridization-chemiluminescent detection (13). Briefly, three 20 µl aliquots of the PCR reaction were spotted onto 3 different nylon membranes using a dot-blot apparatus. After DNA denaturation, neutralization and fixation to the nylon membrane, dot-blots were each hybridized with a parasite-specific nonradioactive probe (100 ng/ml hybridization solution) as suggested by the supplier (3). DNA hybrids were detected by the alkaline phosphatase-based chemiluminescent reaction as described (12, 13).

RESULTS

Estimation of the sample size indicated that a total of 942 samples was sufficient for determination of hemoparasite prevalence rates by serology (23) ; however, since this study was subsampled, the total number of blood samples analyzed was 421.

The multiplex PCR assay detected, in clinically apparently healthy cattle, animals carrying one, two or the three hemoparasites. Figure 1 shows the picture of a representative autoradiograph obtained by doing the PCR assay on the bovine blood samples. The positive responses in the multiplex PCR assay were 281, 253 and 251 for an overall prevalence infection rate of 66.7, 60.1 and 59.6 % for *B. bigemina, B. bovis* and *A. marginale*, respectively.

Percentages of distribution of single- or multiple-infected cattle are shown in table I. Cattle carrying the three hemoparasites showed the highest prevalence (34.9 %), followed by *B. bigemina* infected cattle (12.2 %) and

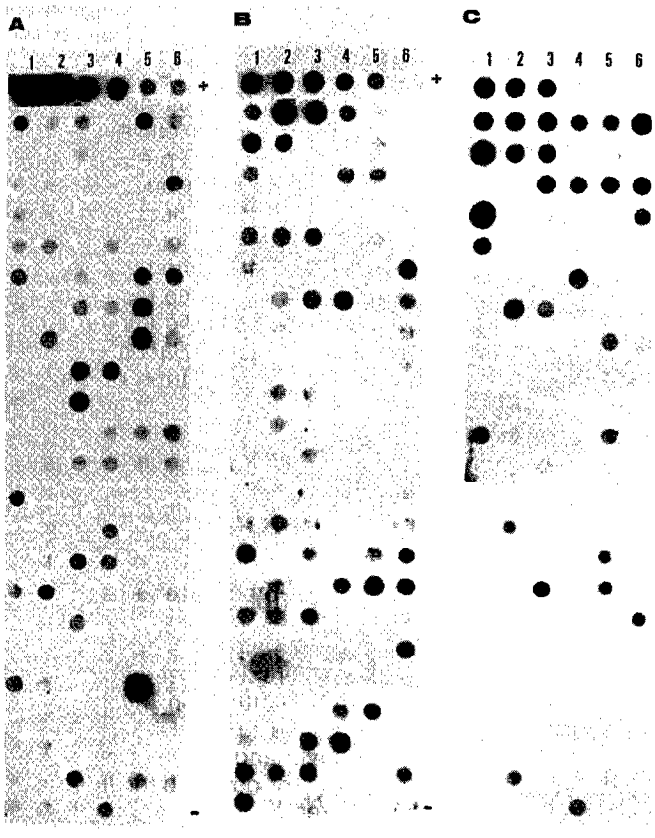

Figure 1 : Analysis of PCR products by dot blot nucleic acid hybridization from bovine blood field samples. First row: Numbers 1-6) positive controls (0.1 % to 0.000001 % infected erythrocytes). Next rows : Blood samples from Yucatan, Mexico. Last row: Numbers 5-6) negative controls (normal bovine blood). Panel A: Dot blots hybridized with B. bigemina*-specific DNA probe. Panel B : Dot blots hybridized with* B. bovis*-specific DNA probe. Panel C : Dot blots hybridized with* A. marginale*-specific DNA probe.*

TABLE I PCR-reactors distribution of bovine blood samples.

	B. bigemina	*B. bovis*	*A. marginale*	Total	%
1)	+	+	+	147	34.9
2)	—	+	+	41	9.7
3)	+	—	+	36	8.5
4)	+	+	—	47	11.3
5)	—	—	+	27	6.4
6)	—	+	—	18	4.2
7)	+	—	—	51	12.2
8)	—	—	—	54	12.8

+ = specific hybridization with species specific DNA probe.

B. bigemina/B. bovis-infected cattle (11.3 %). Cattle harboring both protozoan and rickettsial organisms showed a prevalence rate of 9.7 % for *B. bovis/A. marginale*, and 8.5 % for *B. bigemina/A. marginale* combination. Cattle singly infected with *B. bovis* or *A. marginale* had the lowest prevalence values (4.2 % and 6.4 %, respectively). Only 12.8 % of the cattle tested were PCR-assay negative for all of the three hemoparasites detected in this study.

According to the different age groups the prevalence rates for *A. marginale, B. bigemina* and *B. bovis* are summarized in tables II, III and IV, respectively. The results indicate that the number of positive reactors to *A. marginale* was very similar for cattle aged < 3 months to 36 months (around 50 % prevalence rate), whereas the prevalence rate to the rickettsia increased to 76 % in adult animals over 3 years of age.

High prevalence rates of *Babesia* infection (60-70 %) were observed for young cattle regardless of the infecting protozoan species. The prevalence of *Babesia bigemina* infection in adult cattle slightly declined; however, a relati-

TABLE II Response distribution to* Anaplasma marginale *of blood samples PCR-analyzed by age in months.

	Group	PCR (—)	PCR (+)	Total	% (+)
1)	< 3 months	37	42	79	53.1
2)	3-9 months	36	46	82	56.1
3)	9-18 months	43	47	90	52.2
4)	18-36 months	30	39	69	56.5
5)	> 36 months	24	77	101	76.2

TABLE III Response distribution to* Babesia bigemina *of blood samples PCR-analyzed by age in months.

	Group	PCR (—)	PCR (+)	Total	% (+)
1)	< 3 months	22	57	79	72.1
2)	3-9 months	28	54	82	65.8
3)	9-18 months	25	65	90	72.2
4)	18-36 months	28	41	69	59.4
5)	> 36 months	37	64	101	63.3

Table IV Response distribution to* Babesia bovis *of blood samples PCR-analyzed by age in months.

Group	PCR (-)	PCR (+)	Total	% (+)
1) < 3 months	31	48	79	60.7
2) 3-9 months	30	52	82	63.4
3) 9-18 months	24	66	90	73.3
4) 18-36 months	24	45	69	65.2
5) > 36 months	59	42	101	41.5

vely low prevalence rate for *Babesia bovis* (41.5 %), was determined for cattle over 3 years of age.

When the results were analyzed to determine the Daily Probability Infection (DPI) rate adjusted to 9 months of age for bovine babesiosis, it was found that DPI was 0.004 for *B. bigemina*, whereas for B. *bovis* the DPI was 0.003, indicating a field situation of enzootic instability for bovine babesiosis (17).

DISCUSSION

It has been postulated that use of DNA based-assays for the direct detection of hemoparasites in bovine blood samples would have many advantages over serologic techniques. For example, carrier animals would be identified based upon the presence of an organism (2, 10).

A colorimetric DNA probe-based assay to detect *B. bigemina* was recently reported (11). The DNA probe utilized was able to detect parasitemias of 0.001 % in 200-500 µl volumes of packed cells. It was found, however, that the analytical sensitivity of the *B. bigemina* DNA probe appeared to be too low for its utilization in widespread epidemiological surveys (23), since carrier cattle infected with *B. bigemina* may have peripheral blood parasitemia levels below the limit of detection of this nonradioactive DNA probe (12). The same situation could be applicable for the *B. bovis*-infected carrier cattle (13, 17), and *A. marginale*-infected carriers (1, 4,10). A PCR-based assay with an elevated analytical sensitivity could overcome some of the previously reported constraints. Moreover, detection of multiple infective agents could be accomplished and facilitate the use of a PCR-DNA based assay in epidemiological studies (13). Overall prevalence infection rates for *A. marginale, B. bovis* and *B. bigemina* (77, 44 and 60 %, respectively) have been serologically determined in bovine serum samples obtained from the same cattle of the region (22). There were discrepancies between prevalence infection rates determined in this study using the PCR assay, compared to those reported using serological procedure (22). The Multiplex PCR assay identifies hemoparasite DNA in blood samples, whereas the ELISA (*A. marginale*) and the IFAT (*Babesia* sp.) assays identify antibody in serum samples. However, the overall prevalence rate determined for bovine babesiosis in this study (> 60 %), was similar to the 69 % obtained 14 years ago in a herd of cattle (20). This observation confirmed the endemic status of bovine babesiosis in the area.

One of the advantages of the Multiplex PCR assay over the conventional serological assays was the identification of the presence of the hemoparasites in the youngest group of animals. Detection of colostral antibodies reactive in serological tests, preclude the utilization of this type of assays to determine the infection rate in calves 3-4 months old (17). The estimated *Babesia* infection rates for animals less than 9 months old obtained in this study indicate that the risk of the occurrence of bovine babesiosis outbreaks exists in animals of this geographical region (17). In addition, the concept of inverse age resistance in bovine babesiosis (6) is corroborated in this study, because despite a relatively large number of calves already infected at or around 3 months of age, the presentation of clinical babesiosis in this group was not observed (22). Furthermore, the identification, at the species level, of *Babesia*-infected animals within a population, would facilitate the control measures decision-making process by animal health officers. Thus, this information is very useful when selecting a procedure for specific immunization of cattle, particularly in zones in which a *Babesia* species may be considered more important in terms of causing disease outbreaks (5, 17, 30).

CONCLUSION

In conclusion, the Multiplex PCR procedure for the detection of *B. bigemina, B. bovis* and *A. marginale* infection in carriers is proposed as a valuable tool for epidemiological studies especially in regions where the three hemoparasite concomitantly infect cattle. Cattle from the Yucatan state of Mexico were determined to have high infection rates for *B. bigemina, B. bovis* and *A. marginale*. This study confirmed the endemicity of bovine babesiosis and bovine anaplasmosis in the area.

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FIGUEROA (J.V.), ALVAREZ (J.A.), RAMOS (J.A.), VEGA (C.A.), BUENING (G.M.). Use of multiplex polymerase chain reaction-based assay to conduct epidemiological studies on bovine hemoparasites in Mexico. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 71-75

A study was conducted to test the applicability of a Polymerase Chain Reaction (PCR)-based approach for the simultaneous detection of the bovine hemoparasites *Babesia bigemina*, *B. bovis* and *Anaplasma marginale*. Bovine blood samples from cattle ranches of a previously determined enzootic zone in the Yucatan Peninsula of Mexico, were collected from peripheral blood and processed for PCR analysis. Blood samples were subjected to DNA amplification by placing an aliquot in a reaction tube containing oligonucleotide primers specific for DNA of each hemoparasite species. The PCR products were detected by Dot-Blot nucleic acid hybridization utilizing nonradioactive, species-specific, digoxigenin PCR-labeled DNA probes. Four hundred twenty one field samples analyzed by the multiplex PCR-DNA probe assay showed 66.7 %, 60.1 % and 59.6 % prevalence rates for *B. bigemina*, *B. bovis* and *A. marginale*, respectively. The multiplex PCR analysis showed that animals with single, double or triple infection could be detected with the parasite specific DNA probes. The procedure is proposed as a valuable tool for the epidemiological analysis in regions where the hemoparasite species are concurrently infecting cattle.

Key words : Cattle - *Babesia bigemina* - *Babesia bovis* - *Anaplasma marginale* - Epidemiology - Serological survey - Dot-Blot - DNA probe - Mexico.

FIGUEROA (J.V.), ALVAREZ (J.A.), RAMOS (J.A.), VEGA (C.A.), BUENING (G.M.). Uso de ensayos basados en reacciones en cadena de polimerasas múltiples, para estudios epidemiológicos en hemoparásitos de bovinos, en México. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 71-75

Se llevaron a cabo estudios para probar la utilidad de los métodos de reacciones en cadena de polimerasas (PCR), en la detección simultánea de *Babesia bigemina*, *Babesia bovis* y *Anaplasma marginale*, hemoparásitos de los bovinos. Se colectaron muestras de sangre periférica, de bovinos originarios de ranchos escogidos en zonas enzoóticas de la Península de Yucatán, en México, las cuales se analizaron por PCR. Las muestras sanguíneas se sometieron a una amplificación del ADN, mediante una reacción en tubo de un alicuota con un primer de un oligonucleótido específico para el ADN de cada especie de hemoparásito. Los resultados de las PCR se detectaron gracias a la hibridación de ácidos nucleícos por Dot-Blot, con probadores de ADN marcados con digoxigenina-PCR, no radioactivos, específicos para cada especie. El análisis de las cuatrocientas veintiún muestras mostró prevalencias de 66,7 p.100, 60,1 y 59,6 p.100 para *Babesia bigemina*, *Babesia bovis* y *Anaplasma marginale*, respectivamente. El análisis por PCR múltiple mostró que los animales con infecciones únicas, dobles o triples pueden ser detectados con los probadores específicos de ADN. Este procedimiento podría ser valioso para los análisis epidemiológicos en aquellas zonas en las cuales las infecciones de ganado son frecuentes.

Palabras claves : Bovino - *Babesia bigemina* - *Babesia bovis* - *Anaplasma marginale* - Epidemiología - Encuesta serologica - Dot-Blot -Sonda de ADN - México.

Recommendations for African horse sickness vaccines for use in nonendemic areas

J.A. House [1]

HOUSE (J.A.). Recommandations pour l'utilisation de vaccins contre la peste équine dans des régions non endémiques. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 77-81

La peste équine (PE) est causée par des orbivirus et transmise par des *Culicoides*; elle détermine une mortalité jusqu'à 95 p. 100. Le but d'un programme de lutte et d'éradication est d'empêcher la propagation du virus par le vecteur biologique. Les mesures de lutte comprennent l'abattage des animaux infectés, la mise en étable étanche aux insectes des animaux suspects d'infection, et la vaccination. La vaccination a joué un rôle clef dans l'éradication lorsque la PE est apparue en dehors de l'Afrique. Des vaccins vivants modifiés ainsi que des vaccins inactivés ont été utilisés pour la lutte contre la PE. Pour être acceptable un vaccin doit être : sans danger, efficace et disponible. Le vaccin ne doit causer ni maladie, ni virémie, et le virus vaccinal ne doit pas redevenir virulent lors de passages sur des équidés sensibles. Il doit protéger contre la mort et contre les signes cliniques et, très important, doit prévenir une virémie chez les équidés vaccinés exposés au virus virulent. La méthode pour éprouver l'immunité à la PE par inoculation d'un virus virulent est commentée. Le vaccin doit être facilement disponible, soit par une production régulière dans des installations répondant aux normes internationales, soit dans une banque de vaccin. Des banques de stocks de vaccins vivants modifiés ou de vaccins inactivés concentrés permettent de disposer d'un vaccin contre la PE lors d'épizooties futures. Un test diagnostique a été développé récemment pour distinguer les animaux vaccinés d'animaux infectés naturellement, et fournit de l'information utile aux services officiels pour le contrôle de la PE.

Mots-clés : Peste équine - Contrôle de maladies - Vaccin inactivé - Vaccin vivant modifié - *Culicoides* - Vecteur de maladie - Abattage d'animaux.

THE DISEASE

African horse sickness (AHS) is an arthropod-borne, noncontagious, viral disease of Equidae that causes high mortality in horses, with decreasing mortality in mules, donkeys, and zebras. There are nine serotypes of AHS virus (AHSV) which form a subgroup in the genus *Orbivirus* in the family Reoviridae. African horse sickness is endemic in subsaharan Africa, but the disease has occurred outside of Africa on several occasions. In infected animals, AHSV develops to greatest concentrations in the spleen, lungs, and lymph nodes. Pathological changes in the lungs, heart, and blood vessels account for the clinical and necropsy findings. There are classically four clinical forms of AHS :

- The pulmonary or acute form has a clinical course of 5 to 7 days. Mortality can reach 95 %. Affected animals show rapid, distressed respiration within a few hours of death and die by literally drowning in their own fluids. Necropsy lesions include severe pulmonary edema and hydrothorax.

- The cardiac or subacute form has a course of 5 to 15 days. Mortality ranges from 50 to 90 % and affected animals may have edema of the supraorbital fossa (considered pathognomonic) and edema of the eyelids, conjunctiva, and/or subcutaneous tissues. Lesions observed at necropsy include edema of subcutaneous tissues, edema along the ligamentum nuchae, edema of the intermuscular fascia (particularly in the neck), hydropericardium, and occasional necrosis of the myocardium, especially of the papillary muscles.

- The mixed form, the most common form of the disease, is characterized by combination of the clinical signs and pathological lesions of the pulmonary and cardiac forms. Mortality ranges from 50 to 90 %.

- The horse sickness fever form is a mild form of the disease. By definition, animals recover from this form of the disease following a febrile period of approximately 5 days.

The prevalence of the disease is dependent on the competence and concentration of the vectors. *Culicoides* have been shown to be the most significant biological vectors in nature, but mosquitoes have biologically transmitted the disease under experimental conditions. Control measures include slaughter of infected animals, vaccination, and housing animals suspected to be infected in insect-proof stalls.

REVIEW OF VACCINES

The first vaccines developed for AHS were modified live vaccines (MLV) of adult mouse brain (AMB) origin. These vaccines have been and are used extensively throughout Africa. They were used to help control the AHS pandemic in the Middle East during 1958-63. However, during that outbreak, vaccine related cases of encephalitis in donkeys and horses were reported in Israel and India (11,

1. USDA, APHIS, VS, NVSL, Foreign Animal Disease Diagnostic Laboratory, Box 848 Greenport, New York 11944, États-Unis.

13). The AMB vaccines caused encephalitis in guinea pigs inoculated intranasally (3). Recently, the AMB vaccines have been associated with encephalitis and chorioretinitis in production workers exposed to aerosols of these vaccine viruses (14). The AMB original vaccine for AHSV serotype 4 (AHSV-4) had poor immunogenicity and was eliminated from more recent polyvalent AHS AMB vaccines (4). Due to the outbreak of AHS in Spain, we performed studies to evaluate the safety and efficacy of the AMB AHS vaccine seeds that were available at the Foreign Animal Disease Diagnostic Laboratory. These seeds were tested because diplomatic relations with South Africa were unfortunately not favorable, making the availability of more current vaccines questionable. There was a spectrum of response to the AHS AMB vaccines ranging from a failure to induce any antibody to induction of solid protective immunity (table I). It is especially interesting to note that ponies inoculated with vaccines for serotypes 6 and 7 did not develop any neutralizing antibody after vaccination. Following challenge inoculation, these ponies developed a primary response and survived challenge inoculation. This may indicate that cell-mediated immunity plays a role in protection from AHS. It is also of interest that these ponies had a long febrile period after vaccination (7 to 14 days). Although data on viremias following vaccination are not available, it is likely that there would be enough virus present to infect vectors. Such viremic Equidae infected simultaneously with a second strain or serotype of AHSV could foster genomic reassortment.

TABLE I Responses of ponies vaccinated with AHS AMB vaccines.[a]

Vaccine by serotype number (#ponies)	Days of fever following vaccination	Virus neutralizing antibody after vaccination	Immune response after challenge	Result of challenge
1(1)	3	No	Primary	Died 26 DPC[b]
4(1)	2			Died 21 DPC
5(2)	4, 7			Died 9 DPC
6(2)	7, 12	No	Primary	Survived
7(1)	14			
2(1)	2	No	Anamnestic	Survived
8(1)	3			
3(1)	3	Yes	None	Survived
9(1)	9			

[a] *Information is extracted from reference 6.*
[b] *Days post challenge.*

In 1978, ERASMUS reported the development of cell culture origin MLV vaccines for AHS derived from nonvirulent large plaque variants of AHSV (4). Currently, vaccines for 8 of the 9 serotypes of AHSV are produced by this procedure and used in South Africa. The AHSV-6 vaccine is considered to induce adequate cross-protective immunity to AHSV-9, so no vaccine for AHSV-9 is used. The vaccines are administered as 2 quadravalent vaccines consisting of serotypes 1, 3, 4, and 5 and 2, 6, 7, and 8. A monovalent vaccine for AHSV-4 was produced at the Onderstepoort facility for use in the Spanish outbreaks of AHS 1987 through 1990. Information on the safety and efficacy of these vaccines is not available from the literature.

Following a request from Spanish veterinary officials, we inoculated 3 ponies with the South African monovalent AHSV-4 MLV vaccine (5). None of the 3 ponies had any clinical reaction to the vaccine; and, as noted in table II, following challenge inoculation, none developed any clinical sign attributable to AHS. The virus detected in the blood of pony 2 following intravenous challenge inoculation with AHSV-4 Spain 87 is likely residual challenge virus from the intravenous inoculation of challenge virus. Pony 3 had a prolonged and substantial viremia ; the levels (up to $10^{4.8}$ $TCID_{50}$/ml blood) would likely be adequate to infect insect vectors. It is estimated that a viremia of 10^4 infectious particles/ml blood is adequate to infect a significant proportion of vectors with bluetongue virus (10). Since the number of ponies examined was small, it is not possible to predict the incidence of this viremia under field conditions.

TABLE II Viremia in ponies vaccinated with AHS 4 MLV vaccine (tissue culture origin) following challenge inoculation[a].

Pony identity	Viremia following challenge inoculation
1	None
2	$10^{2.8}$ $TCID_{50}$ per ml of blood on day 3
3	$10^{3.1}$ to $10^{4.8}$ $TCID_{50}$ per ml of blood from days 3 to 11

[a] *Information is extracted from reference 5.*

Backpassage information on commercially produced AHS MLVs is not available. A small plaque AHSV-4, isolated from wild AHSV, did not cause clinical signs of AHS when 10^8 $TCID_{50}$ were inoculated intravenously in 4 ponies (5). No virus was isolated from blood samples collected daily following inoculation. The inoculation induced antibody in all 4 vaccinated ponies and protective immunity in the 2 ponies that were challenge inoculated with AHSV-4 Spain 87. In a backpassage study, daily heparinized blood samples from 2

ponies inoculated with the small plaque virus were respectively pooled; and each pool was inoculated into 1 pony. One of the backpassage ponies died of AHS 35 days after inoculation with the pooled blood. It is difficult to extrapolate the implications of this study to field conditions since the likelihood of vectors becoming infected from an animal without a detectable viremia is quite remote. However, the apparent return to virulence of the backpassaged virus raises a valid question about the stability and safety of MLV AHS vaccine viruses.

Recently, an inactivated AHSV-4 vaccine was commercially produced. Information on the production method and efficacy was reported by DUBOURGET (2). This vaccine has not had extensive use under field conditions to date. Laboratory studies were conducted with one and two doses of vaccine (7,8). Table III summarizes the responses of vaccinated ponies. After challenge inoculation viremia occurred in one vaccinate that received one dose of vaccine. The virus level in the blood was 10^3 infectious particles per ml of blood. This was about 10^1 $TCID_{50}$ below the level considered to be the threshold level (10^4 infectious particles/ml blood) estimated to infect a significant number of *Culicoides* with bluetongue viruses (10).

CONSIDERATIONS FOR FUTURE GUIDELINES FOR AHS VACCINE PRODUCTION

An acceptable vaccine should be : pure, safe, potent, efficacious and available. This paper will address all of these but purity (freedom from contaminants). Guidelines and procedures for AHS cell culture origin MLVs have been presented by the Office International des Epizooties (OIE) (12).

There are several areas to consider for future guidelines for AHS vaccine development, production, and testing. A more complete set of requirements for vaccine production and control could be based upon the master seed principle for virus stocks and cell cultures (1). In addition, good manufacturing procedures for containment and prevention of cross contamination of products could be included (1). However, the development and implementation of these guidelines and test systems would require a considerable investment. It is unlikely that commercial firms would spend the funds to validate such tests because of the small market share of these vaccines in nonendemic areas.

Information on backpassage for the tissue culture-derived MLVs is not available. Backpassage information on MLVs is useful to regulatory officials deciding on the use of vaccines in non-endemic areas. The febrile response of ponies following vaccination with some of the AHS AMB vaccines is of concern because it indicates that these vaccines may produce notable viremias. The lack of clinical response and of detectable viremia following vaccination with cell culture MLVs makes it unlikely that backpassaging via vector infection would occur under field conditions. However, it would be useful to determine whether the vaccine viruses reverted to virulence under controlled experimental backpassage conditions.

Following challenge inoculation, an ideal vaccine for arthropod-borne diseases should prevent a viremia capable of significant vector infection. For AHS, this thre-

TABLE III Responses of ponies vaccinated with commercial inactivated African horse sickness vaccine[a].

Pony response	1 Dose regime[b]		2 Dose regime[c]	
	Vaccinates	Controls	Vaccinates	Controls
Immune response after vaccination	8/9	NA[d]	5/5	NA
Anamnestic response after challenge inoculation	7/9	NA	0/5	NA
Viremia $\geq 10^{2.5}$ MLD_{50} per ml of blood following challenge inoculation	1/9	3/3	0/5	2/2
Clinical signs from challenge inoculation	3/9	3/3	0/5	2/2
Fever from challenge inoculation	3/9	3/3	0/5	2/2
Survived challenge inoculation	9/9	0/3	5/5	0/2

[a] *Equipest™ Rhône Mérieux, Lyon, France.*
[b] *Information is extracted from reference 8.*
[c] *Information is extracted from reference 7.*
[d] *= Not applicable.*
[e] *= Mouse lethal doses 50%.*

shold value has yet to be determined and promises to be difficult to estimate. Work with wild vectors is extremely complex ; colonized insects may provide a means of estimating the level.

The experimental challenge system for arthropod-borne viruses is often not representative of a natural challenge. An intravenous challenge is usually given for AHS, while the natural route of infection is by superficial bites of *Culicoides*. The subcutaneous route of inoculation of challenge virus results in a 2 to 3 day longer incubation before clinical signs appear. Information on the intradermal route of inoculation of AHSV is not available even though this route is closest to the natural route of infection. Regarding the source of the virus, challenge using infected *Culicoides* is not feasible on a routine basis due to the expense of maintaining infected *Culicoides*. As well, there would likely be a wide range of variability in the percentage of insects infected and in the actual dosage of challenge virus administered. One advantage of the *Culicoides* propagated virus that cannot be duplicated by virus produced in cell culture or suckling mouse brain virus, is that it probably represents naturally occurring populations of virus. Challenge virus derived from the blood of viremic horses may also closely resemble populations of virus occurring in nature. Viremic horse blood is preferable for challenge to viruses derived from cell culture or suckling mouse brain as these may represent selected populations of AHSV. Unfortunately, tissue culture or suckling mouse brain viruses are the most easily obtained and the most commonly used challenge viruses.

The master seed principle for vaccine production (1) centers around the development of master seed stocks for production such as virus, bacterial or cell culture master seeds. Once certified for production use, these seeds represent the starting point for production of vaccines. The highest certified passage from the master seed represents the highest passage that can be used for production. Generally for MLV vaccines, the immunogenic potential of a vaccine is determined by performing an immunogenicity test to certify the minimal immunizing dose of vaccine virus able to protect 19 of 20 vaccinated animals following exposure to challenge virus. To release a vaccine for distribution, the vaccine must have adequate virus to account for losses during shipment and storage. The "release titer" (that accounts for potency) for a vaccine virus is normally determined by adding $10^{1.5}$ to $10^{2.0}$ $TCID_{50}$ of virus to the protective dose validated in the immunogenicity test. The release titer for AHS MLVs, based upon the master seed principle has not been determined, although a recommended release titer for tissue culture MLVs is stated as 10^5 plaque forming units of virus per dose (12).

Currently, there is no standardized laboratory potency test for inactivated AHS vaccines. A sucrose gradient method of estimating the amount of AHSV particles, similar to that used for the estimation of intact viral particles in foot-and-mouth disease vaccines, has been used (2). Inoculation of laboratory animals (mice or guinea pigs) followed by an assessment of the serological response is a potential biological assay. These potency tests would have to be correlated with immunity induced in vaccinated Equidae.

African horse sickness vaccine must be available for emergency use; and it is preferable that it be produced in a laboratory operating under international standards. The OIE is striving to develop and improve standards for diagnostic techniques and vaccine production (12). International implementation of such standards could allow the use of vaccines in AHS free areas under emergency conditions. Standards for these laboratories are not yet accepted internationally, but as more diseases are controlled and eradicated, the need for these standards and facilities becomes increasingly apparent.

Cryopreservation of MLVs or inactivated vaccines in a vaccine bank provides previously safety and potency tested vaccines for rapid finishing and shipment. A commercial inactivated vaccine concentrate for AHS-4 is currently cryopreserved by Rhône Mérieux (Dr. M. LOMBARD, personal communication).

The ability to differentiate AHS vaccinated from naturally infected animals is important, particularly for officials conducting a control and eradication program. The commercial inactivated vaccine does not induce antibodies to nonstructural proteins 2 and 3 (NS2 and NS3). This characteristic allows a Western blotting procedure to be used to differentiate antibodies induced with inactivated vaccines from those induced with MLV vaccines or field virus (9).

CONCLUSIONS

Vaccines for protecting animals against AHS should be safe, efficacious, and available. Safety of MLV's can be shown by backpassage studies. Efficacy of vaccines can be demonstrated by protection against clinical disease but more importantly by reducing viremia in challenged animals to a point below that necessary to infect insect vectors. A means of continuous availability must be developed from internationally approved laboratories. A recently developed diagnostic test for AHS using Western blotting can differentiate animals vaccinated with a commercial inactivated AHS vaccine from ones naturally infected or given MLV vaccine. This provides regulatory officials with useful information for the control of AHS.

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HOUSE (J.A.). Recommendations for African horse sickness vaccines for use in nonendemic areas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 77-81

African horse sickness (AHS), which causes mortality up to 95 %, is caused by orbiviruses and is transmitted by *Culicoides*. The goal of a control and eradication program for AHS is to prevent the spread of the virus via the biological vector. Control measures include slaughter of infected animals, housing of suspected infected animals in insect-proof stalls, and vaccination. Vaccination has played a key role in eradication when AHS occurred outside of Africa. Both modified live vaccines (MLV) and inactivated vaccines have been used to control AHS. An acceptable vaccine should be : safe, efficacious, and available. The vaccine should not cause disease or viremia, and the vaccine virus should not revert to a virulent virus upon backpassage in susceptible Equidae. The vaccine should protect against death and clinical signs and, most importantly, should prevent viremia in vaccinated Equidae following exposure to virulent AHS virus. The challenge inoculation system for assessing immunity to AHS is discussed. The vaccine should be readily available, implying that it is either in routine production in facilities that meet internationally accepted guidelines for biological production facilities or in a vaccine bank. Banking of cryopreserved stocks of MLV or concentrates of inactivated vaccines is a means of having AHS vaccine available for future epizootics. A recently developed diagnostic test to differentiate vaccinated from naturally infected animals provides regulatory officials with useful information for the control of AHS.

Key words : African horse sickness - Disease eradication - Inactivated vaccine - Modified live vaccine - *Culicoides* - Vector - Slaughtering.

HOUSE (J.A.). Recomendaciones para el uso de vacunas contra peste equina en zonas no endémicas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 77-81

La peste equina (AHS) presenta tasas de mortalidad de hasta 95 %. Es producida por un orbivirus y transmitida por un *Culicoides*. La finalidad de toda campaña de erradicación y de control de AHS es la prevención de la dispersión del virus mediante el vector biológico. Las medidas de control incluyen el sacrificio, la estabulación de los animales sospechosos en establos adaptados contra la protección de insectos y la vacunación. La vacunación ha jugado un papel importante en la erradicación contra la peste equina, cuando ésta se ha presentado fuera del continente africano. Para el control se han utilizado tanto vacunas vivas (MLV), como inactivadas. La vacuna adecuada debe ser : segura, eficaz y disponible. No debe provocar ni la enfermedad, ni viremia y el virus vaccinal no debe adoptar una forma virulenta durante el pasaje por un equino susceptible. La vacuna debe proteger contra la muerte y los síntomas clínicos, pero sobre todo, debe prevenir la viremia en los equinos vacunados después de una exposición a la forma virulenta del virus del AHS. Se discute el método para probar el sistema de inoculación para asegurar la inmunidad. La vacuna debe ser facilmente accesible, lo cual implica que debe de encontrarse en los bancos de vacunas, o que las facilidades de producción rutinaria deben seguir las normas aceptadas a nivel internacional para la producción biológica. Una forma de asegurar la disponibilidad de la vacuna contra AHS, es la crio-conservación de stocks de MLV o de concentrados de vacunas inactivadas. El desarrollo reciente de una prueba diagnóstica que permite la diferenciación entre una forma de infección vaccinal de la forma natural, permite la determinación de patrones reguladores, de gran utilidad para el control de AHS.

Palabras claves : Peste equina - Erradicación de enfermedad - Vacuna inactivada - Vacuna viva - *Culicoides* - Vector - Sacrificio.

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Role of interferons in infectious diseases in the bovine species : Effect on viruses and rickettsias

TOTTÉ (Ph.), DE GEE (A.L.W.), WÉRENNE (J.). Le rôle des interférons dans les maladies infectieuses du bovin : leurs effets sur les virus et les rickettsies. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 83-86

Le traitement aux interférons (IFN) fournit une protection chez le bovin contre certaines infections virales expérimentales. L'efficacité du traitement a été démontrée contre des infections par le virus de la vaccine et par un rotavirus. En revanche, des infections par le virus de l'herpès bovin, BHV1 (cause de la rhinotrachéite et partiellement du complexe de la fièvre de transport), ne sont pas inhibées par IFN. Le rôle d'IFN dans la résistance des bovins à *Cowdria ruminantium* a été étudié au Zimbabwe. Une bonne corrélation a été trouvée entre la production d'IFN par l'animal après l'infection et sa résistance contre la rickettsie. Cela pourrait indiquer un rôle d'appui des interférons et d'autres cytokines.

Mots clés : Bovin - Interféron - Rickettsiales - Virus - Rotavirus - Herpèsvirus bovin - *Cowdria ruminantium* - Résistance aux maladies.

INTRODUCTION

The authors were, about 10 years ago, able to evaluate the protective activity of recombinant interferons against viral infections in calves (2, 3, 7, 8, 9). Success was obtained in vaccinia and rotavirus infections. But as observed by us and confirmed by others, Interferon was shown to be unable to inhibit the replication of bovine herpes virus 1, even if immunomodulatory effects of the injected interferon appeared to reduce the mortality of superinfection with Pasteurella.

The observation of the authors with rotavirus indicates that the infection itself is able in some situations (infection with extremely high amount of virus (5)) to induce endogenous interferon that exerts an inhibitory effect against the pathogenic effect of the virus. This was the first indication that an interplay of cytokines could play an important role in the natural resistance of cattle against infections.

As interferons have been shown in mouse and human systems to play an important role in the resistance against obligate intracellular parasites, including Rickettsiales (1) the authors undertook an *in vitro* study some years ago to evaluate the importance of this system in cowdriosis .

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MATERIALS AND METHODS

In vivo Cowdria infection, observation of symptoms and of interferon induction

During a vaccination campaign, a group of heifers was infected with *Cowdria ruminantium*, blood from infected goats, injected intravenously, without any antibiotic treatment. Rectal temperature was recorded daily and blood was collected for determination of interferon activity by the classical method of reduction of the cytopathogenic effect of VSV (4).

2-5A Synthetase titration

2-5A Synthetase activity was assayed in the cytoplasmic fraction of the cells. The cytoplasmic fraction was prepared as follows :

- the cells were washed twice with buffer A (NaCl 140 mM, Tris HCl pH 7.5, 35 mM) after trypsinization (all steps at 4 °C) ;

- cells were broken with a Dounce homogeniser in buffer C (10 mM Tris HCl pH 7.5, 1.5 mM magnesium acetate, 1mM DTT, 1 mM benzamidine, 100 µM PMSF) with glycerol 10 % and Triton X100, 0.5 % ;

- lysates were centrifuged for 15 min at 13,000 g in an Eppendorf microcentrifuge and the supernatant containing the cytoplasmic fraction of the cells was stored at -70 °C ;

- 2-5A Synthetase was assayed by the procedure reported before (6). Essentially, incubation of the extract was performed in a reaction mixture containing (in 15 ml volume), 35 mM magnesium acetate, 2 mM fructose-1.6 biphosphate, 1mM DTT, 8 mM ATP, 8 µCi ^{3}H ATP, 15 mM Hepes buffer and 15 µg/ml dsRNA was added to the sample (15 µl of reaction mixture and 10 µl of sample) ;

- the tritiated 2-5(A)n are separated from ^{3}H ATP by ion exchange on DEAE Paper (DE 81 from Whatman) after treatment of the mixture reaction with the bacterial alkaline phosphatase.

The radioactivity of the 2-5A synthetized is determined by liquid scintillation. The results are expressed in pmoles of ATP incorporated per hour and per µg of protein .

Interferons used

Recombinant bovine interferons used for *in vitro* were kindly supplied to us by Dr A. Shafferman, Israel Institute of Biological Research (BoIFN αC and BoIFN αD), or by Dr R. STEIGER, CIBA-GEIGY (BoIFNα1, and BoIFNγ). The human interferon, IFN α2 was a gift from Dr. C. WEISSMANN, Zürich University.

RESULTS AND DISCUSSION

Correlation between resistance to *Cowdria ruminantium* and IFN induction

In the experimentation undertaken with a group of animals that were infected with the rickettsia and not treated with antibiotics, we observed, as expected, a number of death from the disease. Some animals however resisted the infection without treatment. All of these showed an early induction of an antiviral activity corresponding to interferon, soon after infection, before the rise of temperature. This is in contrast to what was observed with animals which did not survive the infection : they did not produce significant amounts of interferon before the rise in temperature.

The figure 1 presents in function of time after the *Cowdria* infection, the production of interferon as measured by its antiviral effect and the daily temperature as symptom of the infection, in one surviving animal and in one animal that died of the infection, compared to the control. All the animals in each group present the same pattern, and the data presented here are typical. The nature of the interferon has been further characterized. Most of the antiviral activity could be ascribed to IFNα using antibodies (the data, not shown here will be published elsewhere). However we cannot exclude the induction of other cytokines: some evidence indicating that a small part of the antiviral activity is not "species specific", is in favor of such an interpretation. Moreover IFNγ which is not maintained in the circulation for a long period, would not be easily detected if produced locally.

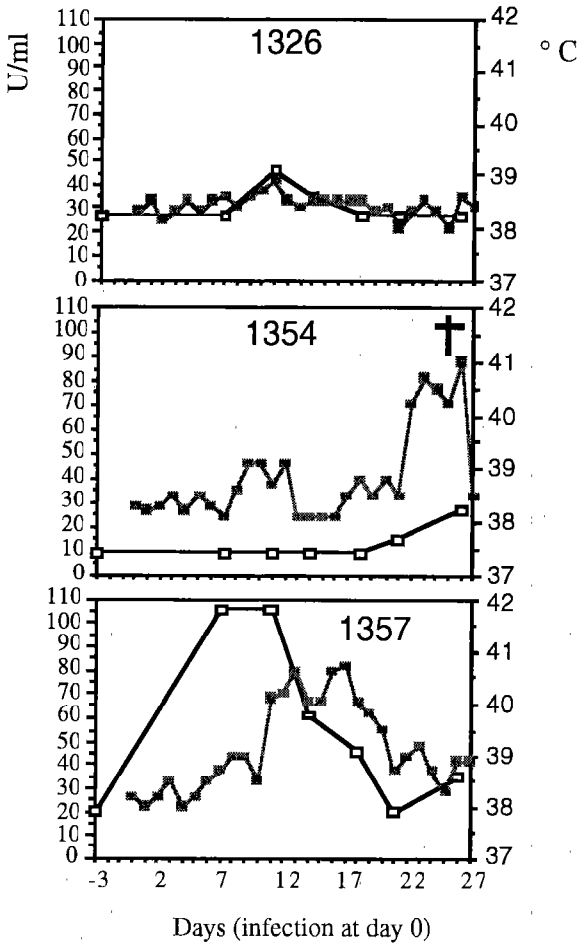

Figure 1 : Circulating antiviral activity (□) and rectal temperature (■) of C. ruminantium *infected cattle. Animal no. 1326 was included as a non infected control. Animal no. 1354 died of heartwater and animal no. 1357 survived the infection.*

Induction of 2-5A Synthetase by interferons

Interferon is acting on cells through its interaction with membrane receptors activating a signal transduction mechanism which ends up by turning on a number of genes coding for proteins playing a role in its mechanism. Twenty proteins have been detected, and among the best known of these proteins, there is a protein kinase and the 2-5A Synthetase. An inhibition in translation of viral message could result from the activity of those proteins. The kinase is involved at the level of initiation while it has been shown that 2-5A Synthetase, by the product of its enzymatic activity 2-5A is activating an RNAse. It was shown for some viruses that one of those enzymes is the main factor in their inhibition. Both of these induced proteins require the presence of dsRNA to have their pathway activated. It should therefore be pointed out that other mechanisms, not yet well understood exist.

However, we have shown that 2-5A Synthetase is a good marker to show that interferon has transmitted its message to the cell. When elevated in the cell the activity of 2-5A Synthetase indicates that the interferon system was activated.

Figure 2 shows the kinetics of induction *in vitro* of synthetase in bovine kidney cells by bovine interferons (2 IFNα and 1 IFNγ). There is some difference between the interferons, in the level of activity induced for a given antiviral

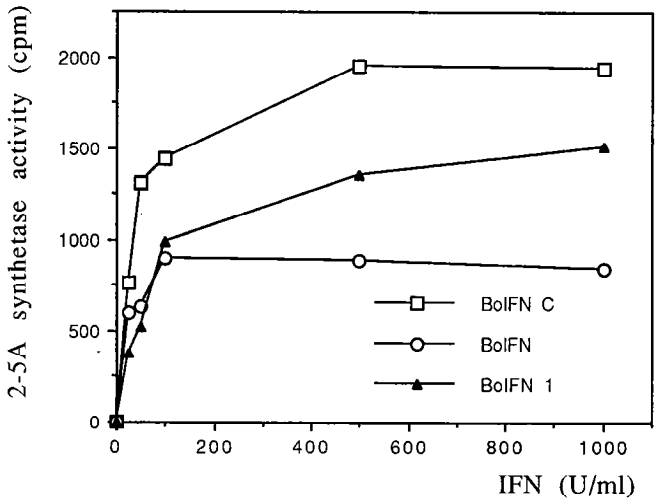

Figure 2 : Kinetics of 2-5A Synthetase induction by bovine interferons in bovine kidney cells.

activity, but they are all active (maximum activity being reached with an amount of interferon as low as 100 U/ml).

Figure 3 shows the 2-5A Synthetase inducing activity in the same cells of the plasma from calves that have been deliberately infected, compared to the activity of different recombinant IFNs. This confirms that the antiviral activity observed in the infected animals is most probably interferon for a large part.

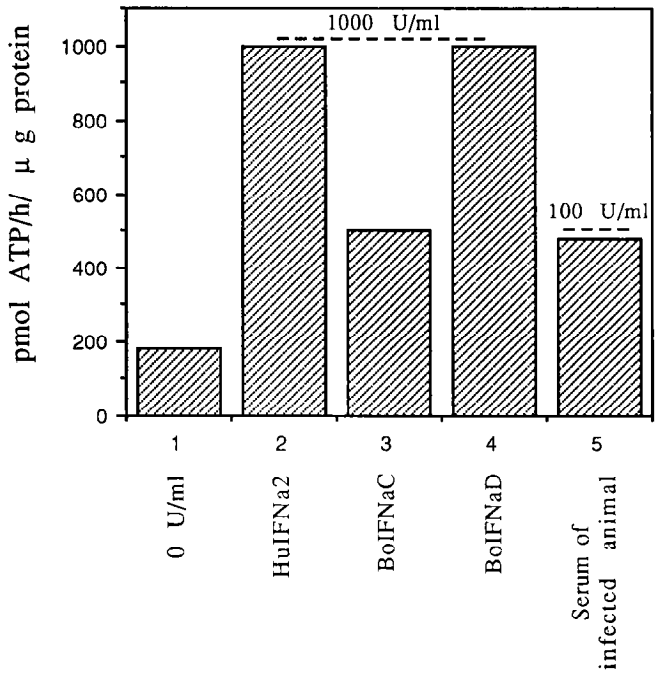

Figure 3 : Induction of 2-5A Synthetase by recombinant interferons and by the serum of an infected animal.

The authors do not imply however that the anti-*Cowdria* activity of interferon results from the activity of 2-5A. It should indeed be pointed out that bovine umbilical endothelial cells (BUEC) are insensitive to the anti-*Cowdria* activity of IFN, while bovine microvasculature endothelial cells (BMC) are very sensitive, both cells responding similarly to IFN for the antiviral activity and for the 2-5A Synthetase induction (see the other paper, "Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells").

CONCLUSION

The observations of the authors, taken together, indicate strongly that the interferon system, and probably other cytokines as well, play a key role in the natural resistance to the infection by *Cowdria ruminantium* as it does for other intracellular parasites.

While we do not imply that interferon will be part of the medication one could apply on a large scale to fight Cowdriosis, they pave the way towards understanding the mechanisms of resistance against the rickettsia, which could be of value to develop more adequate vaccines. An attenuated variant of *Cowdria* which would be a good inducer of interferon could be a good candidate for a vaccine. This is a hypothesis which would be worthwhile to test since we possess the necessary tools for such a venture.

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TOTTÉ (Ph.), DE GEE (A.L.W.), WÉRENNE (J.). Role of interferons in infectious diseases in the bovine species : Effect on viruses and rickettsias. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 83-86

Successful protection was obtained with interferon treatment in experimental viral infections in the bovine species in a number of cases. The efficacy of the treatment against vaccinia virus infection and against rotavirus infection have been demonstrated. On the contrary, bovine herpes virus 1 (BHV 1- causing rhinotracheitis and part of the shipping fever complex) infections were not inhibited by interferon (IFN). The authors have undertaken a study in cattle in Zimbabwe to assess the role of interferon in the resistance of the animals to *Cowdria ruminantium*. A good correlation between production of interferon by the animal following the infection, and the resistance of the animals against the rickettsia was demonstrated. This pointed out the possible "adjuvant" role of interferons and other cytokines.

Key words : Cattle - Interferon - Rickettsiales - Virus - Rotavirus - Bovine herpes virus - *Cowdria ruminantium* - Disease resistance.

TOTTÉ (Ph.), DE GEE (A.L.W.), WÉRENNE (J.). Papel del interferón en las enfermedades infecciosas en las especies bovinas : efecto sobre virus y rickettsias. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 83-86

En varios casos de infecciones virales experimentales en especies bovinas, se obtuvo una protección adecuada con el tratamiento con interferón. Anteriormente se demostró la eficiencia del tratamiento contra la infección por el virus vaccinia y contra la infección por rotavirus. Por el contrario, las infecciones por herpesvirus bovino 1 (BHV 1, agente causal de la rinotraqueitis y parte del complejo de fiebre de transporte ("shipping fever")), no fueron inhibidas por el interferón (IFN). En Zimbabwe, se llevo a cabo un estudio en ganado bovino, con el fin de demostrar el papel del interferón en la resistencia de los animales a *Cowdria ruminantium*. Se demostró una buena correlación entre la producción de interferón por parte del animal después de la infección y la resistencia a la rickettsia. Esto indica un posible papel de colaboración por parte del interferón y otras citoquinas.

Palabras calves : Bovino - Interferón - Rickettsiales - Virus - Rotavirus - Herpesvirus bovino - *Cowdria ruminantium* - Resistancia a la enfermedad.

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Immunization of dogs with Q fever vaccines : comparison of phase I, II and phase I CMR *Coxiella burnetii* vaccines

WILLIAMS (J.C.), PEACOCK (M.G.), RACE (R.E.). Immunisation de chiens avec des vaccins contre la fièvre Q : comparaison entre des vaccins de *Coxiella burnetii* de phase I, phase II et du RCM de phase I. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 87-94

Des vaccins contre la fièvre Q ont été testés sur des chiens de races croisées en utilisant des cellules entières de *Coxiella burnetii* inactivées à la formaline dans la phase I (CEI) ou la phase II (CEII), ou le résidu obtenu par extraction par chloroforme/méthanol (RCM) de cellules en phase I. Le vaccin CEI mélangé (1:1) à l'adjuvant incomplet de Freund (AIF) a provoqué des réponses immunitaires humorales aux antigènes des phases I et II, comme il a été mesuré par le test de microagglutination. Le vaccin RCM mélangé (1:1) à l'AIF a engendré des titres d'anticorps spécifiques aux antigènes de phases I et II plus élevés que le vaccin CEI. Le vaccin CEII a produit seulement des anticorps contre des antigènes de phase II. La durée d'un érythème et d'une induration, après des tests dermiques avec des antigènes de *Coxiella burnetii,* fait penser à une immunité cellulaire. Bien que des granulomes aient été observés, seulement avec les vaccins CEI et CEII, aucun des antigènes utilisés dans le test dermique n'a provoqué des abcès aux points d'injection. En revanche, les ganglions axillaires qui drainent le point d'injection des vaccins ont développé chez tous les chiens des abcès stériles drainants après 19 à 24 jours pour les vaccins CEI et RCM, et 104 jours pour le vaccin CEII. Les abcès se sont résolus moins de 30 jours après leur première apparition. Les réponses des lymphocytes du sang, des ganglions axillaires et mésentériques et de la rate, au Con A, à la PHA et aux antigènes utilisés, 222 jours après la vaccination, étaient variables. Les lymphocytes des organes divers ont répondu à un ou plus des antigènes de rappel et aux deux mitogènes, en l'absence ou la présence d'indométhacine. Bien que ces vaccins contre la fièvre Q aient provoqué une immunité humorale et cellulaire, des abcès stériles drainants ont été provoqués, soit par les antigènes, soit par l'AIF. Les résultats des tests dermiques font penser que le vaccin RCM est le meilleur choix comparé aux vaccins CE, étant donné l'absence de formation tardive de granulomes par le premier. D'autres études seront nécessaires pour déterminer l'origine des réactions indésirables et pour évaluer l'efficacité des vaccins contre la coxiellose des chiens.

Mots clés : Chien - *Coxiella burnetii* - Fievre Q - Vaccin - Immunisation - Réponse immunitaire - Technique immunologique - Immunité cellulaire.

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life Sciences-National Research Council. The facilities are fully accredited by the American Association for accreditation of Laboratory Animal Care.

INTRODUCTION

Coxiella burnetii causes Q fever in humans (3) and coxiellosis in animals (5). Coxiellosis in companion animals and livestock is a risk factor for the acquisition of Q fever (23). Epidemiologic and epizootiologic studies show asymptomatic and/or clinical cases of Q fever to be sporadic and linked with exposure to domestic animals (7, 11). Human infection is acquired from aerosols of *C. burnetii* during parturition in domestic animals such as Bovidae *(Bos taurus* (cattle), *Capra hircus* (goat), *Ovis aries* (sheep)) (17), Felidae *(Felis domesticus* (cat)) (9), and of Canidae *(Canis familiaris* (dog)) after eating the liver of Cervidae (deer) (6), and of wild Leporidae (rabbit) (10). Among pet owners and their contacts airborne Q fever often accounts for significant morbidity and occasional mortality in humans residing in urban, rural and feral settings.

Vaccination of humans and animals is recommended for the prevention and control of Q fever (12) because of the ubiquity of *C. burnetii* in wild and domestic animals. The immunization of humans with formalin-inactivated phase I *C. burnetii* (Henzerling strain) is efficacious (8), but booster injections cannot be safely given because of the likely induction of granuloma which may form sterile abscesses. Various Q fever vaccines are effective in decreasing the shedding of C. *burnetii* in milk, birthing fluids and tissues of animals (13). In this report, we have compared the immunogenicity and pathogenicity of phase I whole-cell (WCI), phase II WC (WCII), and phase I CMR vaccines in mixed breed dogs.

MATERIALS AND METHODS

Vaccines

Coxiella burnetii were grown in the yolk-sac of fertile hen's eggs, separated from host components, inactivated with formalin and prepared as vaccine (20, 21). Extraction of lyophilized phase I Ohio WC with C:M (4:1) was done to produce the CMR vaccine (19).

Immunization

Mixed breed dogs (3 male and 3 female) weighing roughly 23±2 kg were pre-bled and injected with vaccine as follows. On day 0, six dogs (two per vaccine) were injected i.v. with 50 µg (dry weight) of WCI or WCII or CMR in 1 ml of saline, and 50 µg (2 x 25 µg) s.c. in the region of the axillary nodes with vaccine suspended (1:1) in Freund's incomplete adjuvant (FIA) (Difco Laboratories, Detroit, MI, USA). On day 14, each dog was bled and injected i.m. in the left rear upper leg with 100 µg of homologous vaccine suspended in FIA. Animals were evaluated daily for signs of adverse reactions (*i.e.*, physical abnormalities, erythema, induration and abscess) at the injection site up to 221 days following vaccination.

Animals, previously fasted for 24 hours, were anesthetized with sodium pentobarbital (Nembutal sodium-Abbott, Chicago Ill.) administered intravenously (50 mg/5 lbs body weight). Three-fourths of the calculated dose was given rapidly and the rest as needed to induce a surgical plane of anesthesia. The animals necks were shaved and then thoroughly scrubbed with Phisohex disinfectant soap and alternately swabbed several times with 95 % alcohol and zephiran chloride (Sterling Drug Co. New York, N.Y. USA). Blood was then collected and the dogs simultaneously exsanguinated by jugular transection. Prescapular and mesenteric lymph nodes and spleen sections were immediately obtained aseptically. About one gram of each tissue was dissociated by carefully forcing the cells through a stainless steel screen while immersed in physiologic buffered balanced salt solution (PBBS). The cells were washed three times with PBBS and viability determined by trypan blue exclusion. These unfractionated cells were adjusted to a concentration of 5 x 10^6/ml in RPMI 1640 (Grand Island Biological Co., Grand Island, New York) containing 5 % autologous serum and 1 µg/ml gentamicin.

Immunological assays

Humoral anti-*C. burnetii* antibodies were evaluated by a microagglutination assay (MAA) (4). The response of splenic and nodal (axillary and mesenteric) lymphocytes to recall antigens (WCI, WCII, and CMR at 0, 1, 10, and 50 µg per ml) were evaluated. Whole blood was diluted 1:30, a dilution previously determined to be optimal (data not shown), in the RPMI medium without serum and containing 1 µg/ml gentamicin. The effect of indomethacin (IND) (16) (1, 10, and 50 µg per ml) on dog lymphocyte proliferation assay (LPA) was assessed in the absence and presence of the recall antigens, and in the absence and presence of concanavalin A (Con A) (0.1, 1, and 10 µg per ml), and phytohemagglutinin (PHA)(1:20, 1:200, and 1:400 dilution) at 37°C in a 5 % CO_2 incubator for 5 days. After 4 days incubation in microtiter plates, 1 µCi of [^{3}H]TdR (specific activity, 5 Ci/mmol ; Amersham Corp., Arlington Heights, Ill.) was added to each well and incubation was continued for another day. [^{3}H]TdR labelled DNA was collected on micro-fiber class filters and the CPM per filter was determined.

Responses of nucleated cells under various experimental conditions were expressed as stimulation indices (SI) as follows : SI = (CPM in IND and/or mitogen and/or recall antigen stimulated cells ÷ CPM in unstimulated cells - background CPM).

Skin test procedure

Dermal hypersensitivity testing consisted of the i.d. injection of 0.1 ml of vaccine at various dilutions. The right side of each dog was shaved from the midline between the legs and down the side to a position that accommodated three rows of the diluted vaccines. Animals vaccinated with WCI or CMR were skin tested with 10, 1, 0.1, and 0.01 µg of WCI and CMR, and 100, 10, 1, and 0.1 µg of WCII. Animals vaccinated with WCII were skin tested with only WCII at 100, 10, 1, and 0.1 µg. The diameter in mm of erythema was measured with a ruler. The skin thickness in mm of induration was measured with a skin calliper.

RESULTS

Adverse reactions

Between days 19 and 24 after the injections of the WCI or CMR vaccines, the dogs began limping on their left rear leg. Significant swelling (i.e. ≥ 5 mm) was noted at the i.m. and s.c. injection sites. Slight erythema was present over the injection sites. Animals injected with WCII developed similar lesions 104 days afterwards. Within another 24 h after observing the lesions all of the swollen areas had formed abscesses, which erupted and began to drain. The fluid collected from each of the abscesses did not have an odor and no microorganisms were observed by standard bacteriological techniques. To prevent secondary infection, all of the animals were treated with injectable penicillin and streptomycin. The lesions resolved spontaneously within 30 days.

Humoral immune response

The temporal sequences of anti-phase I and anti-phase II antibodies after the injections of vaccine were compared (table I). Animals did not have titers to the antigens prior to vaccination. WCI vaccinated animals developed antigen specific antibodies to both phase I and phase II antigens. Application of the skin test antigens caused an increase in antiphase II antibodies in only one dog. WCII vaccinated animals developed antigen-specific antibodies

TABLE I Humoral immune response of mixed breed dogs to Q fever vaccines.

Vaccine	Days after vaccination and skin test[a]		Microagglutination titer			
			PhII	PhI	PhII	PhI
	Dog 1	*Dog 2*	*Dog 1*		*Dog 2*	
Phase I	0	0	< 2	< 2	< 2	< 2
	14	14	128	128	128	64
	24	24	128	128	256	128
	32	32	128	128	128	128
	95	95	32	64	32	64
	220[b]	220	32	32	64	64
	227	227	32	64	64	64
	234	234	64	64	256	64
	Dog 3	*Dog 4*	*Dog 3*		*Dog 4*	
Phase II	0	0	< 2	< 2	< 2	< 2
	14	14	512	< 2	1024	< 2
	24	27	512	< 2	512	< 2
	32	125[c]	512	< 2	64	< 2
	95	132	8	< 2	64	< 2
	220[c]	138	16	< 2	512	< 2
	227		32	< 2		
	234		64	< 2		
	Dog 5	*Dog 6*	*Dog 5*		*Dog 6*	
CMR	0	0	< 2	< 2	< 2	< 2
	14	14	256	32	1024	16
	27	27	128	32	2048	1024
	125[b]	125	128	4	256	16
	132	132	64	64	128	64
	138	138	512	256	512	1024

[a] *Day that animals were bleed for the detection of humoral immune responses to* Coxiella burnetii.
[b] *Animals skin tested with 11.1 µg phase I, 111.1 µg phase II and 11.1 µg CMR.*
[c] *Animals skin tested with 111.1 µg phase II.*

to only phase II antigen. Application of the skin test antigen caused a marked increase in anti-phase II antibodies in both dogs. CMR vaccinated animals developed antigen-specific antibodies to both phase I and phase II antigens. The antibody titers induced by the CMR vaccine were markedly greater than those induced by the WCI vaccine. Application of the skin test antigens caused a marked increase in both anti-phase I and anti-phase II antibodies in both dogs.

In vitro cellular immune responses

Cellular immune responses were evaluated in vitro 221 days after vaccination. The effect of IND (0, 1, 10, 50 µg/ml) on the lymphocytes of a normal dog was determined in the presence of Con A (0, 0.1, 1.0, 10 µg/ml), PHA (0, 1:20, 1:200, 1:400), WCI, WCII and CMR (all antigens at 0, 1, 10, 50 µg/ml).

Blood lymphocytes

The normal dog lymphocytes responded optimally to the mitogens, Con A and PHA, and IND at 1.0 µg, 1:20 dilution and 1.0 µg, respectively. The mitogenic activity of the antigens revealed no change from 0 to 50 µg for WCI in the presence or absence of IND, but both CMR and WCII induced an optimum response at 1.0 µg with IND at 1.0 µg. The lymphocytes from vaccinated animals responded optimally with the same concentrations of mitogen and antigen combinations.

Splenic and lymph node lymphocytes

The normal dog lymphocytes responded optimally to the mitogens, Con A and PHA, and IND at 1.0 µg, 1:20 dilution, and 1.0 µg, respectively. The antigens had optimal mitogenic activity at 1.0 µg in the presence of IND at 1.0 µg. The lymphocytes from vaccinated animals responded

similarly to mitogen and antigen combinations. Was compared the SI for each of the animal's lymphocytes without IND and with 1.0 µg IND (table II). Although the lymphocytes from each vaccinated animal responded with different SI values to mitogens and antigens in the presence or absence of IND, the animal's lymphocytes responded with a 2-fold or greater increase in activity to at least one of the recall antigens. The observed variability between dog lymphocytes and organs was expected from these mixed breed dogs.

TABLE II Comparison of **in vitro** *cellular immune response of dogs vaccinated with Q fever vaccines*

Animal mitogen or antigen	Response as SI without/with indomethacin			
	Whole blood	Spleen	Axillary nodes	Mesenteric nodes
Control				
Con A	38.4 / 81.5	22.4/24.5	66.4/99.7	242.8/348.7
PHA	13.4/37.4	15.9/28.5	19.8/23.7	172.8/181.4
CBOI	1.2/1.3	0.3/2.8	1.1/3.0	1.1/1.7
CMRI	0.6/1.2	0.5/4.7	1.1/4.1	1.2/1.3
Phase II	0.7/1.1	0.4/1.8	0.7/1.6	1.1/0.9
Phase I Vac				
Con A	15.7/16.5	6.4/1.1	40.5/158.1	265.5/211.5
PHA	8.1/10.1	10.0/4.6	41.0/74.2	187.6/67.6
CBOI	0.7/1.0	5.2/4.2	5.6/5.3	2.5/1.0
CMRI	0.7/1.0	6.3/6.9	7.4/4.8	3.3/0.8
Phase II	0.6/1.0	4.0/3.9	3.7/6.7	0.8/1.3
Phase I Vac				
Con A	5.9/1.3	30.5/1.2	4.7/0.3	70.3/0.9
PHA	4.0/1.1	0.6/0.6	4.0/1.3	10.5/0.7
CBOI	0.4/0.5	0.4/1.1	1.4/1.0	2.0/1.6
CMRI	1.0/0.4	0.3/1.4	0.7/0.9	0.9/2.1
Phase II	0.7/0.5	0.9/0.9	1.0/1.3	1.0/1.1
CMR Vac				
Con A	15.1/18.1	0.4/2.5	62.4/55.9	85.9/290.4
PHA	10.9/6.7	3.2/6.5	36.5/38.8	73.1/155.6
CBOI	0.5/1.2	3.3/4.2	3.3/2.4	0.5/1.8
CMRI	0.6/0.9	3.6/5.0	3.3/2.6	0.8/1.8
Phase II	0.7/0.4	2.1/6.0	3.7/2.9	0.8/1.0
Phase II Vac				
Con A	10.7/14.4	22.0/6.5	114.9/67.1	339.1/338.4
PHA	9.8/9.6	19.1/8.5	27.0/18.0	78.1/48.7
CBOI	0.4/1.2	2.0/1.5	1.2/0.5	0.9/0.6
CMRI	0.6/0.3	6.4/2.4	1.0/0.8	0.5/1.0
Phase II	0.4/1.8	1.2/2.2	2.6/1.0	1.0/0.6
Phase II Vac				
Con A	18.8/0.2	6.6/1.0	36.6/0.9	54.6/1.1
PHA	7.0/1.1	2.2/0.9	11.6/1.1	10.2/2.2
CBOI	0.7/1.1	1.9/1.0	0.8/1.3	1.1/1.2
CMRI	0.6/1.3	1.0/1.0	1.2/1.0	0.3/1.1
Phase II	0.5/1.1	0.5/1.0	0.6/0.8	1.3/0.7

In vivo skin test responses

As a test of their ability to elicit skin test responses, the vaccine antigens were tested in each of the dogs (tables III to VII). No erythema was observed when saline was used at the injection site. Therefore, any erythematous reaction was graded as positive. Because of the variation in skin thickness measurements any change of ≥ 20 mm was graded as significant. Animals vaccinated with WCI showed early (4 h) and late (96 h) erythema, and only late induration responses to all three skin test antigens. Animals vaccinated with WCII showed early and late erythema, and only late induration responses to WCII skin test antigen. The other two antigens were not tested in WCII vaccinated animals because the authors did not want to complicate the humoral immune response profile by injecting animals with phase I antigens. Animals vaccinated with CMR showed only early erythematous, and only early induration responses, but no late responses with all three skin test antigens.

TABLE III Comparison of skin test reponses in dog No. 1 vaccinated with the WCI vaccine.

Hour after skin test	Skin test antigen	Response (mm erythema/induration)			
		10	1	0.1	0.01
0	PHASE I	0/73	0/72	0/90	0/130
4		21/75	0/75	0/93	0/130
24		0/86	0/79	0/90	0/130
48		0/79	0/75	0/91	0/130
72		0/80	0/79	0/86	0/130
96		30/99	0/94	0/84	0/130
120		0/84	0/84	0/93	0/130
0	PHASE II*	0/69	0/69	0/75	0/81
4		15/74	0/77	0/79	0/103
24		0/82	0/79	0/79	0/86
48		0/76	0/71	0/77	0/96
72		0/84	0/76	0/78	0/93
96		150/130	72/100	0/83	0/85
120		0/101	0/85	0/83	0/85
0	CMR	0/57	0/66	0/70	0/85
4		77/74	0/79	0/78	0/88
24		0/79	0/76	0/81	0/96
48		0/69	0/69	0/77	0/93
72		0/76	0/79	0/81	0/94
96		156/130	49/76	0/85	0/83
120		0/117	0/86	0/83	0/74

* *The skin test dose was 100, 10, 1, and 0.1 rather than 10, 1, 0.1, and 0.01 µg.*

TABLE IV Comparison of skin test reponses in dog No. 2 vaccinated with the WCI vaccine.

Hour after skin test	Skin test antigen	Response (mm erythema/induration)			
		10	1	0.1	0.01
0	PHASE I	0/45	0/45	0/45	0/53
4		100/56	104/59	36/60	0/78
24		150/49	49/49	0/46	0/69
48		0/47	0/49	0/57	0/69
72		0/52	0/49	0/52	0/71
96		140/64	0/61	0/56	0/69
120		0/54	0/55	0/53	0/66
0	PHASE II*	0/42	0/42	0/45	0/83
4		100/54	216/55	0/56	0/98
24		0/49	0/42	0/46	0/68
48		0/47	0/45	0/50	0/63
72		0/59	0/50	0/51	0/69
96		110/65	0/52	0/52	0/53
120		0/57	0/46	0/46	0/49
0	CMR	0/36	0/36	0/42	0/44
4		110/57	156/58	12/50	6/50
24		9/43	84/48	0/46	0/50
48		0/46	0/47	0/45	0/46
72		0/52	0/46	0/46	0/51
96		156/71	49/61	0/52	0/51
120		0/51	0/62	0/53	0/43

* *The skin test dose was 100, 10, 1, and 0.1 rather than 10, 1, 0.1, and 0.01 µg.*

TABLE V Comparison of skin test reponses in dog No. 3 vaccinated with the CMR vaccine.

Hour after skin test	Skin test antigen	Response (mm erythema/induration)			
		10	1	0.1	0.01
0	PHASE I	0/49	0/48	0/52	0/57
4		36/68	4/61	0/55	1/62
24		24/58	0/54	0/54	0/59
48		0/51	0/50	0/49	0/57
72		0/58	0/53	0/51	0/56
96		0/57	0/53	0/49	0/51
120		0/56	0/53	0/52	0/54
0	PHASE II*	0/49	0/54	0/57	0/60
4		48/80	20/75	0/60	0/61
24		42/66	0/62	0/58	0/61
48		0/62	0/61	0/57	0/60
72		0/73	0/61	0/56	0/57
96		0/71	0/68	0/54	0/53
120		0/69	0/59	0/56	0/56
0	CMR	0/48	0/51	0/54	0/56
4		48/71	0/74	0/74	0/62
24		0/53	0/55	0/62	0/64
48		0/50	0/53	0/51	0/54
72		0/60	0/52	0/55	0/57
96		0/61	0/53	0/56	0/56
120		0/59	0/53	0/56	0/55

* *The skin test dose was 100, 10, 1, and 0.1 rather than 10, 1, 0.1, and 0.01 µg.*

TABLE VI Comparison of skin test reponses in dog No. 4 vaccinated with the CMR vaccine.

Hour after skin test	Skin test antigen	Response (mm erythema/induration)			
		10	1	0.1	0.01
0	PHASE I	0/63	0/66	0/76	0/101
4		72/91	24/86	0/91	0/100
24		6/72	0/79	0/79	0/96
48		ND**	ND	ND	ND
72		ND	ND	ND	ND
96		ND	ND	ND	ND
120		ND	ND	ND	ND
0	PHASE II*	0/57	0/69	0/89	0/107
4		18/82	0/71	0/88	0/97
24		15/76	0/71	0/84	0/97
48		ND	ND	ND	ND
72		ND	ND	ND	ND
96		ND	ND	ND	ND
120		ND	ND	ND	ND
0	CMR	0/55	0/64	0/87	0/100
4		72/80	2/78	0/90	0/104
24		30/74	0/75	0/97	0/130
48		ND	ND	ND	ND
72		ND	ND	ND	ND
96		ND	ND	ND	ND
120		ND	ND	ND	ND

* *The skin test dose was 100, 10, 1, and 0.1 rather than 10, 1, 0.1, and 0.01 µg.*
** *Not done. The dog died inadvertently.*

TABLE VII Comparison of skin test reponses in dogs No. 5 and 6 vaccinated with the WCII vaccine.

Hour after skin test	Skin test antigen	Response (mm erythema/induration)			
		100	10	1	0.1
Dog 5					
0	PHASE II	0/51	0/48	0/55	0/56
4		63/62	0/60	18/63	6/59
24		15/52	0/51	0/56	0/62
48		0/48	0/48	0/52	0/52
72		0/52	0/50	0/52	0/53
96		36/71	0/54	0/53	0/54
120		0/55	0/53	0/54	0/56
Dog 6					
0	PHASE II	0/54	0/54	0/59	0/61
4		4/60	0/58	0/58	0/58
24		0/66	0/59	0/59	0/59
48		0/64	0/58	0/58	0/60
72		0/91	0/62	0/54	0/57
96		130/130	56/130	30/105	0/63
120		0/130	0/130	0/124	0/59

DISCUSSION

The enzootic cycles of C. *burnetii* infection among wild and domestic animals suggest that the only practical method of controlling the spread of the microorganism to susceptible end hosts (humans) is through vaccination of secondary reservoir hosts. Safe, immunogenic and efficacious phase I *C. burnetii* vaccines have been developed for Q fever and coxiellosis. In previous studies, scientists have noted that dogs play the role of sentinel animals because they share the home and the surrounding environment with humans (18). Carnivorous pet animals such as cats and dogs are involved in the transmission of *C. burnetii* to humans. Although eradication of *C. burnetii* from the environment is not practical, vaccination of pet animals is a method of controlling this infection in animals and preventing the dissemination of the microorganism to humans. We have presented results which indicate that Q fever vaccines may be used to immunize dogs. The level of immunity induced by these Q fever vaccines may protect dogs against *C. burnetii* infection.

A comparison of phase I Q fever vaccines in dogs has revealed that WCI and CMR induced significant humoral and cellular immune responses to phase I and phase II *C. burnetii* (tables I to VI). WCII vaccine only induced humoral responses to phase II antigen (table I), and the animals exhibited cellular immune responses to phase I and phase II antigens (table II). Although the experimental groups were small, the CMR vaccine induced higher antigen-specific antibody levels than the WCI and WCII vaccines (table I), and primed lymphocytes responded just as well as WCI and WCII vaccinated animals (table II).

Granulomas are part of the natural pathology of Q fever and coxiellosis. The induction of dermal granulomas by Q fever antigens in humans and guinea pigs is a sensitive method to detect CMI and possible adverse vaccine reactions in previously sensitized humans (1) and animals (2). The dermal skin responses of dogs sensitized with WCI, WCII and CMR vaccines have been tested (tables III to VII). The time course of erythema and induration after skin test with *C. burnetii* antigens are suggestive of immunity and granuloma formation, respectively. The early (4 hours) erythema responses were obtained with all skin test antigens regardless of the sensitizing vaccine antigen. Late (96 h) erythema responses were obtained with all skin test antigens in animals sensitized with WCI or WCII. Only the CMR vaccine sensitized animals to respond with an early induration.

Important distinctions between skin test responses were noted in the animals sensitized with the WC vaccines and the CMR subunit vaccine (table VIII). Animals sensitized with WCI or WCII vaccines developed only late induration reactions to the skin test antigens. Neither the late erythema nor the late induration reactions were obtained after skin testing the dogs vaccinated with CMR. These skin test reactions are similar to those obtained by vaccinating and skin testing guinea pigs with CMR (2). The CMR has retained the same immunogenic potential for dogs as shown for mice and guinea pigs (19, 20, 22). The humoral and cellular immune response of dogs vaccinated with CMR suggests that the CMR determinants which protect mice and guinea pigs from lethal *C. burnetii* infection may also protect dogs.

TABLE VIII Comparison of skin test reactions after vaccinating with Q fever vaccines.

Vaccine	Skin test response	Skin test activity*		
		Phase I	Phase II	CMR
Phase I	Erythema			
	Early	yes	yes	yes
	Late	yes	yes	yes
	Induration			
	Early	no	no	no
	Late	yes	yes	yes
Phase II	Erythema			
	Early	NT**	yes	NT
	Late	NT	yes	NT
	Induration			
	Early	NT	no	NT
	Late	NT	yes	NT
CMR	Erythema			
	Early	yes	yes	yes
	Late	no	no	no
	Induration			
	Early	yes	yes	yes
	Late	no	no	no

** Any erythema was graded as yes. Induration with a ≥ 20 mm change was graded as yes.*
*** NT = Not tested.*

The severe adverse reactions noted for dogs in this study were not observed in mice, guinea pigs, sheep and goats in previous studies (19, 20, 22). Either the antigens or the FIA or the combinations induced adverse immune responses unique to dogs. FIA is known to stimulate macrophages, promote uptake of antigen, enhance antibody formation, and induce granuloma formation, but not to induce CMI (15). Phase I WC and CMR also possess adjuvant-like activities which enhance non-specific resistance to various pathogens and stimulates lymphokine production in mice (14). The phase I vaccines in FIA induced abscesses earlier (i.e., by day 19 to 24) than the phase II vaccine in FIA (i.e., by day 104). This suggests that the *C. burnetii* antigens may play a role in the induction of abscesses in dogs. Even though all three vaccines induced adverse reactions, the CMR vaccine did not induce the late skin test induration reactions characteristic of WCI vaccines that induce granulomas or sterile abs-

cesses in sensitive human recipients. CM extraction was shown to remove the determinants that cause granulomas in guinea pigs (2). Moreover, the reconstitution of CMR with the CM extracted lipids restores the granuloma inducing capability of the CMR to induce severe reactions in mice (20). Perhaps the CMR-oil-and-water emulsion restored this activity in dogs.

CONCLUSION

The Q fever vaccines tested in this study were immunogenic, inducing both humoral and cellular immune responses. The CMR vaccine induced greater humoral antigen-specific antibody responses than did the WCI and WCII vaccines. Cellular immunity was induced by all three vaccines as evidenced by *in vitro* lymphoproliferative assays and by *in vivo* skin testing. The CMR vaccine as skin test antigen did not induce the late induration responses which are characteristic of *C. burnetii* WCI and WCII vaccines. Although all three vaccines caused induration and sterile draining abscesses, the abscesses resolved within 30 days. These unacceptable adverse reactions could have been induced by FIA, the antigens or the combinations. There remain further study to determine the source of these adverse reactions and to evaluate the effectiveness of these vaccines against coxiellosis in dogs.

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WILLIAMS (J.C.), PEACOCK (M.G.), RACE (R.E.). Immunization of dogs with Q fever vaccines : comparison of phase I, phase II and phase I CMR *Coxiella burnetii* vaccines. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 87-94

Q fever vaccines were tested in mixed breed dogs by vaccinating them with formalin-killed *Coxiella burnetii* whole cells (WC) in either phase I (WCI) or phase II (WCII), or the chloroform : methanol residue (CMR) subunit of phase I cells. Phase I vaccines mixed (1:1) with Freund's incomplete adjuvant (FIA) induced humoral immune responses to phases I and II antigens as measured by microagglutination assay. The CMR vaccine mixed (1:1) with FIA induced greater antigen-specific antibody levels to both phases I and II antigens than the corresponding WCI vaccine. The WCII vaccine induced antibody responses to only phase II antigens. The time course of erythema and induration after skin testing with *C. burnetii* antigens were suggestive of cell-mediated immunity (CMI). Although granulomas were observed with only WCI and WCII, none of the skin test antigens induced abscesses at the injection site. In contrast, axillary nodes draining the vaccine injection site developed sterile draining abscesses in all dogs by days 19 to 24 for the WCI and CMR, and day 104 for the WCII vaccines. The abscesses had resolved within 30 days after first appearance. Responses to Con A and PHA and recall antigens of lymphocytes from the blood, axillary and mesenteric nodes, and spleen at 222 days after vaccination were variable among dogs. Lymphocytes from various organs responded to one or more of the recall antigens and to both mitogens in the absence or presence of indomethacin. Although these Q fever vaccines induced humoral and CMI, either the antigens or FIA caused sterile draining abscesses. The skin testing results suggest that the CMR vaccine is a better alternative than the WC vaccines because of the lack of late granuloma formation by CMR. There remains further studies to determine the source of the adverse reactions and to evaluate the effectiveness of the vaccines against coxiellosis in dogs.

Key words : Dog - *Coxiella burnetii* - Q fever - Vaccine - Immunization - Immune response - Immunological techniques - Cell mediated immunity.

WILLIAMS (J.C.), PEACOCK (M.G.), RACE (R.E.). Inmunización en perros con vacunas de fiebre Q : comparación de vacunas de *Coxiella burnetii* en la fase I, fase II y del résiduo cloroformo/metanol (RCM) en la fase I. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 87-94

Se probaron vacunas contra fiebre Q en perros de razas cruzadas, mediante vacunación con células completas de *Coxiella burnetii* (CC), muertas en formalina, tanto en la fase I como en la fase II, o con células de sub-unidades de residuos de cloroformo/metanol de la fase I. Mediante mediciones por ensayos de microaglutinación, se determinó que las vacunas fase I mezcladas (1:1) con adyuvante de Freund incompleto (AFI), indujeron respuestas inmunes humorales con antígenos de las fases I y II. La vacuna RCM mezclada con AFI (1:1) indujo niveles más elevados de antígenos específicos y anticuerpos, que la CCI correspondiente, tanto con antígenos de la fase I como de la II. La vacuna CCII indujo respuestas de anticuerpos solamente con antígenos de la fase II. La duración del eritema y la induración en piel después de la prueba con antígenos de *C. burnetii,* sugieren la presencia de inmunidad celular. A pesar de que se observaron granulomas con la CCI y la CCII, ninguno de los tests antigénicos en piel indujeron abscesos en el sitio de inyección. Sin embargo, los nódulos axilares que drenaban el sitio de inyección, sí desarrollaron abscesos estériles de drenaje en todos los perros, entre el día 19 y 24 para CCI y RCM y al día 104 para las vacunas CCII. Los abscesos desaparecieron aproximadamente 30 días después de la aparición. Doscientos veintidós días post vacunación, las respuestas a Con A y PHA y la tasa de antígenos linfocitarios de llamado en la sangre, los nódulos axilares y mesentéricos y del bazo, fueron variables en todos los perros. En varios órganos, los linfocitos respondieron a uno o a varios antígenos de llamado y a ambos mitógenos, tanto en presencia como en ausencia de indometacina. A pesar de que estas vacunas contra fiebre Q inducen inmunidad humoral y celular, se observan abscesos estériles de drenaje, producidos ya sea por el antígeno o por el AFI. Los resultados de los tests en piel sugieren que la vacuna RCM es una mejor alternativa que las vacunas WC, debido a la ausencia de formacion tardía de granuloma por parte del RCM. Se recomienda la realización de estudios posteriores, con el fin de determinar el origen de las reacciones secundarias y de evaluar la eficiencia de la vacuna contra la coxielosis en perros.

Palabras claves : Perro - *Coxiella burnetii* - Fiebre Q - Vacuna - Inmunización - Respuesta inmunitaria - Técnica inmunológica - Inmunidad celular.

Poster

A prospective survey of *Leishmania infantum* in a cohort of dogs in an endemic area of South of France*

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VIDOR (E.), BISSUEL (G.), DUBREUIL (N.), MOREAU (Y.). Un suivi de *Leishmania infantum* dans une cohorte de chiens dans une région endémique du sud de la France. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 95

Afin d'évaluer la faisabilité d'un essai de vaccin contre la leishmaniose sur le terrain, une cohorte de 50 chiens mâles de race Beagle, non infectés, a été introduite dans un foyer actif de leishmaniose canine, afin de déterminer l'incidence sérologique et clinique. La prospection a été faite dans la région des Cévennes à 460 m d'altitude, dans un site sélectionné pour des considérations écologiques (densité des phlébotomes attendue, hygrométrie, chiens affectés dans les environs du chenil). Les chiens ont été maintenus dans 2 enclos de 25 animaux chacun. Ils ont été examinés mensuellement par inspection clinique et par sérologie, entre le 17 mai 1989 et mi-1991. Un diagnostic parasitologique a été entrepris à chaque cas douteux ou suspect de leishmaniose. Après deux ans, 70 p. 100 des chiens étaient séropositifs pour *Leishmania infantum* et 20 chiens montraient le tableau typique de leishmaniose canine, dont 16 sont morts. Les résultats sont encourageants. Ils permettront de mieux comprendre l'histoire naturelle de la leishmaniose chez les chiens et nous donnent un site parfait pour des essais sur le terrain de vaccins potentiels.

VIDOR (E.), BISSUEL (G.), DUBREUIL (N.), MOREAU (Y.). A prospective survey of *Leishmania infantum* in a cohort of dogs in an endemic area of South of France. *Revue Élev. Méd. vét. Pays trop.*, 1993. **46** (1-2) : 95

In order to evaluate the feasibility of *Leishmania* vaccine trial in the field, a 50 naive dog cohort was introduced in an active focus if canine leishmaniasis for determination of serological and clinical incidence. The survey was carried out in the Cevennes area at 460 m high a place selected for ecological considerations (expected sandfly, density, hygrometry, affected dogs near the kennel). The 50 Beagle males were divided into two pens of 25 where they were kept. Clinical examination and serological follow-up were done every month from May 17, 1989, to mid-1991. Parasitological diagnosis was made in each doubtful or suspicious case of leishmaniasis. Two years after the beginning of the study, 70 % of dogs were seropositive against *Leishmania infantum* and 20 dogs displayed typical picture of canine leishmaniasis, among which 16 died. The results are encouraging. They will permit better understanding of natural history of leishmanisis in dogs and will give us a perfect site for field clinical trials of potential vaccines.

VIDOR (E.), BISSUEL (G.), DUBREUIL (N.), MOREAU (Y.). Estudio prospectivo de la *Leishmania infantum* en una cohorte de perros, en una zona endémica al sur de Francia. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 95

Con el fin de conocer la posibilidad de realizar una prueba de campo, para evaluar una vacuna contra *Leishmania*, se introdujo una cohorte de 50 perros sanos en un foco activo de leishmaniosis canina, con el objetivo de determinar la incidencia clínica y serológica. El estudio se llevó a cabo en la zona de Cevennes, a 460 m sobre el nivel del mar. El sitio fue seleccionado por las condiciones ecológicas (densidad de moscas esperada, higrometría, perros afectados cerca de la perrera). Los 50 machos beagle se mantuvieron en dos encierros con 25 animales cada uno. El examen clínico y el seguimiento serológico realizaron cada mes, desde el 17 de mayo de 1989 hasta mediados de 1991. El diagnóstico parasitológico obtuvo para cada caso sospechoso o dudoso de leishmaniosis. Dos años después del inicio del estudio, 70 p. 100 de los perros se mostraron seropositivos contra *Leishmania infantum* y 20 perros mostraron un cuadro típico de leishmaniosis canina, de los cuales murieron 16. Los resultados son positivos y permitirán una mejor comprension de la historia natural de la leishmaniosis en perros, proporcionándo al mismo tiempo un excelente sitio para ensayos clínicos de campo para la evaluación de vacunas potenciales.

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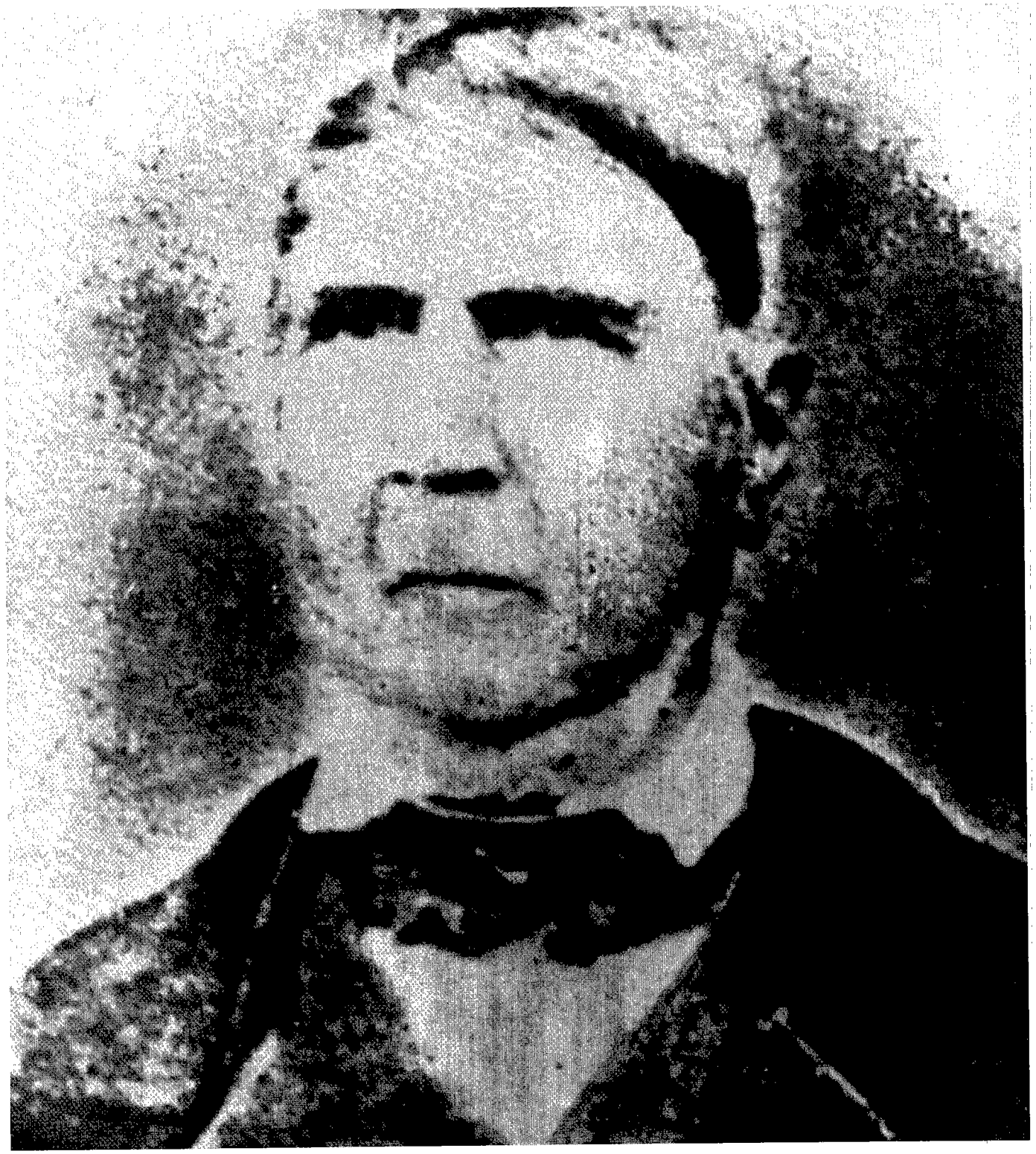
** Ce texte, dont seuls les résumés sont publiés dans ce volume, a fait l'objet d'un poster.*

Retour au menu

Session
Cowdriose

E. V. Cowdry in 1925 described the causative organism of heartwater Rickettsia ruminantium *in the tissues of ticks and animals.*



Louis Tricharad in 1838 described a disease "Nintas" which is thought to be heartwater.

The significance of recent highlights in heartwater research*

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BEZUIDENHOUT (J.D.). L'importance des acquisitions récentes de la recherche sur la cowdriose. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 101-108

Beaucoup des objectifs de la recherche sur la cowdriose, identifiés jusqu'à présent, ont été atteints pendant la décennie passée. Certains acquis, tels que la mise au point de sondes ADN spécifiques pour *Cowdria* et l'atténuation de l'organisme, sont encore au stade expérimental, mais d'autres, comme la culture *in vitro*, sont déja des procédures bien établies dans nombre de laboratoires. Des techniques sérologiques sont plus généralement utilisées depuis que d'autres méthodes et d'autres sources d'antigènes sont disponibles. Néanmoins, des réactions croisées avec *Ehrlichia* continuent à compliquer l'interprétation des données épidémiologiques. Les études biochimiques pour identifier, isoler et caractériser les protéines antigéniques de *Cowdria* ont mis en évidence des protéines immunodominantes bien définies, qui pourraient convenir à des tests sérologiques. Malgré ces progrès importants de la recherche, les méthodes pratiques pour le diagnostic de la maladie et la lutte contre elle n'ont pratiquement pas changé. La lutte est toujours basée soit sur une lutte intensive contre les tiques, soit dans certains cas sur la création et le maintien de la stabilité endémique. La méthode d'infection et de traitement utilisant du sang infecté ou le traitement prophylactique par des tétracyclines continuent à constituer l'essentiel de la lutte contre la maladie en Afrique du Sud. La cowdriose reste une menace pour le continent américain tant que *Amblyomma variegatum* sera présent dans la région des Caraïbes, et l'éradication de la tique semble indiquée.

Mots clés : Cowdriose - *Cowdria* - Tique - *Amblyomma variegatum* - Sonde à ADN - Culture *in vitro* - Technique immunologique - Antigène - *Ehrlichia* - Protéine - Tétracycline - Afrique - Afrique du Sud - Caraïbes.

INTRODUCTION

I should like to start out by thanking the organizers of the conference for the invitation to speak at this symposium. I regard it as a great honour and appreciate the opportunity to participate.

The title of my talk namely *The significance of recent highlights in heartwater research*, is hopefully broad and vague enough to allow me to speak on what ever I think is recent or what I regard as highlights and to rely on my own interpretation of what the significance of these findings may be. I fully realise that it may differ from the interpretation of some of you present here today and I hope this will come out in the discussions to be held later during the week.

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* Texte introductif à la session Cowdriose.

During the 1986 workshop on heartwater, held in the Kruger National Park in South Africa a great deal of time was spent discussing the most urgent needs for future research (6). This was done mainly to structure future research on heartwater and to assist scientists in identifying gaps in our knowledge should they be interested in studying the disease. The majority of these goals were also recognized by CAMUS and BARRÉ in their very valuable review of 1982 (13).

This paper briefly looks at those goals again and judges the progress or lack thereof that took place since the proceedings of the previous workshop were published in 1987. It should therefore not be regarded as a complete review but merely a discussion on the progress made since the last workshop with regard to the goals set (table I). The most important findings will be highlighted and their significance briefly discussed.

DISCUSSION

The organism

Due to the fact that a great deal of studies on heartwater in the future will still depend on a constant supply of organisms from cell cultures, it is important that special effort should be made to address the remaining problems in this regard such as, quantification of infection, stabilizing and preservation of infection and even possible cloning of stocks. Information on the growth requirements of endothelial cells and *Cowdria* organisms in culture is also needed urgently. The establishment of a cell line that will support the growth of the Kümm stock should also speed up comparative studies between stocks. It will also be interesting to know whether the *Ehrlichia*-like organism which has apparently transformed to a *Cowdria*, will be able to grow in culture (16, 17).

DNA studies on *Cowdria* are beginning to pay off as is evident from various reports on the subject. Genomic libraries were constructed (1) and DNA probes developed (36, 58, 62) and applied to demonstrate *Cowdria* antigen in various tick and animal tissues. The significance of this is that although a DNA recombinant vaccine for cowdriosis is not imminent, work toward this goal has begun. One should guard against unrealistic expectations regarding

TABLE I A summary of the progress made on heartwater research since the 1986 workshop held in South Africa.

Goals set at workshop	Achievements	References
Re : **THE ORGANISM** Morphology of various stocks in culture	No comparative morphological studies were performed. However, no obvious differences were noticed.	(35)
Developmental cycle	*Cowdria* appears to have a *Chlamydia*-like developmental cycle.	(35)
Identification of antigenic proteins	Immunodominant antigenic proteins were identified. Western blot technique failed to demonstrate any soluble antigens in culture medium.	(30, 48) (56)
Improved methods to stabilize and preserve the organism	Successful lyophilization of infected : — mouse tissue (Kümm & Welgevonden) — sheep blood (Welgevonden & Ball 3) — cell cultures (Welgevonden)	 (23) (23) (7)
Standardization of culture techniques	Much more standardized now but still more of an art than a science.	(5, 10, 11, 61)
Cloning and characterization of suitable cell lines	Nothing reported.	
Cloning and characterization of *Cowdria* isolates	Nothing reported. A difficult but great challenge.	
Attenuation of organisms in cell cultures	Spontaneous attenuation of the Senegal stock at Utrecht and confirmation at Guadeloupe. Other stocks did not attenuate.	(29, 38)
Establishment of more suitable cell lines, especially of ovine and caprine origin	A number of suitable endothelial cell lines of bovine and ovine origin have been established, and more than 15 different *Cowdria* stocks were cultivated in these cells.	(5, 8)
Purification of dense forms	Nothing specifically reported on the methods of purification. Infectivity of reticulate and elementary (electron dense) bodies of the Welgevonden stock were tested in mice.	(32)
Isolation of plasmids from dense forms	Nothing reported.	
Development of DNA probes	A number of *Cowdria* specific DNA probes were developed. Some were more sensitive than others. The one, pCS20, was used to demonstrate *Cowdria* antigen in ticks and animal tissues.	(36, 58, 62)
Establishment of a *Cowdria* metabolic unit	Nothing reported.	
THE HOST Reservoir status of wild and domestic animals Genetic resistance	A real breakthrough by workers who demonstrated intermittent infectivity of blood for long periods after infection. More work was done on genetic resistance in Creole Guadeloupean goats. An attempt to establish an inbred line of mice resistant to *Cowdria* failed.	(2, 12) (40) (21)
Pathology : Pathogenesis Identification of antigen antibody complexes	Cr32 specific epitopes were demonstrated on the surface of elementary bodies by immuno-gold labelling with a specific monoclonal antibody. Immunohistochemical staining of *Cowdria* in tissue sections were achieved	(32) (9)

TABLE I A summary of the progress made on heartwater research since the 1986 workshop held in South Africa (continued).

Goals set at workshop	Achievements	References
Role of vaso-active substances in increased permeability	Nothing reported.	
Quantification of infectivity	A mouse lethal dose assay was developed to test the infectivity of the Kwanyanga stocks of *C. ruminantium* in goats and ticks.	(25)
Diagnostic methods		
Rapid test for diagnosis of heartwater in live animals	DNA probe detected *Cowdria* in infected sheep blood.	(36)
Easier technique to obtain brain tissue from dead animals	Collection of brain material from dead animals using a hammer, 15 cm nail and a syringe with needle was described.	(37)
Serological techniques		
Differentiation between *Cowdria* and *Ehrlichia*	Cross reactions between *Cowdria* and most *Ehrlichia* spp. are present no matter what source of antigen is used, even in the competitive ELISA with a monoclonal antibody against the Cr32 protein.	(19)
	Complete absence of cross immunity between *E. phagocytophila* and *Cowdria.*	(34)
Comparison of serological tests	Tests were compared and good correlations found in sensitivity and specificity, however there are :	(39)
	— differences in the ease of antigen production and use,	
	— cross reactions with something other than *Cowdria.*	(19)
Isolation of monoclonal antibodies	A few monoclonal antibodies were produced.	(32)
Immunity		
Elucidation of non specific immunity in young animals	Confirmation that there is no difference in the resistance of calves born to cows fully susceptible to heartwater from that of calves bred in a heartwater endemic area.	(20)
Intra uterine infection	1 suspected case reported.	(55)
Passive immunity	All effects to neutralize infectivity of *Cowdria* by exposure to immune sera failed.	(59)
Nature of immunity	Conclusive evidence that immunity to heartwater in mice is largely cell-mediated.	(18)
Antigenic diversity and cross immunity between stocks	Marked antigenic diversity exists between stocks. Antigenicity of stock sometimes varied in different host species. No single stock has yet been identified that will protect against all other stocks. Not even a combination of stocks seems to enhance immunity.	(22, 24, 31, 33, 50)
THE VECTORS		
Distribution, biology and ecology		
Training of tick taxonomists	Not aware of any definite commitments made in this regard.	
Ecological studies on vectors of cowdriosis	In Zimbabwe the results of studies on the role of CO_2 and attraction/aggregation/attachment pheromone in host finding were utilized to improve sampling techniques and to develop a novel method for tick control. The ecology and biology of *A. variegatum* in the Caribbean especially with regards to the factors responsible for the spread of the tick were studied in depth.	(41, 43, 44, 63)
	Wild hosts of *A. hebraeum* and *A. marmoreum* were identified in South Africa.	(3) (47)
Development of a tick vaccine against *Amblyomma*	Limited success with midgut and nymphal tissues.	(15, 27, 52)

TABLE I A summary of the progress made on heartwater research since the 1986 workshop held in South Africa (continued).

Goals set at workshop	Achievements	References
Development of methods to determine the infection rate in ticks	Three additional methods for determining the infection rate have been published.	(25, 42, 62)
Sources of infection for ticks/ reservoir status of hosts/transovarial transmission	See also above. Additional findings, demonstrating the constant absence of organisms in ovarian tissues, indicates that transovarial transmission is highly unlikely to occur or play a role in the epidemiology of the disease.	(4, 42, 57, 62)
Characterization of *Cowdria* stages in the salivary gland	*Cowdria* is seldom seen in the salivary glands of infected ticks and therefore very difficult to obtain for further studies.	(26)
CONTROL ***Treatment*** Wonder drug for treatment	Rifampicin is effective against *Cowdria* infection but has not been registered for treatment.	(45)
Screening of compounds on infected cell cultures	Nothing reported.	
Improved supportive treatment	Nothing reported.	
Vaccination Improved live vaccine	Subcutaneous implant of doxicycline to control vaccination reactions (Doximplant*).	(46)
Easier route of administration	Nothing reported.	
Development of a DNA recombinant vaccine	Nothing reported.	
Development of a method to increase natural immunization through increasing the infection rate of ticks	Nothing reported. However, use of the aggregation/attachment pheromone to increase transmission rates to livestock proposed.	(42)

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the utilization of these probes as diagnostic tools, especially in certain countries in Africa. Under these conditions, farmers and stockmen get to know the disease so well that they seldom bother to have the diagnosis confirmed. The chances that they will pay for such a diagnosis is remote. Also the absence of a proper infrastructure makes the collection and testing of material, no matter what the origin may be, very difficult.

On the other hand, it is realised that DNA probes will prove invaluable in elucidating heretofore unaccessible epidemiological aspects of the disease. Such information especially the exact infection rate in ticks and the factors that influence it is important for the construction of epidemiological models which will ensure effective control strategies. The application of biotechnology for the diagnosis and control of ticks and tick-borne diseases was recently reviewed (51).

The host

In the case of host related studies there are three aspects of which recent findings have great significance. Firstly, the demonstration of a prolonged intermittent carrier state in domestic and some wild animals provides the evidence that was needed to explain how enzootic stability is created and maintained in heartwater endemic areas. Secondly, the persistent cross reactions with *Ehrlichia* in all serological tests, even in the case of the competitive ELISA which makes use of a monoclonal antibody directed specifically against the Cr32 protein (19). These findings, unfortunately, cast a shadow over all serological results of tests or epidemiological studies performed in areas where the presence of *Ehrlichia* can not be excluded. Serological surveys will therefore remain of little value until such time that the two organisms can be diffe-

rentiated accurately. Thirdly, but perhaps most importantly, are the findings that there exist marked antigenic differences between the different stocks of *Cowdria*. This stands in sharp contrast with earlier findings where no such differences, with the exception of the murinotropic stocks, could be demonstrated. There is presently not a single stock available that will give full protection against all other stocks. It appears that not even a combination of stocks will enhance the immunity (22). A further important finding is that there now exists conclusive evidence that in mice at least, immunity to heartwater is largely cell-mediated (18). This is probably also true for other animal species and will have a profound effect on the type of molecular engineered vaccine that should be developed for effective control of the disease.

The vector

Since the last workshop, vector related aspects of the disease received a surprising amount of attention and which has really produced a remarkable amount of new information. An additional experimental vector of heartwater, the American reptile tick A. *dissimile*, was identified (28). Demonstration of the infection in ticks was done using a variety of methods (25, 36, 42, 63). This no doubt is the beginning of accurate methods to determine the infection rate in ticks and which is so badly needed for the development of epidemiological models who would form the basis of more effective control strategies. A great deal of research was conducted on the role of CO_2 and attraction/aggregation/attachment pheromones in host finding. The results of these studies were utilized to improve sampling techniques for nymphs and adults of *A. hebraeum* and *A. variegatum* and for the development of a novel method for tick control (41, 43, 49, 60). The biology and ecology of *A. variegatum* in the Caribbean were studied in great detail (3). These results, to a large extent, make it possible to explain and predict the way and rate in which the vector tick and the disease have spread in that region.

Control

Despite the significant progress on almost all aspects of the disease, the treatment and control of the disease remain virtually unchanged. Apart from a real lack of information on the pathogenesis which hampers the development of improved methods for supportive treatment the lack of progress on control is ascribed to a concentration of research on new potential areas of diagnostics probes and biochemical aspects of the organism while improvement of control measures currently in use were left aside in the meantime. However the recent findings regarding attenuation of at least one stock of *Cowdria* , successful freeze-drying and improved culture techniques could, within a relatively short period of time, be combined to replaced the present blood vaccine.

CONCLUSION

Many of the goals set at the 1986 workshop were addressed during recent years and a great deal of additional information became available, some of it of real significance. The overall significance of these findings is that a strong scientific basis has been laid on which sound strategies for future control measures can be built (53, 54).

This explosion of knowledge is timely because of the ever increasing threat that the disease may spread from the Caribbean to the American mainland. Apart from a solid understanding of the epidemiological factors that are involved in this unfortunate situation, an efficient dead vaccine is what is urgently needed to protect the susceptible animal population in those threatened areas or countries.

It is becoming more and more clear that the two parts in the world where heartwater poses a problem or a threat, namely Africa and the Caribbean, need different control strategies. In the case of the Caribbean heartwater will remain a real threat to the American mainland for as long as the disease is present on any island in the region. Moreover the tick on its own or together with its capability to transmit dermatophilosis is already a major threat to the cattle population in Latin America and certain parts of the USA. Eradication of the tick rather than control of the disease appears to be the option of choice. Conclusions from research on Guadeloupe on *A. variegatum* are that eradication of the tick appears technically difficult but possible, economically profitable but socially completely utopian (14).

In Africa the eradication of the disease is not possible and therefore the strategy to limit the impact of the disease should rather focus on the creation of enzootic stability. In this instance a living vaccine would perhaps be as good or even better than a non-living vaccine because of its potential to multiply in the host and to increase the infection rates in ticks. High infection rates in ticks are one of the cornerstones of the concept of enzootic stability.

It is therefore clear that, although there are definite areas of research that overlap between these two control strategies such as the development of methods to determine the infection rate in ticks accurately and to identify carrier and immune animals, the emphasis on other aspects may differ sharply. For enzootic stability it is very important not to have a highly susceptible animal population and the identification of breeds or populations of animals with a high resistance to the disease is thus an important aspect for future research.

When it comes to the eradication of the disease, it would have been better to work with highly susceptible animals in order to monitor the presence of the disease more easily. Methods to ensure effective tick control are of course much more critical in an eradication program than in the case of enzootic stability where tick control is aimed only at controlling excessive tick numbers in order to prevent tick worry.

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J.D. Bezuidenhout

BEZUIDENHOUT (J.D.). The significance of recent highlights in heartwater research. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 101-108

Many of the objectives identified earlier, with regard to research on cowdriosis, have heen achieved during the past decade. Some contributions such as the development of *Cowdria* specific DNA probes and the attenuation of the organisms are still in the experimental stage but others, such as *in vitro* cultivation, are already well established practices in many laboratories. Serological techniques are now more widely used since other methods and other sources of antigen became available. However, persistent cross reactions with *Ehrlichia* still complicate the interpretation of epidemiological data. Biochemical studies to identify, isolate and characterize antigenic proteins present in *Cowdria* organisms revealed definite immunodominant proteins that could prove to be suitable antigens for serological tests. Despite these significant research advances, practical methods to diagnose and control the disease have remained virtually unchanged. Control is still based on either intensive tick control or in some cases on the establishment and maintenance of endemic stability. The infection and treatment method using infected blood or prophylactic treatment with tetracyclines, remains the backbone of disease control in South Africa. Heartwater will remain a threat to the American mainland for as long as *Amblyomma variegatum* is present in the Caribbean and eradication ot the tick from that region seems indicated.

Key words : Heartwater - *Cowdria* - Tick - *Amblyomma variegatum* - DNA probe - *In vitro* culture - Immunological technique - Antigen - *Ehrlichia* - Protein - Tetracycline - Africa - South Africa - Caribbean.

BEZUIDENHOUT (J.D.). Importancia de descubrimientos recientes en la investigación de la cowdriosis. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 101-108

La mayor parte de los descubrimientos concernientes a la cowdriosis se llevaron a cabo durante el siglo pasado. Algunos aspectos, como el desarrollo del ADN específico para *Cowdria* o la atenuación de los organismos, se encuentran aún en un estadio experimental. Otros, como el cultivo *in vitro*, son prácticas ya establecidas en muchos laboratorios. El uso de las técnicas serológicas ha aumentado con el acceso a otros métodos y otras fuentes de antígenos. Sin embargo, las reacciones cruzadas persistentes con *Ehrlichia*, dificultan la interpretación de los datos epidemiológicos. Mediante estudios bioquímicos de identificación, aislamiento y caracterización de las proteínas antigénicas presentes en los organismos del género *Cowdria*, se han encontrado proteínas inmunodominantes, que podrían actuar como antígenos adecuados para los tests serológicos. A pesar de estos avances, los métodos prácticos de diagnóstico y control de la enfermedad no han cambiado en forma importante. El control se basa principalmente en el control intensivo de la garrapata y, en algunos casos, en el establecimiento y mantenimiento de una estabilidad endémica. El método de infección y tratamiento, mediante el uso de sangre infectada, o el tratamiento profiláctico con tetraciclinas, son los principales métodos de control de la enfermedad en Sudáfrica. Mientras *Amblyomma variegatum* se mantenga presente en el Caribe, la cowdriosis representará una amenaza para el Continente Americano, por lo que se recomienda la erradicación de la garrapata de esta región.

Palabras claves : Cowdriosis - *Cowdria* - Garrapata - *Amblyomma variegatum* - Sonda de ADN - Cultivo *in vitro* - Técnica inmunológica - Antígeno - *Ehrlichia* - Proteína - Tetracilina - Africa - Sudáfrica - Caribe.

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STVM-93

Heartwater in Guadeloupe and in the Caribbean

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Une enquête séro-épidémiologique sur la cowdriose dans les Petites Antilles a été organisée en 1992, de la Grenade jusqu'à Saint-Martin. Un échantillon de 1 p. 100 des ruminants a été choisi au hasard et les sérums ont été analysés par ELISA indirect. Le pourcentage de sérums positifs était de 30 p. 100 à la Guadeloupe, 25 p. 100 à Antigua, 2,2 p. 100 à Saint-Martin, 1,3 p. 100 à St.Kitts et Nevis, 3,8 p. 100 à Montserrat, 1,7 p. 100 à Dominique, 1,5 p. 100 à Sainte-Lucie, 1,5 p. 100 à Saint-Vincent, 3,5 p. 100 à la Barbade, 2,9 p. 100 à la Grenade et de 7 p. 100 à la Martinique. On sait que la population de ruminants de la Guadeloupe et d'Antigua est infectée par la cowdriose. Le pourcentage faible de sérums positifs et l'absence de cas cliniques dans les autres îles suggèrent fortement que les sérums positifs dans ces îles sont problablement imputables à des réactions croisées non-spécifiques entre *Cowdria* et d'autres micro-organismes (peut-être *Ehrlichia*), qui restent à être identifiées. Cependant, il convient de porter une attention particulière aux pourcentages relativement élevés de moutons positifs à la Martinique (15 p. 100) et à Montserrat (11 p. 100).

Mots clés : Bovin - Ovin - Cowdriose - Epidémiologie - *Cowdria ruminantium* - Test ELISA - Maladie transmissible par tiques - *Amblyomma variegatum* - Guadeloupe - Caraïbes.

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INTRODUCTION

Thirteen years after the diagnosis of heartwater (12) and 10 years after the beginning of the research program in Guadeloupe, where do we stand ? What is the exact distribution of heartwater in the Lesser Antilles ?

The distribution of heartwater was previously determined using different methods:

- collection of adult *Amblyomma variegatum* on cattle and inoculation of tick supernatant into susceptible goats (1) : the islands of Guadeloupe, Marie-Galante and Antigua appeared to be infected (2, 3). This was confirmed in Guadeloupe and Antigua by post mortem diagnosis on the brain of animals which had died from heartwater ;

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- serological survey using IFAT on Kümm antigen (4) : the islands of St. Lucia, Martinique, St.Kitts & Nevis, and St.Martin were suspected to be infected because at least 5 % of the ruminants were seropositive for *Cowdria.* With an IFAT using the Gardel stock cultivated on endothelial cells as antigen, the only suspected islands with seropositive reactions over 5 % were Martinique and St.Martin (8) ;

- by testing the same sera collected in 1983 using a competitive ELISA (CELISA) followed by confirmation of positive reactions by Western blotting (11), the islands of Dominica, Grenada, Martinique, Montserrat, St.Kitts, St.Lucia, St.Martin and St.Vincent were suspected to be infected. However, the islands of Grenada and St.Vincent were not infested by *A. variegatum* and the question of the specificity of the different tests arose.

The development of an indirect ELISA (9) and of a regional project to eradicate the tick, led to a new sero-epidemiological survey in the Lesser Antilles. The objective of the survey was to determine the distribution of heartwater in the Lesser Antilles and in particular to further investigate whether the disease actually occurs in the suspected islands or not.

MATERIAL AND METHODS

Experimental design

The survey was carried out between February and September 1992 in collaboration with the veterinary services of the Lesser Antilles. Eleven islands were surveyed : Grenada, St.Vincent, Barbados, St.Lucia, Martinique, Dominica, Guadeloupe, Montserrat, Antigua, St.Kitts & Nevis, St.Martin. One percent of the overall ruminant population estimated at 200, 000 heads was sampled. The islands being divided into districts, parishes or municipalities, a cluster sampling technique was applied : 1 % of herds in each area was randomly selected and all animals within selected herds were sampled. In each herd, data were collected on the farming system including tick control methods. The breed, sex, age, presence of ticks were recorded for each sampled animal. In addition, each island was classified according to its status regarding the infestation by the tick : 0 = tick not reported, 1 = tick reported but not established, 2 = tick very limited in distribution and under control, 3 = tick limited in distribution, 4 = tick widespread.

Serological test

All sera were tested for the presence of antibodies to *Cowdria ruminantium* using an indirect ELISA (9).

RESULTS AND DISCUSSION

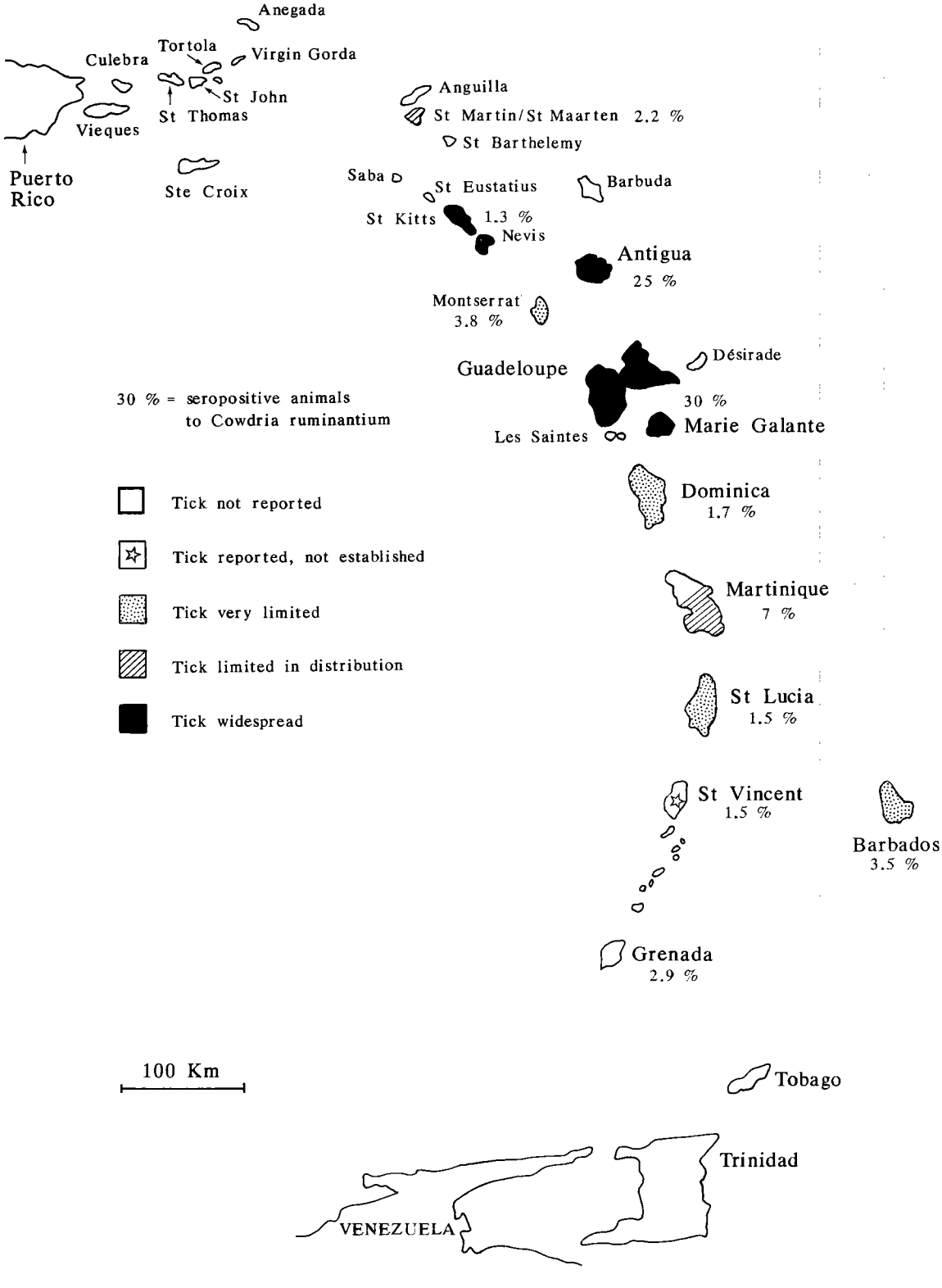
A total of 3,579 sera were collected in 11 different islands and tested for the presence of antibodies to *Cowdria ruminantium.* The results are presented in table I and map 1. Two groups of islands were identified on the basis of serological results :

- Antigua and Guadeloupe were found to have a high percentage of seropositive animals (25 and 30 % respectively). Both Antigua and Guadeloupe have a long history of

TABLE I Percentage of sera from domestic ruminants with antibodies to **C. ruminantium** *detected by indirect ELISA.*

Islands	Number seropositive/total tested (%)				Tick	Conclusion
	Cattle	Goats	Sheep	Total		
Antigua	13/102 (13)	27/76 (35)	20/65 (31)	60/243 (25)	4	HW
Barbados	3/51 (6)	1/47 (2)	3/100 (3)	7/198 (3.5)	2	0
Dominica	3/102 (3)	0/109 (0)	2/88 (2)	5/299 (1.7)	2	0
Grenada	1/60 (1.7)	3/43 (7)	1/101 (2)	5/204 (2.9)	0	0
Guadeloupe	112/592 (19)	156/295 (53)	—	268/887 (30)	4	HW
Martinique	9/297 (3)	4/99 (4)	27/176 (15)	40/572 (7)	3	?
Montserrat	0/91 (0)	0/81 (0)	10/89 (11)	10/261 (3.8)	2	?
St. Kitts & Nevis	0/50 (0)	0/47 (0)	2/56 (2)	2/153 (1.3)	4	0
St. Lucia	6/191 (3)	0/87 (0)	0/134 (0)	6/412 (1.5)	2	0
St. Martin	0/27 (0)	0/29 (0)	2/22 (6)	2/89 (2.2)	3	0
St. Vincent	3/159 (2)	0/37 (0)	1/65 (1.5)	4/261 (1.5)	1	0

Tick : 4 widespread ; 3 limited in distribution ; 2 very limited in distribution ; 1 reported, not established ; 0 not reported.



Map 1 : Distribution of A. variegatum *ticks in the Lesser Antilles and percentage of seropositive animals to* C. ruminantium.

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infestation with the tick and both have experienced clinical cases of heartwater (2, 12) ;

- in the other 9 islands except Martinique, less than 4 % of the sera were positive. This correlates well also with an apparent absence of clinical cases of cowdriosis. However, 11 and 15 % of sheep sera were found to be positive in Montserrat and Martinique respectively, raising the question of the presence of undetected cowdriosis or serological crossreactivity between *Cowdria* and other micro-organisms such as *Ehrlichia* (5, 7). The hypothesis of the presence of an *Ehrlichia* species was already explored in Martinique. In previous surveys (4), 10 % of the sera collected on ruminants in this island were positive for *Cowdria*. However, when a pool of 300 adult *Amblyomma variegatum* collected on these animals were ground and inoculated into a splenectomized Friesian heifer and an intact sheep, no fever, parasitemia or seroconversion to *Cowdria* were observed, indicating that positive sera were not due to *Cowdria* and if an *Ehrlichia* species occurs, it is not transmitted by *A. variegatum*. In the 9 above mentioned islands, the percentage of positive sera was significantly higher in sheep (3.3 %) and in cattle (2.2 %) than in goats (0.8 %). This could be explained by the presence of *Ehrlichia ovina* in sheep and *E. bovis* in cattle. The presence of E. *bovis* has been reported in Brazil (10) but *E. ovina* has never been isolated in the region. Thus the presence of these parasites in the Caribbean needs to be confirmed. In particular, in Montserrat and Martinique, isolation might be attempted by inoculating fresh blood collected from seropositive animals into susceptible ones followed by the isolation of the rickettsiae *in vitro*.

In addition to serological and clinical evidence that cowdriosis is absent from the surveyed islands except Antigua and Guadeloupe, it appears that in several islands, the populations of ticks on livestock are not high enough to allow the transmission of heartwater. Thus, no *A. variegatum* were reported in Grenada. In St.Vincent, only two male *A. variegatum* were reported in 1988 but no more since that time. St. Lucia, Dominica, Montserrat, Barbados have limited foci which are being controlled. During this survey, no *A. variegatum* were found in Dominica and Barbados, one engorged female was found in St.Lucia and one bovine infested by males was observed in Montserrat. Only St. Martin, St. Kitts & Nevis had abundant tick populations on domestic ruminants. However, seroprevalences were very low in these last islands.

The results of this survey were somewhat different from those of previous surveys. The comparison of the results between the different surveys are presented in table II. The different serological tests used (IFAT on Kümm antigen or infected endothelial cells, competitive or indirect ELISA) and the different periods of blood collecting (1982-84, 1992) may account for these differences. A recent comparison of 5 serological tests (6) showed the good sensitivity of all the tests used and the extensive cross-reactions with putative *Ehrlichia* species revealed with all 5 tests. This indicates that the influence of the serological method used was probably not essential to explain the differences between serological results. In contrast, in several islands the situation regarding the infestation by *A. variegatum* has changed between the 2 surveys :

- in Barbados, *A. variegatum* ticks were not present

TABLE II ***Comparison between results of different seroepidemiological surveys on heartwater in the Lesser Antilles.***

Islands	Inoculation of ticks		Serological surveys (% sero+)					Tick Infestation	Conclusion
	No of ticks	Results	IFAT Kümm	IFAT Gardel	CELISA	W. Blot	ELISA		
Antigua	500	1+/5	4.3	ND	4.9	ND	25.0	4	HW
Barbados	0	ND	0.8	ND	ND	ND	3.5	2	0
Dominica	28	0/2	3.2	ND	24.0	21.3	1.7	2	0
Grenada	0	ND	2.5	ND	5.4	3.6	2.9	0	0
Guadeloupe	2 086	12+/19	24.3	ND	38.6	ND	30.0	4	HW
Martinique	500	0/6	10.7	5.9	6.7	4.2	7.0	3	?
Montserrat	0	ND	1.0	ND	2.6	2.6	3.8	2	?
St. Kitts & Nevis	225	0/4	8.0	3.3	10.6	4.6	1.3	4	0
St. Lucia	212	0/4	4.7	4.2	4.5	ND	1.5	2	0
St. Martin	107	0/6	10.3	7.5	3.2	3.2	2.2	3	0
St. Vincent	0	ND	2.6	ND	9.9	6.1	1.5	1	0
Years of sampling	1982 - 1984		1982 - 1984				1992		

ND : not determined.

during the first survey but are now established at a very low level ;

- in Dominica, ticks appeared in 1983, were present at a relatively low level during the first survey but are now under control ;

- in Martinique, ticks were very abundant in 1982, and are now very rare in spite of their wide distribution ;

- in Montserrat, ticks were absent during the first survey, were very abundant the following years but are now under control ;

- in St.Lucia, ticks were numerous during the first survey but are now under control.

These modifications of tick populations between 1982 and 1992 are likely to be responsible for the differences in the results.

CONCLUSION

The present survey strongly suggest that heartwater is absent from Dominica, Grenada, St.Kitts & Nevis, St.Lucia, St.Martin and St.Vincent. Further research is needed to confirm the absence of heartwater in Montserrat and Martinique.

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A sero-epidemiological survey for heartwater was organized in 1992 in the Lesser Antilles, from Grenada to Saint Martin. Blood from about one percent of the ruminant livestock of the islands was randomly sampled and the sera were tested with an indirect ELISA. The percentage of positive sera was found to be 30 % in Guadeloupe, 25 % in Antigua, 2.2 % in St.Martin, 1.3 % in St.Kitts & Nevis, 3.8 % in Montserrat, 1.7 % in Dominica, 1.5 % in St.Lucia, 1.5 % in St.Vincent, 3.5 % in Barbados, 2.9 % in Grenada and 7 % in Martinique. Ruminants from Guadeloupe and Antigua are known to be infected with heartwater. The low percentage of positive sera and the absence of clinical cases in the other islands strongly suggest that positive sera in these islands are probably due to non-specific cross reactions between *Cowdria* and other micro-organisms (possibly *Ehrlichia*) which remain to be isolated. In particular, the high percentages of positive sheep sera in Martinique (15 %) and Montserrat (11 %) should be further investigated.

Key words : Cattle - Sheep - Cowdriosis - Epidemiology - *Cowdria ruminantium* - ELISA - Tickborne disease - *Amblyomma variegatum* - Guadeloupe - Caribbean.

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En 1992, se organizó un estudio sero epidemiológico para la cowdriosis en las Antillas Menores, desde Grenada hasta San Martín. Se recolectaron al azar muestras sanguíneas de alrededor 1 p. 100 del hato de las islas, las cuales se examinaron mediante el ELISA indirecto. El porcentaje de sueros positivos fue de 30 p. 100 en Guadalupe, 25 p. 100 en Antigua, 2,2 p. 100 en San Martín, 1,3 p. 100 en San Kitts & Nevis, 3,8 p. 100 en Monserrat, 1,7 p. 100 en Dominicana, 1,5 p. 100 en Santa Lucía, 1,5 p. 100 en San Vicente, 3,5 p. 100 en Barbados, 2,9 p. 100 en Grenada, 7 p. 100 en Martinica. Se sabe que los rumiantes de Guadalupe y Antigua están contaminados con cowdriosis. En cuanto a las otras islas, el bajo porcentaje de sueros positivos y la ausencia de casos clínicos sugiere que los sueros positivos encontrados en éstas, podrían deberse a reacciones cruzadas no específicas entre *Cowdria* y otros microrganismos (posiblemente *Ehrlichia*) los cuales aún no han sido aislados. Se recomienda un estudio posterior, principalmente para explicar los porcentajes positivos en Martinica (15 p. 100) y Monserrat (11 p. 100).

Palabras claves : Bovino - Ovino - Cowdriosis - Epidemiología - *Cowdria ruminantium* - ELISA - Enfermedad transmitida por garrapatas - *Amblyomma variegatum* - Guadalupe - Caribe.

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Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by indirect ELISA

MARTINEZ (D.), COISNE (S.), SHEIKBOUDOU (C.), JONGEJAN (F.). Détection d'anticorps contre *Cowdria ruminantium* dans le sérum de ruminants domestiques par ELISA indirect. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 115-120

Un test ELISA (enzyme-linked immunosorbent assay) a été mis au point pour la détection d'anticorps contre *Cowdria ruminantium* dans le sérum de ruminants domestiques. Les micro-organismes cultivés dans des cellules bovines endothéliales ombilicales ont été utilisés comme antigène. Lorsque la culture a montré une lyse à 90 p.100, le surnageant a été centrifugé, soniqué et appliqué sur des plaques microtitres en polystyrène. Des anticorps ont été détectés à partir de 9 jours après immunisation expérimentale de chèvres. La sensibilité de l'ELISA, calculée sur 73 sérums de ruminants, se trouvait entre 97,3 et 98,6 p.100. La spécificité globale du test était de 97 p.100 (N = 2925). Néanmoins, la spécificité était beaucoup plus basse pour les ovins (94,4 p.100, N = 881) que pour les caprins (98,6 p.100, N = 651) et les bovins (97,8 p.100, N = 1393). Des réactions croisées, qui peuvent expliquer certaines des réactions faussement positives, ont été trouvées entre l'antigène de Cowdria et des sérums contre *Ehrlichia bovis* (1 bovin positif sur 2 infectés expérimentalement) et *E. ovina* (2 moutons positifs sur 2 infectés), mais non pas contre *E. phagocytophila*. Les variabilités intra- et inter-test étaient respectivement de 7,5 et de 7,8 p.100, ce qui montre une bonne reproductibilité de l'ELISA.

Mots clés : Ruminant - Bovin - Caprin - Ovin - *Cowdria ruminantium* - Anticorps - Test ELISA - Antigène - Sérum - *Ehrlichia bovis* - *Ehrlichia ovina* - *Ehrlichia phagocytophila*.

INTRODUCTION

The development of serodiagnostic tests for cowdriosis has long been hampered by the inability to cultivate *Cowdria ruminantium in vitro*. A capillary flocculation test (13) and a complement fixation test (5) using crude extracts of brains from infected animals lacked sensitivity. An indirect fluorescent antibody test (IFAT) was described by DU PLESSIS (6) who used mouse peritoneal macrophages infected with a mouse infective stock of *Cowdria* (Kümm stock) as antigen. The use of primary blood neutrophil cultures as antigen by Logan (21) improved the specificity of the IFAT. The detection of antibodies to *Cowdria* in ruminant sera by ELISA using parasites purified from infected ticks, tissues or blood by chromatography techniques (33) or density gradient centrifugation (28) was also reported. However, the unavailability of sufficient amounts of purified antigens by these techniques resulted in a poor sensitivity of the ELISA.

The cultivation of *Cowdria* in bovine endothelial cells *in vitro* first described by BEZUIDENHOUT *et al.* (1), provided a convenient source of antigen for use in serology. Since that time, several laboratories have developed IFAT on endothelial cells infected by *Cowdria* (34, 15, 23). Recently, partly because of high backgrounds obtained in an indirect ELISA, a competitive ELISA (CELISA) using a monoclonal antibody to the immunodominant antigenically conserved 32 kilodalton protein of *C. ruminantium* was described (16).

In this study, the development and the evaluation of an indirect ELISA aimed at being used as a single dilution test for the detection of antibodies to *C. ruminantium* in the serum of goats, sheep and cattle, is described.

MATERIALS AND METHODS

Sera

In Guadeloupe, 30 goats and 1 sheep were inoculated intravenously with 5 ml of supernatant of a bovine umbilical endothelial cell culture (BUE) infected with either the virulent Gardel stock (31), or an attenuated Senegal stock (MARTINEZ, unpublished) of *Cowdria.* Animals infected with the attenuated rickettsiae were not treated whereas those inoculated with a virulent stock were treated with oxytetracyclin (15 mg/kg/day IV for 2 or 3 days) as soon as their rectal temperature rose above 40 °C. Sera were collected at regular interval and preserved at - 20 °C until use. Eight goat sera, 2 cattle sera and 2 sheep sera were raised at Utrecht (The Netherlands) against the Senegal (14), the Welgevonden (7), the Kwanyanga (22) and the Lutale (14) stocks of *Cowdria* by the same technique. In addition, 10 sera from cattle, 10 sera from sheep and 2 sera from goats experimentally infected with either the Ball 3 (12), the Breed (8), the Kümm (6), the Kwanyanga, the Mali (19), the Mara (9) and the Welgevonden stocks of *C. ruminantium* were provided by the Onderstepoort laboratory, Republic of South Africa (10).

Negative sheep, goat and cattle sera were collected in regions without *Amblyomma* ticks and therefore free of

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cowdriosis (France, The Netherlands, Islands of Les Saintes in the West Indies), or in areas infected with A. *variegatum* but where heartwater has never been identified (Mayotte, 11 islands of the Lesser Antilles). These populations of negative sera were used for the calculation of the specificity of the test.

Experimental *Ehrlichia* infections

Infections with *Ehrlichia bovis* were monitored in 2 calves, *E. ovina* in 2 sheep and *E. phagocytophila* in one goat. Clinical manifestation of these experimental infections have been described in more detail elsewhere (17). The isolate of *E. bovis,* which caused moderate but prolonged fever in Friesian calves, originated from Kenya (26). The isolate of *E. ovina* from Turkey caused prolonged fever periods in Dutch sheep, which eventually recovered spontaneously. Finally, *E. phagocytophila* , causal agent of tick-borne fever of ruminants, was isolated from cattle on the North Sea Island of Ameland, The Netherlands (32). All isolates were stored in liquid nitrogen as infected stabilates, cryopreserved with 10 % DMSO before being used in this study. Aliquots of 2 ml deep-frozen blood were rapidly thawed and inoculated intravenously. The animals were monitored by taking their daily rectal temperature and clinical inspection. Sera were collected from all experimental animals once a week.

Antigen preparation

C. ruminantium (Gardel stock) was cultivated in bovine umbilical endothelial cell (BUE) cultures as previously described (23). When approximately 80 % of the cell monolayer was lysed, the remaining adherent cells were scraped, mixed with the supernatant and centrifuged at 2500 g for 15 min. The antigen was constituted by the pellet resuspended in sterile PBS and disrupted by 5 cycles of sonication (30 s each) interspersed by 1 min interval in an ethanol-dry ice bath. The antigen concentration was expressed in protein content which was determined by the method of Bradford.

ELISA procedure

An indirect ELISA method was used (11). Microplates (Nunc immuno-modules) were coated overnight at 37 °C with 5 µg/ml of antigen in carbonate-bicarbonate buffer 0.1 M, pH 9.5 (100 µl per well). For the assay, the plates were washed 3 times with phosphate buffer saline 0.1 M, pH 7.2, supplemented with 0.1 % tween 20 (PBS-tween, solution used for all washings). To each well was added 100 µl of test serum diluted in PBS-tween added with 3 % skimmed cow milk (Régilait). The optimal working dilutions of sera determined by checkerboard titration were found to be 1/800 for cattle, 1/400 for sheep and 1/400 for goats. The plates were incubated at 37 °C for 1 hour and washed 5 times. Rabbit anti-goat, anti-cattle or anti-sheep IgG conjugated to horse radish peroxidase optimally diluted in the blocking buffer was then added (100 µl per well) and the plates incubated at 37 °C for 1 hour. After 5 washings, each well was filled with 100 µl of citrate buffer 0.1 M, pH 5.5, containing 0.5 mg/ml O-phenylene diamine and 3 µl/ml of 9 % H_2O_2. The enzymatic reaction was stopped after 30 min of incubation at room temperature by adding 50 µl of 2N H_2SO_4 per well and the absorbance was read at 495 nm.

Sensitivity and specificity of the ELISA

To use the ELISA as a single dilution test, the threshold between positive and negative sera was determined at optimum working dilution of serum and conjugate for cattle, sheep and goats. The frequency distribution of absorbances of negative populations of sera were determined. Although these distributions were slightly skewed to the right, they were approximated normal distributions and the cut-off point value was fixed at mean absorbance of negative sera + 2.58 SD (t = 2.58 for $P < 0.01$, 24). The sensitivity and specificity of the ELISA were then calculated using these threshold values.

Precision of the ELISA

Intra and inter-assay variability were assessed using goat sera. Twelve replicates of 8 sera, the mean absorbance of which ranged between 0.28 and 1.1, were tested in each plate. One set of 3 identical plates was used each day for 3 days. The precision of the test was expressed in residual coefficient of variation (CV %) after computation of data using general linear procedures (29).

RESULTS

In the indirect ELISA described in this study, non specific binding of undesirable proteins was low (absorbance around 0.06) and the ratio between positive and negative sera was high, ranging between 3 and 20. Specific antibodies were detected between 9 and 19 days post-inoculation in all sera (n = 31) of animals experimentally infected with the Gardel and the Senegal stocks of *Cowdria* in Guadeloupe. The overall sensitivity of the ELISA calculated with the sera from Guadeloupe in addition to experimental sera from The Netherlands and the Republic of South Africa was 100 %. However, when field sera from RSA which were considered positive on the basis of the resistance of the animals to challenge were taken into account, the sensitivity of the ELISA ranged between 97.3 % and 98.6 % (table I).

TABLE I Sensitivity of the ELISA.

Animal specie	Origin of sera	No. of sera	No. positive sera	Sensitivity
Cattle	Rep. South Afr. Netherlands	10 2	10 2	100
Goats	Rep. South Afr. Netherlands Guadeloupe	10[1] 8 30	8/9 8 30	95.8/97.9
Sheep	Rep. South Afr. Netherlands Guadeloupe	10 2 1	10 2 1	100
Total general		73	71/72	97.3/98.6

[1] *Eight sera were collected in the field and considered positive on the basis of the survival of the animal to challenge, the other 2 sera were from experimental infection.*

The results on the specificity of the test are summarized in table II. The overall specificity calculated with 2,925 sera was 97 % indicating an overall percentage of false positive sera of 3 %. However, if we consider the 3 species separately, the specificity was far lower for sheep (5.6 % of false positive sera) than for goats and cattle (1.4 % and 2.2 % of false positive sera respectively). Because of the existence of positive sera in areas infected with *A. variegatum* but free of cowdriosis, the cross-reactivity with *Erlichia* species some of which are known to be transmitted by this tick *(E. bovis)*, was investigated. A seroconversion was revealed by the ELISA on *Cowdria* antigen in 1 out of 2 cattle inoculated with *E. bovis* (figure 1) and in 2 sheep inoculated with *E. ovina* (figure 2). No crossreaction was observed in 1 sheep infected with *E. phagocytophila* (figure 2).

As shown in figure 3, the intra-plate variability expressed in CV % decreased when the absorbance of the serum increased. The values of intra and inter-assays variability were 7.5 % and 7.8 % respectively, indicating that within plate variations represented the main part of the variability.

TABLE II Specificity of the ELISA.

Animal specie	Origin of sera	No. of sera	No. positive sera	Specificity
Cattle	Rep. South Afr. France Netherlands Mayotte West Indies	5 30 10 320 1028	1 (20) 0 (0) 0 (0) 5 (1.6) 25 (2.4)	80 100 100 98.4 97.6
	Total for cattle	1393	31 (2.2)	97.8
Goats	Rep. South Afr. Les Saintes (FWI) West Indies	5 67 579	0 (0) 1 (1.5) 8 (1.4)	100 98.5 98.6
	Total for goats	651	9 (1.4)	98.6
Sheep	Rep. South Afr. Netherlands Les Saintes West Indies	5 10 35 831	0 (0) 0 (0) 1 (2.9) 48 (5.9)	100 100 97.1 94.2
	Total for sheep	881	49 (5.6)	94.4
Total general		2 925	89 (3)	97.0

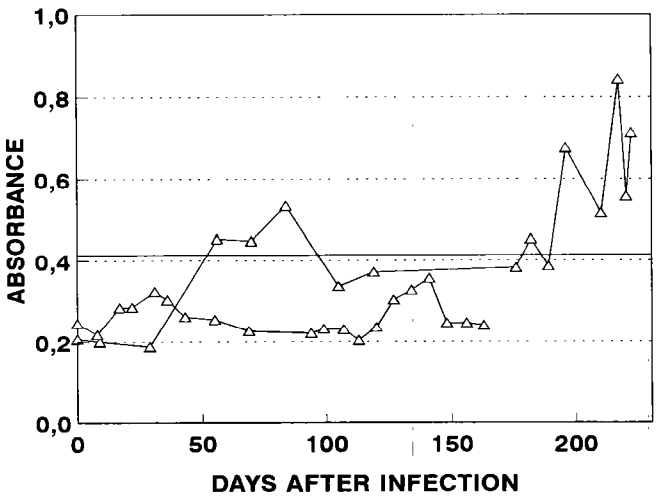

Figure 1. Kinetic of antibody levels of 2 cattle inoculated with Ehrlichia bovis *as determined by ELISA on* Cowdria *antigen.*

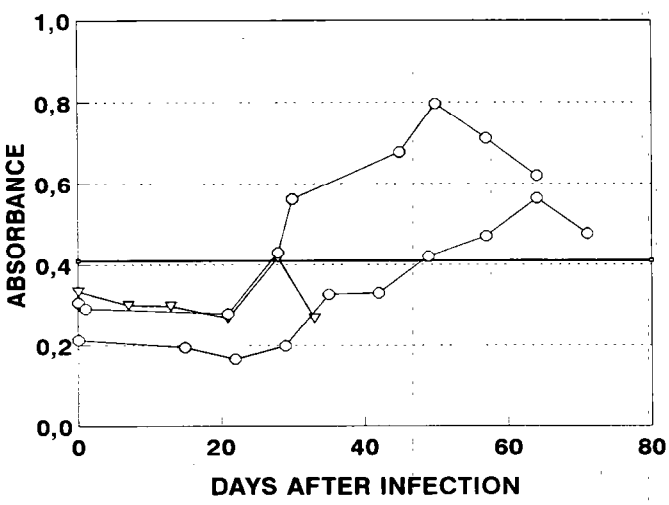

Figure 2 : Kinetic of antibody levels of 2 sheep inoculated with Ehrlichia ovina *(o) and 1 sheep inoculated with* E. phagocytophila *(Δ) as determined by ELISA on* Cowdria *antigen.*

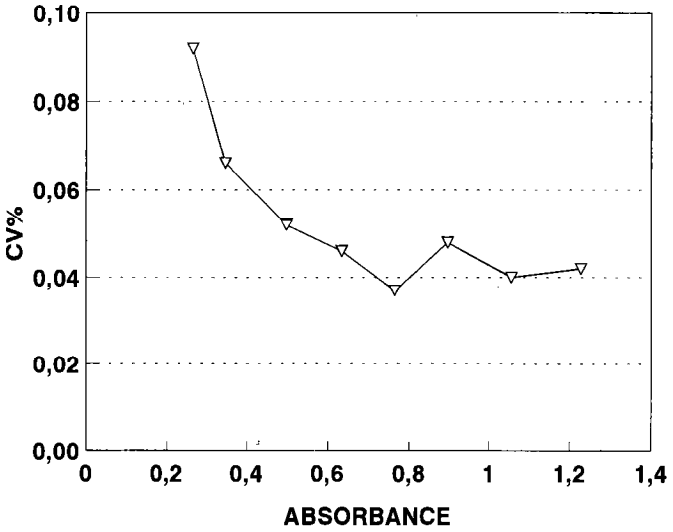

Figure 3 : Evolution of intra-plate variability of the ELISA expressed in residual coefficient of variation, as a function of the absorbance of the serum. Each point represents the mean of 72 measures.

DISCUSSION

In this study a single dilution indirect ELISA was described. Its reproducibility was good compared to other enzyme immunoassays (18). The variability of the measures was lower when the absorbance of the serum increased (figure 3), justifying the utilization of 8 sera ranging from low to high absorbance to evaluate the precision of the method. In contrast to other attempts in developing a similar ELISA, the problems of high backgrounds were easily overcome by using a convenient blocking agent such as skimmed cow milk.

The sensitivity was good, the ratio between positive and negative sera ranging between 3 and 20. The overall sensitivity calculated with 73 sera from sheep, goats and cattle varied between 97.3 % and 98.6 % (table I). However, amongst these 73 positive sera, the sera which were found negative (1 or 2 depending on the assay) were collected in the field from goats which were considered immune on the basis of their resistance to challenge (10). However, resistance to a virulent infection does not constitute an absolute criterion of previous contact with the parasite since populations of goats within a breed can acquire a genetically determined resistance under selective pressure. Thus, populations of creole goats from Guadeloupe isolated from an endemic area of cowdriosis for more than 10 years and reared in tick free conditions expressed a degree of resistance of 50 % (25). If only experimental sera are taken into account, antibodies to *Cowdria* were detected in 100 % of the samples between 9 and 19 days after inoculation. The time necessary for the first antibodies to be detectable is in agreement with SEMU *et al.* (30). The existence of serotypes (15, 23) had no significant influence on the sensitivity of the ELISA which was able to detect antibodies in sera raised against 11 different stocks of *Cowdria* from all parts of Africa and from the Caribbean.

The calculation of the specificity revealed an overall percentage of false positive sera of 3 %. Previous studies conducted in the Caribbean have shown the existence of positive sera in islands where cowdriosis has never been identified in spite of active research (3). These positive sera identified for the first time by CAMUS (2) using IFAT performed on Kümm antigen were confirmed by IFAT on BUE cells infected with *Cowdria* (23) and by CELISA (27). The presence of seropositive ruminants in several islands from the Caribbean where cowdriosis probably does not occur, was confirmed in this study using the indirect ELISA (table II). In contrast, no positive serum was recorded amongst 50 sera from Europe. The specificity was good when the test was used on cattle or goat sera (97.8 % and 98.6 % respectively), but was only 94.4 % on sheep. The false positive sera were not uniformely spread in the Caribbean, but located mainly in 2 islands out of 11 tested (4). Since it is likely that heartwater does not exist in these 2 islands, crossreactivity with related microorganisms possibly associated with ticks should be considered. In this respect, crossreactivity with *Ehrlichia equi* and to a lesser degree *E. canis* has previously been shown by LOGAN *et al.* (20). As a matter of fact, in our study, crossreactivity between antisera to *Ehrlichia bovis* or *E. ovina,* and *Cowdria* antigen was revealed by the indirect ELISA. In contrast, there was no crossreaction with *E. phagocytophila.* These crossreactions had little influence when using the test to screen large quantities of goat and cattle sera in epidemiological surveys. However, in certain situations, the percentage of false positive sheep sera was too high. Serological data from epidemiological studies should therefore be interpreted carefully in this species in which there is a particular need for a more specific antigen. Besides epidemiological studies, the test proved very simple and useful to follow the antibody response of domestic ruminants in controlled experiments of vaccination.

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MARTINEZ (D.), COISNE (S.), SHEIKBOUDOU (C.), JONGEJAN (F.). Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by indirect ELISA. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 115-120

A solid phase enzyme immunoassay for the detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants was developed by using microorganisms cultivated on bovine umbilical endothelial cells as antigen. When the culture showed 90 % lysis, the supernatant was centrifuged, sonicated and coated on polystyrene microtiter plates. Antibodies were detected as early as 9 days after experimental immunization of goats. The sensitivity of the ELISA calculated with 73 ruminant sera ranged between 97.3 % and 98.6 %. The overall specificity of the test was 97 % (N = 2925). However, the specificity was far lower for sheep (94.4 %, N = 881) than for goats (98.6 %, N = 651) and cattle (97.8 %, N = 1393). Crossreactivity which could explain some of the false positive reactions, was found between Cowdria antigen and sera raised against *Ehrlichia bovis* (1 bovine positive out of 2 inoculated) or *E. ovina* (2 sheep out of 2 inoculated became positive) but not with *E. phagocytophila*. The intra-assay and inter-assay variability were 7.5 % and 7.8 % respectively, indicating a good reproducibility of the ELISA.

Key words : Ruminant - Cattle - Goat - Sheep - *Cowdria ruminantium* - Antibody - ELISA test - Antigen - Sera - *Ehrlichia bovis* - *Ehrlichia ovina* - *Ehrlichia phagocytophila*.

MARTINEZ (D.), COISNE (S.), SHEIKBOUDOU (C.), JONGEJAN (F.). Detección de anticuerpos de *Cowdria ruminantium* en el suero de rumiantes domésticos mediante el ELISA indirecto . *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 115-120

Se desarrolló una fase sólida de inmunoensayo enzimático para la detección de anticuerpos de *Cowdria ruminantium*, en el suero de rumiantes domésticos. Como antígeno se usaron cultivos de microorganismos en células de endotelio umbilical bovino. Cuando se alcanzó una lísis de 90 % en el cultivo, se centrifugó el sobrenadante, se trató por ultrasonido y se cubrió con poliestireno en placas de microtitulación. Los anticuerpos se detectaron 9 días después de la inmunización experimental de las cabras. La sensibilidad del ELISA, calculada con 73 sueros de rumiantes, se localizó en un intervalo de 97,3 a 98,6 %. La especificidad general del test fue de 97 % (N = 2925). Sin embargo la especificidad fue inferior para las ovejas (94,4 %, N = 881) que para las cabras (98,6%, N = 651) y los bovinos (97,8 %, N = 393). Las reacciones cruzadas, que podrían explicar algunos de los falsos positivos, se dieron entre el antígeno de *Cowdria* y los sueros preparados contra *Ehrlichia bovis* (1 bovino positivo de cada 2 inoculados) o *E. ovina* (2 ovejas de cada 2 inoculadas fueron positivas), pero no contra *E. phagocytophila*. Los ensayos de variabilidad intra-test y inter-test fueron de 7,5 % y 7,8 % respectivamente, indicando una buena reproductibilidad del ELISA.

Palabras claves : Rumiante - Bovino - Caprino - Ovino - *Cowdria ruminantium* - Anticuerpo - Test ELISA - Antigeno - Suero - *Ehrlichia bovis* - *Ehrlichia ovina* - *Ehrlichia phagocytophila*.

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Serological diagnosis of heartwater in Zimbabwe. Problems and perspectives *

BARBET (A.), TEBELE (N.), SEMU (S.), PETER (T.), WASSINK (L.), MAHAN (S.). Diagnostic sérologique de la cowdriose au Zimbabwe. Problèmes et perspectives. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 121

Nous avons étudié la valeur potentielle de l'immunoblotting (Western blotting) pour le sérodiagnostic de la cowdriose au Zimbabwe, utilisant des *Cowdria ruminantium* de culture comme antigène. L'anticorps dominant de la réponse des bovins infectés expérimentalement avec des isolats de *C. ruminantium* du Zimbabwe était dirigé contre un polypeptide d'environ 32 kDa. Des sérums de bovins et ovins de Floride étaient tous négatifs contre ce polypeptide, et des sérums de régions endémiques pour la cowdriose au Zimbabwe étaient positifs, ce qui a suggéré la possibilité d'utiliser cette réaction pour le diagnostic. Toutefois, un grand nombre de réactions positives à l'immunoblot a été obtenu en testant des sérums de bovins et d'ovins de régions au Zimbabwe connues pour être indemnes d'*Amblyomma* et de cowdriose. La dilution de ces sérums positifs n'a pas permis de les distinguer de sérums positifs en provenance de régions endémiques. Des moutons de régions indemnes, positifs à la réaction, étaient négatifs pour *C. ruminantium* par PCR, n'étaient pas infectieux pour des tiques, et étaient entièrement sensibles à la cowdriose expérimentale. Il est donc vraisemblable qu'il s'agit de fausses réactions croisées, causées par un organisme apparenté qui existe dans les régions indemnes de cowdriose de Zimbabwe.

BARBET (A.), TEBELE (N.), SEMU (S.), PETER (T.), WASSINK (L.), MAHAN (S.). Serological diagnosis of heartwater in Zimbabwe. Problems and prespectives. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 121

We investigated the potential value of immunoblotting (Western blotting) for serodiagnosis of heartwater in Zimbabwe using cultured *Cowdria ruminantium* as antigen. In experimental infections of cattle, the dominant antibody response against Zimbabwe isolates of *C. ruminantium* was to a polypeptide of approximately 32 kDa. Florida cattle and sheep sera were uniformly negative to this polypeptide, and sera from heartwater-endemic areas of Zimbabwe were positive, suggesting possible use of this reaction in diagnosis. However, on testing cattle and sheep sera from known *Amblyomma* and heartwter-free regions of Zimbabwe a large number of positive immunoblot reactions were obtained. These positive sera could not be distinguished from sera from heartwater-endemic regions by dilution. Sheep from heartwater-free regions showing this reaction were negative for *C. ruminantium* by PCR, did not transmit *C. ruminantium* to ticks and were fully susceptible to heartwater on challenge. It is likely, therefore, that these are false positive cross-reactions caused by a related organism present in heartwater-free areas of Zimbabwe.

BARBET (A.), TEBELE (N.), SEMU (S.), PETER (T.), WASSINK (L.), MAHAN (S.). Diagnóstico serológico de la cowdriosis en Zimbabwe. Problemas y perspectivas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 121

Se estudió el potencial inmunológico del "Western blotting" para el diagnóstico serológico de la cowdriosis en Zimbabwe. El antígeno utilizado fue *Cowdria ruminantium*. En infecciones experimentales de ganado, la principal respuesta de anticuerpos contra los aislamientos de *C. ruminantium* de Zimbabwe, se observó frente a un polipéptido de aproximadamente 32 kDa. La respuesta a este polipéptido fue negativa en ganado proveniente de Florida y en sueros de ovinos, mientras que los sueros provenientes de zonas de Zimbabwe endémicas para la cowdriosis se mostraron positivos, lo cual sugiere el posible uso diagnóstico de esta reacción. Sin embargo, se obtuvieron gran cantidad de respuestas positivas con sueros bovinos y ovinos provenientes de zonas de Zimbabwe libres de *Amblyomma* y de cowdriosis. La dilución no permitió diferenciar estos sueros positivos de aquellos provenientes de zonas endémicas. Las ovejas originarias de las zonas libres de cowdriosis mostraron una respuesta positiva a *C. ruminantium* mediante PCR, no transmitieron *C. ruminantium* a las garrapatas y fueron muy susceptibles a los tests contra la cowdriosis. A pesar de esto, se sospecha que se trata de falsos positivos, de reacciones cruzadas provocadas por un organismo similar, presente en las zonas de Zimbabwe libres de cowdriosis.

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STVM-93

The sero-diagnosis of heartwater : a comparison of five tests

DU PLESSIS (J.L.), BEZUIDENHOUT (J.D.), BRETT (M.S.), CAMUS (E.), JONGEJAN (F.), MAHAN (S.M.), MARTINEZ (D.). Le diagnostic sérologique de la cowdriose : cinq tests comparés. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 123-129

Cinq tests sérologiques, l'ELISA indirect, l'ELISA de compétition, deux tests par immunofluorescence indirecte utilisant des antigènes différents, et la technique de Western blotting, ont été comparés sur des sérums de contrôle négatifs ou positifs pour *Cowdria ruminantium* et des sérums d'animaux de régions indemnes de cowdriose. Aucun des tests ne donnait de réaction positive sur les sérums de contrôle négatifs. En dehors de variations peu importantes dans la sensibilité, il y avait une bonne corrélation entre les 5 tests. Leur spécificité reste contestée, car dans tous les 5 tests, des réactions croisées considérables ont été enregistrées avec des anticorps contre un agent non encore identifié, probablement *Ehrlichia*.

Mots clés : Cowdriose - *Cowdria ruminantium* - Diagnostic - Technique immunologique - Test ELISA - Immunofluorescence indirecte - Western blotting - Antigène - Sérum - Anticorps - *Ehrlichia*.

INTRODUCTION

The indirect fluorescent antibody (IFA) test, in which either infected neutrophil cultures (15), the peritoneal macrophages of mice infected with the Kümm stock of *Cowdria ruminantium* (MIFA) (5) or endothelial cell cultures (CIFA) (17) are used as antigen, has been employed during the past decade to detect antibodies to *C. ruminantium*. Recently a competitive ELISA, in which monoclonal antibodies to a 32-kilodalton *Cowdria* protein are used, has been developed (14).

Due to conflicting reports on the specificity and sensitivity of the IFA test in which infected peritoneal macrophages are used as antigen (17) and the degree of cross-reactions between *C. ruminantium* and *Ehrlichia* (7,13,16), a number of bovine, ovine and caprine sera known to be either negative or positive as well as sera assumed to be positive to *Ehrlichia,* were submitted to 5 tests in 4 different laboratories.

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MATERIALS AND METHODS

Sera

Quadruplicate serum samples were prepared by the Onderstepoort Veterinary Institute and dispatched to 3 other laboratories that had indicated their willingness to participate in the exercise. Known negative control sera were obtained from animals born and reared under tick-free conditions.

Ten positive ovine and the same number of positive bovine sera were collected from sheep and cattle likewise born and reared under tick-free conditions and experimentally infected with either the Ball 3 (10), the Breed (4), the Kümm (1), the Kwanyanga (18), the Mali (15), the Mara 87/7 and Mara 90/20 (9) and the Welgevonden (2) stocks of *C. ruminantium*. The severity of the reactions of the animals was rated as previously described (6): Animals in Category I exhibited a severe febrile reaction of at least 40.5 °C for 3 or more consecutive days accompanied by clinical signs of anorexia and depression and were treated; those in Category II showed a marked febrile response but no other clinical signs and were not treated and those in Category III only a mild to moderate transient febrile reaction.

The positive caprine sera comprised one specimen from a goat infected with the Welgevonden stock, another from a goat immunized with a 31 kDa C. *ruminantium* protein (VAN KLEEF, personal communication) and 8 field specimens collected from goats in a heartwater endemic area. The latter 8 goats were subsequently proved to be immune to challenge with the Mara 87/7 stock.

Sera assumed to be positive to *Ehrlichia* were obtained from cattle and sheep born and raised in regions of southern Africa where *Amblyomma* ticks with certainty do not occur and have never been reported to occur. These sera had earlier on been found positive in the IFA test in which infected mouse peritoneal macrophages were used as

antigen. An agent indistinguishable from *Ehrlichia* has subsequently been isolated from a *Hyalomma truncatum (3)* and both *Rhipicephalus appendiculatus* and *R. evertsi* ticks (unpublished) collected on these farms.

Serological tests

IFA test

The CIFA test in which *C. ruminantium*-infected bovine umbilical endothelial cell cultures were used as antigen, was performed at Guadeloupe as described by MARTINEZ *et al.* (17). In Harare a bovine aorta-endothelial cell culture infected with the Crystal Springs stock of *C. ruminantium* was used as antigen (19). The MIFA test, with infected mouse peritoneal macrophages as antigen, was carried out as previously described (5). The cut-off points were 1:80 for both CIFA tests in the Guadeloupe and Harare laboratories, and 1:20 for the MIFA test. Sera that gave a negative reading at these dilutions were considered negative.

Competitive ELISA (cELISA)

The cELISA was carried out as described previously (14) with minor modifications. A Senegalese isolate of *Cowdria ruminantium* was used to infect bovine umbilical endothelial cell cultures. Sonicates of endothelial cell culture supernatants were applied in 100 µl volumes to the wells of a microtitre plate (Costar) at a concentration of 6µg/ml protein in carbonate-bicarbonate buffer, pH 9.6. The plates were incubated at 37 °C for one hour and then overnight at 4 °C. The plates were then rinsed three times with tap water. Subsequently, serum (final dilution 1:50) and a mouse monoclonal antibody directed against the 32 kDa protein of *Cowdria* (4F10-B4) (final dilution 1:400), both diluted in 1 % milk powder/PBS pH 7.2 /0.05 % Tween-20, were simultaneously applied to the plate in volumes of 100 µl per well. The plates were incubated for 1 h at 37 °C. After washing three times with tap water, peroxidase labeled rabbit-anti-mouse immunoglobulins (Dakopatts) diluted 1:750 in PBS pH 7.2/0.02 % Tween-20/0.25 % gelatin was applied at 100 µl per well and incubated for 1 h at 37 °C. After washing, 100 µl of ABTS substrate solution (Sigma) with hydrogenperoxide was added per well and incubated for 30 min at room temperature. The optical density (OD) was measured at 4:05 nm using a microplate reader.

Indirect ELISA

The antigen for the indirect ELISA (Guadeloupe) was prepared from *C. ruminantium*-infected bovine umbilical endothelial cell cultures (17). When approximately 80 % of the cell monolayer was destroyed, the remaining adherent cells were scraped off, mixed with the supernatant and certifuged at 2500 g for 15 min. The pellet was resuspended in sterile PBS and sonicated 5 times for 30 s in an ethanol-dry ice bath. Microplates were coated overnight at 37°C with 5 µg/ml of antigen in a carbonate-bicarbonate 0.1M buffer, pH 9.5 (100 µl per well). The plates were washed 3 times with phosphate buffered saline (0.1M, pH 7.2), supplemented with 0.1 % Tween-20. To each well was added 100 µl of test serum (diluted 1:800 for cattle, 1:100 for sheep and 1:400 for goats) in PBS-Tween, with 3 % skimmed cow milk as a blocking buffer. The plates were incubated for 1h at 37°C and washed 5 times in PBS-Tween. Horse radish peroxidase conjugated rabbit anti-goat, anti-bovine or anti-sheep IgG, optimally diluted in the blocking buffer, was added at 100 µl per well and the plates incubated for 1h at 37°C. After 5 washings, each well was filled with 100 µl of citrate buffer (0.1M, pH 5.5) containing 0.5 mg/ml 0-phenylene diamine and 3 µl/ml of 9 % H_2O_2. The enzymatic reaction was stopped after 30 min of incubation at room temperature by adding 50 µl of 2N H_2SO_4 and the absorbance read at 495 nm.

Western blots

At Utrecht endothelial cell culture sonicates, similar to the sonicates used for cELISA, were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis on a 12.5 % polyacrylamide gel. Western blotting was carried out essentially as described earlier (12), but electrophoretic transfer was carried out for 1 h at 100 V or overnight at 20 V. Blots were quenched for 1 h in PBS/5 % skimmed milk and incubated for 1h with test serum, or positive or negative control serum, diluted 1:150 in PBS containing 0.02 % Tween-20 and 5 % milk. Bound antibodies were visualized by incubation with rabbit anti-bovine (ovine or caprine) immunoglobulins conjugated with alkaline phosphatase (Sigma) at a dilution of 1:2000. Binding of conjugate was visualized by the addition, after washing, of 100 mM Tris-HCL buffer (pH 9.5), containing 100 mM NaCl, 5 mM MgCl2 buffer with nitroblue tetrazolium (NBT) and 5-bromo-4-dichloro-3-indocyl phosphate (BCIP). The reaction was stopped with a 20 mM Tris-HCL buffer of pH 8.0 containing 5 mM EDTA.

At Harare elementary bodies were harvested from culture supernatants (the Crystal Springs stock of *C. ruminantium* grown in bovine aorta-endothelial cells) by centrifuging at 30 000 g for 30 min at 4 °C. The cells were washed twice in PBS, sonicated for one min and subsequently analyzed on 12 % SDS-PAGE. After gel electrophoresis, electrophoretic transfer was carried out overnight at 20 V and continued the next morning for 1 h at 70 V. The membranes were blocked in 0.25 % gelatin for 2 h and probed overnight at room temperature with sera diluted 1:100. Bound antibodies were visualized by the addition of peroxidase labelled Protein G for 2 h and with 4-CN-peroxidase substrate for 30 min. The reaction was stopped by the addition of tap water. Rainbow molecular weight standards (Amersham) were run on each gel to assess the molecular mass of the *Cowdria* proteins.

RESULTS

The sera were submitted to the cELISA and the Western blot tests in one of the laboratories (Utrecht), to the indirect ELISA and the CIFA tests at Guadeloupe and to the CIFA and the Western blot tests (the cattle sera only) in the Veterinary Research Laboratory, Harare, Zimbabwe. At the Onderstepoort Veterinary Institute the sera were tested in the MIFA test only.

With only one exception, no false positive reactions were recorded and the known negative control sera of all 3 animal species reacted negatively in all 5 tests (tables I, II, III). In the indirect ELISA the absorbance value of one negative control bovine serum (Bovine 3, table II) only just exceeded the cut-off point.

TABLE I Results of 5 serological tests on sheep sera.

Sheep No.	Nature of serum/ *C. ruminantium* stock	Competitive ELISA	Indirect ELISA (5)		Western blot		Reciprocals of IFA titres	
			1st assay	2nd assay	Utrecht	Harare	CIFA/Guadeloupe	MIFA
1	-ive control	3/– (1)	0,143/– (2)	0,100/–	–	–	– (3)	– (4)
2	"	8/–	0,083/–	0,085/–	–	–	–	–
3	"	1/–	0,172/–	0,134/–	–	–	–	–
4	"	8/–	0,202/–	0,148/–	–	–	–	–
5	"	1/–	0,102/–	0,078/–	–	–	–	–
6	*Ehrlichia*	57/+	0,309/+	0,321/+	+	d	80	1280
7	"	83/+	0,554/+	0,521/+	++	+++	–	20
8	"	65/+	0,417/+	0,320/+	+	++	–	5120
9	"	63/+	0,566/+	0,516/+	++	++	320	20480
10	"	25/d (6)	0,501/+	0,468/+	+	+	–	1280
11	Mara 87/7 (2/I) (7)	84/+	0,611/+	0,963/+	++	+++	80	80
12	Welgevonden (8/I)	66/+	0,590/+	0,561/+	d	++	d	320
13	Mali (2/I)	50/+	1,488/+	1,232/+	++	+	640	20480
14	Mara 87/7 (3/I)	33/d	0,377/+	0,371/+	+	+	d	320
15	Kwanyanga (2/I)	43/+	1,581/+	1,212/+	++	++	–	1280
16	Mali (12/I)	57/+	0,810/+	0,833/+	++	+++	160	1280
17	Mara 87/7 (12/I)	45/+	0,261/+	0,238/+	d	d	–	20
18	Ball 3 (12/I)	71/+	0,650/+	0,625/+	++	+	80	1280
19	Kwanyanga (12/I)	37/d	0,552/+	0,530/+	+	++	80	1280
20	Kümm (12/I)	53/+	0,449/+	0,419/+	d	++	320	320

(1) % inhibition/final evaluation ; - = negative
(2) absorbance/final evaluation
(3) -ive at 1 : 80 dilution
(4) -ive at 1 : 20 dilution
(5) working dilution of serum : 1/100 ; cut-off point 1st assay : 0,214, 2nd assay : 0,169
(6) d = doubtful
(7) serum was collected from Sheep 11 2 months after having shown a category I reaction to the Mara 87/7 stock.

TABLE II Results of 5 serological tests on bovine sera.

Bovine No.	Nature of serum/ *C. ruminantium* stock	Competitive ELISA	Indirect ELISA (1)		Western blot		Reciprocals of IFA titres		
			1st assay (2)	2nd assay (3)	Utrecht	Harare	CIFA		MIFA
							Guadeloupe	Harare	
1	-ive control	12/–	0,027/–	0,031/–	–	–	–	–	–
2	" "	8/–	0,032/–	0,047/–	–	–	–	–	–
3	" "	1/–	0,124/+	0,109/+	–	–	–	–	–
4	" "	8/–	0,090/–	0,102/–	–	–	–	–	–
5	" "	1/–	0,062/–	0,060/–	–	–	–	–	–
6	*Ehrlichia*	45/+	0,332/+	0,372/+	+	++++	640	–	1280
7	"	63/+	0,481/+	0,459/+	++	++++	640	320	5120
8	"	20/–	0,195/+	0,186/+	–	d	160	–	320
9	"	16/–	0,100/–	0,122/+	–	–	160	–	20
10	"	30/d	0,195/+	0,215/+	+	++++	80	–	320
11	Mara 90/20 (1/I)	58/+	0,685/+	0,682/+	++	++++	1280	320	5120
12	Mara 87/7 (1/I)	45/+	0,355/+	0,326/+	++	+++	160	320	320
13	Mara 90/20 (1/II)	57/+	0,901/+	0,816/+	++	++++	1280	1280	5120
14	Mara 90/20 (1/III)	61/+	0,649/+	0,532/+	++	++++	2560	320	5120
15	Mara 87/7 (1/II)	67/+	0,643/+	0,596/+	++	++++	5120	1280	5120
16	Mara 87/7 (3/II)	35/d	0,604/+	0,617/+	+	++	640	–	1280
17	Mara 87/7 (6/III)	13/–	0,397/+	0,396/+	–	++	160	–	80
18	Kwanyanga (2/I)	64/+	0,914/+	0,826/+	++	+++	1280	1280	1280
19	Breed (2/III)	13/–	0,386/+	0,337/+	d	+	160	–	20
20	Ball 3 (2/III)	65/+	0,872/+	0,779/+	++	+++	1280	320	80

(1) working dilution of serum : 1/800
(2) cut-off point : 0,108
(3) cut-off point : 0,105

All 10 positive ovine sera from experimentally infected sheep were positive in the indirect ELISA, the Western blot (Harare) and the MIFA test. The latter gave positive titres that varied from 1:20 to 1:20480 (table I). Two and four sera gave either doubtful or negative results with the cELISA and CIFA tests, respectively. In the case of the sera that were positive with both IFA tests, there was wide divergence in the titres recorded in the 2 tests.

There were also 3 and 7 bovine sera from experimentally infected animals that gave negative or doubtful reactions with the cELISA and that were negative with the CIFA test carried out at Harare, respectively. All 10 sera were positive with the other 2 IFA tests and the indirect ELISA (table II). Low titres were recorded with both IFA tests on 2 sera that were negative with the cELISA.

Two and three goat sera were doubtful or negative with the CIFA and cELISA, respectively, while 9 and 10 were positive with the indirect ELISA and the MIFA test, respectively (table III). One serum (Goat 13) that reacted negatively with both the indirect ELISA and the cELISA, was positive in the MIFA at a low titre.

Out of the 30 sera tested positive with one or more of the 4 other tests, 25 were positive with the Western blot technique in one or both of the laboratories, while the other 5 were either doubtful or negative (tables I,II, III). In most of these cases low levels of antibody were recorded with some or all of the other 4 tests. Two of the *Ehrlichia* positive bovine sera were also negative or doubtful in the Western blot carried out both at Utrecht and Harare.

With few exceptions, the 10 sera from animals in all probability infected with *Ehrlichia* reacted positively in all 5 tests. Three of the bovine sera were either doubtful or negative with the cELISA and the Harare CIFA, but it is noteworthy that low titres and low levels of absorbance were recorded on these sera with the other 2 IFA tests and the indirect ELISA.

No results with the IFA test in which infected neutrophils are used as antigen were available, since to our knowledge this test is currently not used in any laboratory. This is probably due to problems encountered with the preparation of the antigen and the difficulty in reading the results (17).

DISCUSSION

It is encouraging that there are now several serological tests for heartwater. The fact that a particular laboratory prefers and successfully carries out a particular test, can probably be attributed to the fact that individual research

TABLE III ***Results of 5 serological tests on goat sera.***

Goat No.	Nature of serum/ *C. ruminantium* stock	Competitive ELISA	Indirect ELISA (2)		Western blot	Reciprocals of IFA titres	
			1st assay	2nd assay		CIFA/Guadeloupe	MIFA
1	-ive control	10/–	0,026/–	0,025/–	–	–	–
2	" "	18/–	0,033/–	0,040/–	–	–	–
3	" "	18/–	0,031/–	0,030/–	–	–	–
4	" "	25/d	0,021/–	0,032/–	–	–	–
5	" "	17/–	0,030/–	0,036/–	–	–	–
6	Welgevonden (1)	77/+	0,710/+	0,750/+	++	1280	5120
7	Welgevonden (2/I)	72/+	0,152/+	0,181/+	++	1280	1280
8	Field serum	82/+	0,175/+	0,256/+	++	1280	5120
9	" "	43/+	0,108/+	0,111/+	+	160	80
10	" "	70/+	0,201/+	0,207/+	+	1280	1280
11	" "	79/+	0,197/+	0,194/+	++	160	1280
12	" "	79/+	0,210/+	0,236/+	++	640	5120
13	" "	17/–	0,032/–	0,038/–	–	–	20
14	" "	23/d	0,068/–	0,092/+	–	80	1280
15	" "	25/d	0,171/+	0,155/+	–	–	320

(1) serum was collected from goat 6 four months after having been immunized with a Cowdria *31 kDa protein.*
(2) working dilution of serum : 1/400 ; cut-off point : 0,089.

workers acquire a special skill in either the preparation of the antigen or the execution of a particular test.

With only one exception no false positive reactions were recorded with any of the 5 tests and all known negative control sera were recorded as such. The absorbance value of the one negative control bovine serum exceeded the cut-off point so slightly that a minor modification of the cut-off point should rectify the matter without affecting the sensitivity of the test.

The most significant finding in this comparative study, in which sera were subjected to 5 serological tests currently used in heartwater research, was that with rare exceptions all 10 sera collected from 5 cattle and 5 sheep in regions where *Amblyomma* does not occur and that were initially tested positive in the MIFA test, were also positive to both ELISA tests, the Western blot technique and the CIFA test. Although agents indistinguishable from *Ehrlichia* were isolated from ticks on these farms (3), these agents must be characterized more fully before identifying them as *Ehrlichia*. They were, however, highly suspicious for *Ehrlichia* and the mere fact, that all 10 sera reacted positively against *C. ruminantium* in some or all of the 5 tests, some to high titres, strongly suggests that, irrespective of the test or the nature of the antigen, antibodies *to Ehrlichia* consistently cross-react with *C. ruminantium.*

Furthermore, on one hand neither the MIFA (1), nor the neutrophil IFA (11), nor the cELISA (14) tests show cross-reactions with other rickettsial agents such as *Anaplasma marginale, Coxiella burnetti, Chlamydia* and *Rickettsia* spp. On the other hand, high antibody titres were recorded with the MIFA test on the sera of control dogs experimentally infected with *Ehrlichia canis* (8). These observations strongly suggest that in the present study the antibodies detected by all 5 tests in the sera of animals from heartwater-free regions were in response to *Ehrlichia.*

Since antibodies to *Cytoecetes phagocytophila* do not cross-react with *Cowdria* in the cELISA test, it was hoped that this test would be able to distinguish between *Cowdria* and *Ehrlichia* (14), but the ovine and bovine sera from *Amblyomma*-free areas were positive in the cELISA. Three bovine sera in all probability were doubtful or negative with this test not because of the absence of cross-reactions, but because low levels of antibody were present in the sera, as shown with both the indirect ELISA and the MIFA tests. The fact that antibodies to *Ehrlichia* compete as strongly with monoclonal antibodies to the *Cowdria*-specific 32 kDa protein as do antibodies to *C. ruminantium,* suggests that this dominant protein is also present in *Ehrlichia.* This is further support of the close relatedness of *Cowdria* and *Ehrlichia (3).* The close relationship between *Cowdria* and *Ehrlichia,* especially *E. canis* and *Ehrlichia chaffeensis,* has recently also been shown by 16S ribosomal DNA sequence analysis (20).

On the one hand the cross-reactions shown by these 10 sera question the specificity of all 5 tests. This would, however, not be the case if more substantial evidence was obtained that *Cowdria* and *Ehrlichia* are antigenically closely related (3, 20).

Although an indication of the sensitivity of the 5 tests can be obtained from the sera expected to contain antibodies, because they were produced in cattle and sheep experimentally infected with *C. ruminantium,* the number of sera used were not adequate for a statistical evaluation of the confidence limits and sensitivity of the different tests. Bearing this in mind and considering that some of the caprine sera were not drawn from experimentally infected animals, it would nevertheless seem that the sensitivity of the Harare CIFA test, the Western blot and to a lesser extent the cELISA was somewhat lower than that of the other IFA tests and the indirect ELISA.

There is unfortunately considerable doubt whether the CIFA test can be applied to sheep sera with confidence. On one hand only 60 % of the positive ovine sera were positive and the titres of 4 of these were significantly lower than those detected with the MIFA test and with the percentage of inhibition absorbance levels of the cELISA and indirect ELISA, respectively. Since, on the other hand, only 2 out of 5 sera presumably positive to *Ehrlichia* were positive in the CIFA test, one could also argue that the latter test is better able to distinguish between *Cowdria* and *Ehrlichia.* The fact that all 5 sera were positive in all 4 other tests, weighs heavily against such a possibility. Furthermore, one of the collaborating laboratories declined to submit the ovine sera to the CIFA test because of inconsistent results.

Although it is true that the preparation of antigen for the MIFA test is fastidious and the reading of the results sometimes laborious (17), it appears to be the IFA test of choice because of its high sensitivity and has an advantage over the use of endothelial cell cultures as antigen, particularly in the case of sheep sera.

Both ELISA tests hold promise as tests for epidemiological field studies in heartwater endemic areas, but their somewhat lower sensitivity compared to the IFA test, particularly in the case of cELISA, has to be borne in mind, since levels of antibody tend to be low in heartwater endemic areas, particularly in the case of cattle exposed to frequent infection through the tick (9).

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Five serological tests, the indirect and competitive ELISA, the indirect fluorescent antibody (IFA) test with 2 different antigens and the Western blot technique were compared and applied to sera that were known to be either negative or positive against *Cowdria ruminantium* or that were collected from animals in heartwater-free regions. No false positive reactions were recorded with any of the tests against the known negative sera. Except for minor variations in the sensitivity of the 5 tests, there was good correlation between them. Their specificity, however, remains in dispute since in all 5 tests extensive cross-reactions were recorded with antibodies in response to an as yet unidentified agent, probably *Ehrlichia*.

Key words : Heartwater - *Cowdria ruminantium* - Diagnosis - Immunological technique - ELISA test - Indirect immunofluorescence - Western blot technique - Antigen - Sera - Antibody - *Ehrlichia*.

DU PLESSIS (J.L.), BEZUIDENHOUT (J.D.), BRETT (M.S.), CAMUS (E.), JONGEJAN (F.), MAHAN (S.M.), MARTINEZ (D.). Diagnóstico serológico de la cowdriosis : comparación de cinco tests. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 123-129

Se compararon cinco tests serológicos, el ELISA competitivo y el indirecto, la inmunofluorescencia indirecta de anticuerpos (IFA), con dos antígenos diferentes y con la técnica del Western Blot. Estos tests se realizaron ya sea con sueros conocidos positivos o negativos contra *Cowdria ruminantium*, o recolectados de animales provenientes de zonas libres de cowdriosis. Ninguno de los tests registró reacciones de falsos positivos para los sueros de seronegatividad conocida. Aparte pequeñas variaciones en la sensitividad de los 5 tests, se observó una buena correlación entre ellos. La especificidad es sin embargo dudosa, debido a que se observaron cantidad de reacciones cruzadas con anticuerpos de un agente no identificado, pero que se sospecha ser *Ehrlichia*.

Palabras claves : Cowdriosis - *Cowdria ruminantium* - Diagnóstico - Técnica inmunológica - Test ELISA - Inmunofluorescencia indirecta - Western Blot - Antigeno - Suero -Anticuerpo - *Ehrlichia*.

The relationship between *Cowdria* and *Ehrlichia* : change in the behaviour of ehrlichial agents passaged through *Amblyomma hebraeum*

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DU PLESSIS (J.L.). Les relations entre *Cowdria* et *Ehrlichia*: changement du comportement d'*Ehrlichiae* passées par *Amblyomma hebraeum*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 131-143

Antérieurement l'auteur a rapporté l'augmentation de la pathogénicité d'un agent ressemblant à une *Ehrlichia*, isolé d'une femelle adulte d'*Hyalomma truncatum*, et par la suite causant une maladie chez les moutons indifférenciable de la cowdriose après des passages chez *Amblyomma hebraeum*. Il s'agit ici d'un phénomène similaire. Un organisme ressemblant à une *Ehrlichia*, observé dans le sang d'un agneau sérologiquement positif et infecté de façon naturelle, a changé de comportement et a pris les caractéristiques de *Cowdria* après passage sur *A. hebraeum*. L'immunité croisée entre des moutons guéris d'infection avec l'organisme transformé et d'autres guéris de plusieurs stocks de *Cowdria ruminantium* confirme la parenté étroite entre l'*Ehrlichia* supposée et *Cowdria*. Deux de sept autres lignées de passages moutons/tiques ont donné des titres élevés d'anticorps à *C. ruminantium* et une résistance à l'épreuve par *C. ruminantium*, ce qui laisse supposer des changements similaires du comportement des agents passés par *Amblyomma*.

Mots clés : Ovin - Bovin - *Ehrlichia* - *Cowdria* - *Cowdria ruminantium* - Infection - *Amblyomma hebraeum* - Tique - Anticorps.

INTRODUCTION

In an earlier preliminary communication it was reported that a putative ehrlichial agent, isolated from an adult *Hyalomma truncatum* tick, collected from cattle in a region of Namibia where *Amblyomma* ticks, the vectors of *Cowdria ruminantium,* do not occur, and subsequently passaged in *Amblyomma hebraeum,* became more pathogenic and elicited a fatal disease closely resembling heartwater in a sheep (4). To attempt a repetition of this phenomenon and to obtain clarity on the factors that determine this change in behaviour, ticks and sheep from regions in the Republic of South Africa (RSA) where *A. hebraeum* does not occur, were collected for further study.

Since 81 % of the cattle on the farm in Namibia from which the infected tick was collected were serologically positive (4) in the indirect fluorescent antibody (IFA) test in which the Kümm stock of *C. ruminantium is* used as antigen (6), the ticks used in the present study were collected from cattle and sheep in regions where in a preliminary survey high percentages of sheep and cattle were serologically positive.

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MATERIALS AND METHODS

Serological survey

Sera collected from sheep and cattle on 18 farms in districts of all 4 provinces of the RSA, known to be free from A. *hebraeum,* were subjected to the IFA test as previously outlined (6). The sera were tested at a dilution of 1:20, except in the case of 3 farms from which seropositive lambs were transferred to the laboratory for tick feeding.

In the case of 8 of these farms the opportunity arose to collect ticks from the animals that were blood-sampled for serum. The absence of *Amblyomma* was verified and specimens of *Rhipicephalus evertsi, Rhipicephalus appendiculatus* and *H. truncatum* were collected. The identity of the ticks was confirmed at the laboratory.

Tick inoculations into mice

Homogenates prepared either from single ticks or from pools of ticks were inoculated into mice as previously described (3). Pools consisted either of 2-3 engorged or partially engorged females or 5-10 males. By means of a homogenizer equipped with a glass cylinder and a teflon piston the ticks were homogenized in 1-2 ml of PBS. The homogenates were centrifuged at 250 g for 5 min and the supernatants added to equal volumes of buffered lactose peptone (BLP).

Two 6-week-old conventional, outbred Swiss mice were injected intravenously (i.v.) with 0.2 ml of each homogenate. Three days later all clinically healthy mice were inoculated intraperitoneally (i.p.) with another 0.2 ml of the corresponding homogenate. Four to 5 weeks later, serum samples were collected from the mice and subjected to the IFA test at a dilution of 1:10.

Sheep-*Amblyomma* passaging of putative ehrlichial agents in tick homogenates

Three passage lines were commenced by inoculating tick homogenates into Dorper sheep reared free from ticks (on concrete) from birth until they were used at 6-9 months of age. Sheep 1 (table I) was injected i.v. with an inoculum consisting of 0.5 ml of each of the tick pools prepared from the *R. evertsi* ticks collected on Farms 1, 9 and 18 (table II). Likewise, Sheep 5 was inoculated with

TABLE I Reactions, antibody response and immunity against challenge of sheep infected with tick homogenates and thereafter with infected ticks at different passage levels.

Farms No.	Sheep No.	Infected ticks	Response		Resistance to challenge		Uninfected ticks
			Febrile reaction	IFA titre	Reaction index	Interval (1)	
	1	*R. evertsi* homogenate	Mild, intermittant(MI)	- ive	27.8	4	1/L/W/14(2)
1,9 & 18	2	50/1/N/89/S1 (3)	18/5/41,3 ; (4) thereafter MI	1 : 20	15.3	6	2/L/W/14
	3	(5)	MI	1 : 20	Not challenged		2/N/W/13
	4	30/2/A/62/S3	MI	1 : 20	17.6	7 1/2	–
4, 6, 9	5	*H. truncatum* homogenate	MI	- ive	32.6	4	I/N/S/68
	6	30/1/A/78/S5	MI ; thereafter 165/4/40,2	1 : 20	27.5	9	2/L/S/110
	7	*R. appendiculatus* homogenate	MI	- ive	Not challenged		1/N/S/11
	8	24/1/A/87/S7	15/5/39,5	-ive	28.5	3	2/N/W/16
	9	8/2/A/72/S8	MI	-ive	21.5	2	3/N/W/17
8	10	20/3/A/83/S9	51/11/40,5	1 : 20	18.6	3	4/L/S/54
	11	50/4/N/63/S10	MI	1 : 80	0	3	5/N/S/26
	12	24/5/A/300/S11	12/5/39,8 ; 52/9/40,5	1 : 20	19	4	6/N/W/15
	13	30/6/A/64/S12	MI ; then 86/3/41,5	1 : 80	23.6	7	7/N/S/121

(1) Interval in months between infection and challenge.
(2) Passage level 1, Wessels (W)/ Spesbona (S) strain of A. hebraeum *larvae (L)/nymphae (N) allowed to feed on Sheep 1, 14 days after having been infected.*
(3) 50 passage level 1 nymphae (N) or adults (A) allowed to feed on Sheep 2, 89 days after having engorged as larvae/nymphae on Sheep 1.
(4) 18/5/41,3 = the febrile reaction of Sheep 2 commenced 18 days after attachment of ticks, lasted for 5 days and attained a maximum temperature of 41,3 °C.
(5) The larvae placed on Sheep 2 failed to engorge and Sheep 3 was infected with 10 ml blood drawn from Sheep 2 at the height of the febrile reaction.

TABLE II Serological response of mice inoculated with tick homogenates.

Farm No.	No. of tick pools	Tick species	Mouse Serology			Sheep No. Table I
			– ive	+ ive, 1/20	+ ive,7 1/20	
1	1	*R. evertsi*	+			1
4	1	*H. truncatum*	+			5
4	1	*R. evertsi*	+			– (1)
6	1	*H. truncatum*		+		5
8	5	*H. rufipes*	+			–
8	5	*R. appendiculatus*		3/5 (2)		7
8	4	*R. evertsi*	+			–
9	1	*H. truncatum*	+			5
9	1	*R. evertsi*		+		1
10	1	*R. evertsi*	+			–
11	1	*R. evertsi*	+			–
18	1	*H. rufipes*	+			–
18	1	*R. appendiculatus*	+			–
18	1	*R. evertsi*	+			1

(1) Tick homogenate not inoculated into sheep.
(2) Mice inoculated with 3 out of 5 tick pools positive.

an inoculum prepared from *H. truncatum* ticks collected on Farms 4, 6 and 9 (table I). Sheep 7 (table I) was infected with an inoculum consisting of 0,5 ml of each of the 3 *R. appendiculatus* pools that had elicited antibodies in mice (table II) and 2,5 ml of an homogenate prepared in 5 ml BLP from the spleens of 3 of the mice that were serologically positive.

At intervals after infection ranging from 11 to 121 days, varying numbers of unengorged *A. hebraeum* larvae or nymphae were allowed to feed on the sheep. Although it was intended that the feeding of the ticks should have coincided with the febrile reaction of the sheep, this was not always feasible, because febrile reactions very often were of short duration and low magnitude and a rise of temperature for one day was often followed by a return to normal the next day. Furthermore, the feeding of larvae was sometimes unsuccessful in that they either did not engorge or did not moult and a second attempt with nymphae could be undertaken only much later (table I, Sheep 5, 8, 11, 13). Hence the wide variation in intervals between infection and the feeding of ticks.

Larvae and nymphae of either the Spesbona or the Wessels strains of *A. hebraeum,* reared free from infection by *C. ruminantium* (13), were used to infest the sheep kept under tick-free conditions (concrete floors, washed daily). When unfed, uninfected nymphae were required, larvae were fed on rabbits and left to moult. Ticks were placed in calico bags glued to the backs of sheep (13). From time to time batches of the uninfected larvae and nymphae used for the sheep/tick passaging, were allowed to feed on naive sheep. None of the sheep developed any febrile reactions, other clinical signs or antibodies detectable with the IFA test. Engorged, infected ticks were collected and left to moult at 80 % relative humidity and 27 °C. Regular counts of adult male ticks that remained attached to the sheep, were carried out.

At intervals after moulting varying between 62 and 300 days, the first generation unengorged, infected nymphae or adults were placed on the next passage level susceptible sheep. In this manner several sheep/tick passages were carried out. Early morning rectal temperatures of the sheep were recorded. At a temperature rise to 40 °C or higher, blood was collected from the ear in a haematocrit tube, centrifuged and a smear prepared from the buffy coat stained in 5 % Giemsa for 50 min.

At monthly intervals serum samples from the sheep were subjected to the IFA test. Pre-infection samples were likewise tested.

Two to 6 months after infection, the sheep were challenged with an i.v. inoculation of 5 ml sheep blood infected with the moderately pathogenic Mara 90/20 stock of *C. ruminantium* (9). No treatment was given and a reaction index (RI) calculated as previously described (7) for each sheep. An additional 10 points were added if the sheep died. A cut-off RI of 24 was determined by infecting 2 susceptible sheep with the Mara 90/20 stock. Both animals developed mild to moderate clinical signs of inapathy and inappetence and recovered without treatment. Animals with a RI below 24 were considered partially immune and those with a RI of 10 or lower as fully resistant.

Mouse tissue from which Omatjenne agent originated

In an attempt to repeat the isolation of the Omatjenne agent (4), 2 further sheep/tick passage lines with the same starting material were carried out. The mouse spleen homogenate prepared from a mouse that had been infected with the liver and spleen of the serologically positive mouse inoculated with the *H. truncatum* homogenate, was used in both cases. In the one passage line, sheep 14 (Table III) was inoculated i.v. with 2 ml spleen homogenate. Nine days later 50 Spesbona nymphae were allowed to feed on the sheep, followed by 3 further passages, in the manner described above. To determine whether the interval between the initial feeding of the ticks and their subsequent feeding on a susceptible sheep is important, Sheep 17 and 21 (Table III) were infected with the same batch of adult ticks that had fed on Sheep 16 as nymphae. Sheep 16 and 20 on one hand and Sheep 18 and 19 on the other were likewise infested with the same batch of adults and nymphae, respectively. Because sheep 15 had developed an encouraging antibody titre and was partially immune to challenge, sheep 23 was infected with 10 ml blood collected from sheep 15 at the height of the febrile reaction. Two further passages were subsequently carried out.

In the other passage line and to mimic as closely as possible the procedure followed during the earlier isolation of the Omatjenne agent (4), 6 mice were inoculated i.v. with the spleen homogenate and 10 Spesbona *nymphae* allowed to feed on each mouse 5-7 days later, making use of a Velcro corset (13). Peripheral blood smears prepared from the tails of 2 mice that died after the ticks had engorged, were stained with Giemsa. The lungs of one of these mice were fixed in 10 % formalin and H & E sections and thin sections for electronmicroscopy prepared according to standard techniques.

Only 5 adult male and one female tick were finally available. They were fed on Sheep 26 (table III), 3 days after 4 uninfected males had been put on the sheep. Since no unengorged larvae were available, Spesbona *nymphae* were fed on Sheep 26 9 days after the 6 infected ticks had attached. Two further sheep/tick passages were carried out.

Serologically positive naturally infected lambs

Three 6-9 month-old German Merino lambs with high IFA titres and that originated from flocks on Farms 4, 5 and 6 where seropositivity percentages of 93-100 had been recorded, were transferred to the laboratory for sheep/tick

TABLE III Reactions of sheep infected with mouse tissue from which Omatjenne agent was isolated and during subsequent sheep/tick passages.

Sheep No.	Infected ticks	Response		Resistance to challenge		Uninfected ticks
		Febrile reaction	IFA titre	Reaction index	Interval	
14	(1)	5/9/40.4 ; thereafter IM	1 : 20	32.6	2	I/N/S/9
15	20/1/A/79/S14	IM ; 33/3/40.9	1 : 1280	7.8	3	2/N/W/35
16	12/2/A/72/S15	IM ; 33/6/40.9	1 : 5120	0	3	3/N/W/22
17	16/3/A/76/S16	13/3/39.5 ; thereafter IM	1 : 1280	6.5	3 1/2	4/L/W/14
18	50/4/N/91/S17	IM	1 : 20	14.4	3	– (2)
19	50/4/N/74/S17	8/8/40.3 ; thereafter IM	1 : 20	14.1	4	–
20	16/2/A/215/S15	IM	1 : 5120	0	2	–
21	12/3/A/232/S16	IM	1 : 20	31.8	4	4/N/W/14
22	24/4/A/59/S21	IM	- ive	26.1	4 1/2	–
23	10 ml blood, Sheep 15	IM	1 : 1280	0	4 1/2	2/N/W/19
24	20/Z/A/153/S23	IM ; 61/7/40.3	1 : 80	32.9	4	3/N/W/101
25	24/3/A/64/S24	IM	1 : 5120	14.5	7 1/2	–
26	(3)	10/10/40.2 ; thereafter IM	1 : 20	34	4	1/N/S/9
27	50/1/A/47/S26	20/9/40.7	- ive	–	–	2/L/S/14
28	200/2/N/36/S27		- ive	–	–	

(1) Sheep 14 infected with mouse spleen homogenate from which Omatjenne agent was isolated.
(2) – = not done.
(3) Sheep 26 infected with adult Amblyomma *ticks that had engorged as nymphae on mice inoculated with mouse spleen from which Omatjenne agent was isolated.*

passaging (Table IV). Upon arrival the animals were treated with a tickicide and housed under tick free conditions. Buffy coat smears prepared from peripheral blood were stained with Giemsa.

Approximately 200 Wessels strain *A. hebraeum* larvae were allowed to engorge on lambs 1 and 2 and 50 Spesbona strain nymphae on Lamb 3. Fifty-eight days later 50 unengorged infected nymphae that had hatched from the larvae were allowed to engorge on sheep 29 and 31 (table IV). Ten Spesbona males that had engorged as nymphae on Lamb 3 52 days earlier were allowed to attach to sheep 34, followed by 12 females 3 days later. Further sheep/tick passages were carried out, serum samples tested and challenges executed as described and recorded in table IV.

Characterization of the Vosloo agent

The infectivity, pathogenicity and immunogenicity of the agent isolated from Sheep 35, hereafter referred to as the Vosloo agent (after the owner of Lamb 3), were determined in sheep, mice and cattle.

Two groups of 10 mice each were inoculated either i.v. or i.p. with 0.3 ml of blood collected in heparinized tube from Sheep 35 on Day 5 of the febrile reaction, added to an equal volume of BLP and stored in liquid nitrogen. Mice that died were autopsied and Giemsa stained smears prepared from the peritoneal cells of one of the mice that had shown clinical signs 12 days after having been infected i.p.

To prepare a stabilate for future use, 10 ml of the stabilate used to infect the mice was inoculated i.v. into a susceptible sheep. On Day 3 of the febrile reaction 100 ml of blood was collected in an equal volume of citrated BLP and aliquots of 10 ml deepfrozen.

Two one-year-old heartwater susceptible South Devoncross oxen were inoculated i.v. with 5 ml of the 2nd stabilate. Early morning rectal temperatures were recorded and both oxen were treated with oxytetracycline* at a dosage of 10 mg/kg live mass on Day 5 of the febrile reaction. One of the oxen that died despite the treatment, was autopsied and a Giemsa stained smear prepared from its brain.

* Terramycin, Pfizer.

TABLE IV Reactions of sheep infected with **A. hebraeum** *ticks fed on seropositive naturally infected lambs and during subsequent sheep/tick passages.*

Lamb/farm No.	Sheep No.	Infected ticks	Response		Resistance to challenge		Uninfected ticks
			Febrile reaction	IFA titre	Reaction index	Interval	
	29	50/1/N/58/Lamb 1 (1)	3/14/40.8 ; thereafter IM	1 : 20	16.9	6	2/L/S/122
1/4	30	50/2/N/52/S29	IM	1 : 80	15.7	5 1/2	3/L/S/8
	31	50/1/N/58/Lamb 2	3/7/41.1 ; thereafter IM	1 : 20	21.8	6	2/N/W/61
2/5	32	30/2/A/66/S31	IM	1 : 80	22.9	7	3/L/S/13
	33	50/3/N/83/S32	8/12/40.1	- ive	29	4	–
	34	22/1/A/52/Lamb 3	IM ; 71/4/40.2	- ive	(2)		2/N/W/19
3/6	35	24/2/A/57/S34	I ; 148/8/42 ; died	1 : 80	–	–	3/L/S/59
	36	50/3/N/104/S35	IM ; 31/6/40.8 ; IM	- ive	–	–	–

(1) 50 passages level 1 Wessels nymphae that had engorged as larvae on Lamb 1/ Farm 4/ 58 days earlier, allowed to feed on Sheep 29.
(2) Died of uraemia due to renal calculi.

TABLE V Cross-challenges between Vosloo agent and several stocks of **C. ruminantium.**

Sheep No.	Sheep blood infected with	Reaction	Challenge stock	Reciprocal IFA titre at challenge	Reaction to challenge
37	Vosloo agent	7/10/41.4 (1) ; T3 x (2)	Welgevonden	5120	No reaction
38	Vosloo agent	7/8/41.5 ; T3 x	Kümm	5120	11/10/41.8 ; 23.1 (4)
39	Vosloo agent	7/10/42 ; T3 x	Ball 3	> 5120	12/4/40.2 ; 2.5
40	Vosloo agent	8/9/41.5 ; T2 x	Mara 87/7	> 5120	No reaction
41	Vosloo agent	8/12/41.7 ; T1 x	Kwanyanga	> 5120	No reaction
42	Vosloo agent	7/11/40.9 ; T1 x	Mali	> 5120	No reaction
43	Vosloo agent	7/8/41.5 ; T2 x	Germishuys	> 5120	11/8/41.2 ; 14.5
44	Vosloo agent	7/10/41.7 ; T2 x	Breed	5120	21/7/39.9 ; 3
45	Ball 3 stock	8/10/41.6 ; T3 x	Vosloo agent	– (3)	12/4/39.9 ; 4.1
46	Ball 3 stock	9/9/41.4 ; T3 x	Vosloo agent	–	12/4/41 ; 9
47	Ball 3 stock	8/12/41.6 ; T2 x	Vosloo agent	–	13/4/40.4 ; 4.5

(1) 7/10/41.4 = The febrile reaction of Sheep 37 commenced on Day 7, lasted for 10 days and attained a maximum temperature of 41.4 °C.
(2) T3 x = Sheep 37 was treated 3 times.
(3) Not tested.
(4) 23.1 = reaction index at challenge.

The cross-immunity between the Vosloo agent and several stocks of *C. ruminantium* was determined by infecting 8 heartwater susceptible sheep i.v. with 5 ml of the 2nd stabilate (table V). The sheep were treated on the 3rd day of the febrile reaction. If there was a further rise in the temperature 2 or more days after the initial treatment, the animals were treated a 2nd and even a 3rd time in some cases. No homologous challenge was given and one month after infection the sheep were challenged with sheep blood stabilates infected with the Welgevonden (3), the Kümm (2), the Ball 3 (12), the Mara 87/7 (9), the Kwanyanga (20), the Mali (19), the Germishuys (7) and the Breed (5) stocks of *C. ruminantium.* Three additional sheep that had been used in the current production of the

heartwater vaccine issued by the institute, were challenged with the 2nd stabilate of the Vosloo agent. No treatment was given and a RI calculated for each sheep.

RESULTS

Serological survey

It is evident from table VI, that high percentages of the sera collected from sheep and cattle in widely distributed regions of the RSA where *A. hebraeum* does not occur, reacted positively in the IFA test. The prevalence was particularly high in sheep and varied from 60-100 % and from 20-93 % in cattle. There were no less than 8 farms on which 100 % of the sheep were positive.

There was no correlation between the serological prevalence and the distribution of the 3 tick species, based on the account by HOWELL *et al* (16). All 3 tick species seemed to be involved, but the seropositivity percentages of 70 and 71-100 recorded on Farms 14 and Farms 1-3, respectively, where either *H. truncatum* or *R. evertsi* occurs in the absence of the other species, suggest that these 2 species are certain hosts to the ehrlichial agents.

Tick inoculations into mice

It can be seen from Table II that the ticks from only 3 out of the 8 farms on which ticks for sheep/tick passaging were collected, elicited an antibody response of low magnitude in mice inoculated with tick homogenates.

TABLE VI Prevalence of IFA test antibodies in sheep and cattle to putative ehrlichial agents in regions of South Africa where **A. hebraeum** *does not occur.*

Province	Farm No.	District	Tick distribution (1)			% Serologically + ive	
			H. truncatum	*R. evertsi*	*R. appendiculatus*	Sheep	Cattle
Transvaal	1	Amersfoort	–	+	–	100	20
	2	Belfast	–	+	–	71	–
	3	Carolina	–	+	–	79	–
	4	Klerksdorp	+	+	–	93	67
	5	Klerksdorp	+	+	–	100	–
	6	Lichtenburg	+	+	–	100	80
	7	Lydenburg	+	+	+	–	37
	8	Piet Retief	–	+	–	100	55
	9	Schweizer-Reneke	+	+	–	100	93
	10	Ventersdorp	+	+	–	100	60
	11	Wakkerstroom	–	+	–	100	33
Cape	12	George	+	+	+	60	40
	13	Jansenville	+	+	–	63 (2)	57
	14	Postmasburg	+	–	–	70	–
	15	Uniondale	+	+	+	0	40
Natal	16	Kokstad	–	+	–	–	25
	17	Utrecht	–	+	+	100	27
Orange-Free State	18	Bloemfontein	+	+	–	–	25

(1) HOWELL et al. *(16).*
(2) Angora goats.

Sheep *Amblyomma* passaging of putative ehrlichial agents in tick homogenates

The febrile and antibody response and resistance to challenge of the sheep infected with tick homogenates and thereafter with ticks infected in subsequent passages, are given in Table I. Febrile reactions were mild and intermittent, the temperature rising for 1-3 days to 39.5-40 °C every 8-16 days. Occasionally the reactions were more severe (Sheep 2, 6, 8, 10, 12, 13). No antibody was detected in Sheep 1 and 5 inoculated with the *R. evertsi* and *H. truncatum* homogenates, but at the subsequent passage levels antibody was detected at low titres.

In the case of the *R. appendiculatus* passage line, antibody was detected only at the 3rd passage level and thereafter at 4 subsequent passage levels. The presence of antibody, even at a titre of 1:20, that react with *C. ruminantium* in the IFA test, proves that these antibodies develop in response to an infectious agent present in the tick homogenates and passaged in the ticks, because the pre-infection serum of all the sheep used in these experiments were consistently negative at a dilution of 1:20.

Sheep 2 and 4 were partially immune against challenge. In the case of the *R. appendiculatus* passage line, Sheep 8 was fully susceptible to challenge, while the animals infected during the 5 subsequent passages were either fully (Sheep 11) or partially immune.

Mouse spleen from which Omatjenne agent originated

Sheep 14 that was infected with the mouse tissue from which the Omatjenne agent had evolved (4), reacted mildly and became seropositive, but was fully susceptible to challenge (Table III). During the subsequent 3 sheep/tick passages, however, there was an increase in antibody levels and resistance to challenge (Sheep 15,16, 17), but in the 4th passage (Sheep 18) and in Sheep 19 on which ticks from the same batch were allowed to feed, there was a distinct decline in both parameters. A repetition in Sheep 20 with the same passage level ticks fed on Sheep 16, gave the same positive result, but the passage in Sheep 21 and in a subsequent passage (Sheep 22) were, however, unsuccessful. Blood from Sheep 15 again resulted in a positive response in Sheep 23, but 2 subsequent passages (Sheep 24 and 25) resulted in a decline of resistance to challenge. Attempts to revert to earlier passage levels, were therefore unsuccessful.

The blood smear prepared from the mice on which *Amblyomma* nymphae had engorged and that were subsequently used to infect Sheep 26 (Table III), revealed several monocytes with colonies of organisms that were indistinguishable from *Ehrlichia* (photo 1). H & E stained histological sections of the lungs of these mice showed an acute interstitial pneumonitis characterized by the presence of numerous activated alveolar macrophages, some of which contained highly suspicious colonies of

Photo 1 : Ehrlichial morula in the monocyte of a mouse (x 5,000).

Photo 2 : Electron photomicrograph of ehrlichial organisms in the lung of a mouse (x 15,000).

organisms in their cytoplasm. Electron microscopy (photo 2) showed that these colonies contained organisms conforming in morphology with *Ehrlichia canis* in pulmonary mononuclear cells (14).

Serologically positive naturally infected lambs

The Giemsa stained blood smears from Lamb 3 was highly suspicious for *Ehrlichia,* although an exhaustive examination of the smear revealed very few inclusions in the monocytes. The ring-like inclusion shown in photo 3 was detectable more often than the morulae (photo 4).

The range of antibody titres recorded on Farms 4, 5 and 6 from which the 3 lambs originated, are shown in Table VII. Titres ranged from 1:320 to as high as 1:5120. Antibodies in Sheep 29 and 31, infected with nymphae

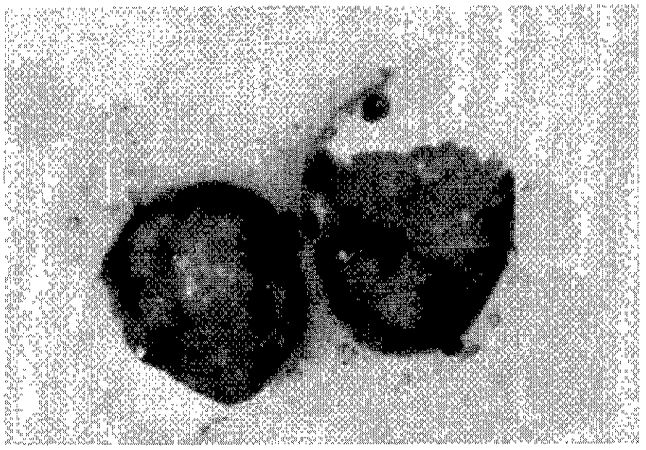

Photo 3 : Ring-like colony of ehrlichial organisms in the monocyte of Lamb 3 (x 5,000).

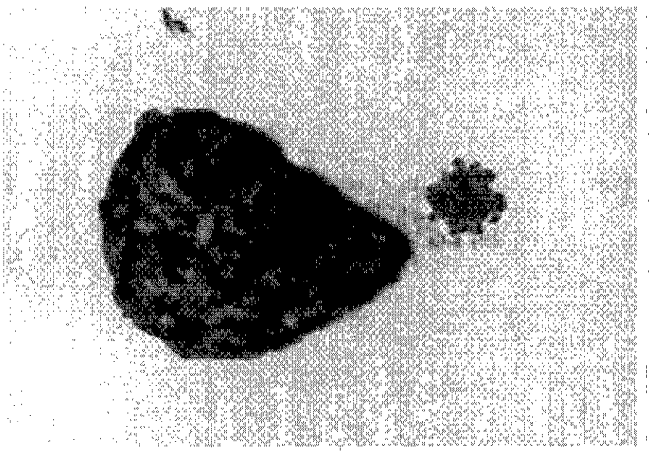

Photo 4 : Ehrlichial morula in the monocyte of Lamb 3 (x 5,000).

TABLE VII IFA test titres of lamb flocks from which 3 naturally infected lambs originated.

Farm No.	Lamb No./ IFA titre	No. of lambs tested	Reciprocal of IFA titre						% Sero-positive
			- ive	20	80	320	1280	5120	
4	1/1 : 320*	22	14	1	3	4	0	0	36
5	2/1 : 5120	22	0	0	0	6	12	4	100
6	3/1 : 80	15	0	1	7	6	1	0	100

* *Lamb 1 had a IFA test titre of 1 : 320.*

that had engorged on Lambs 1 and 2, showed that these ticks had picked up the putative ehrlichial agents from these lambs in their larval stage (Table IV). Sheep 29 and 31 were only partially resistant to challenge and so were Sheep 30 and 32 infected with passage level 2 ticks.

Although Sheep 34 remained seronegative after having been infested with adult ticks that had engorged as nymphae on Lamb 3 (Table IV), the passage level 2 nymphae must have picked up the infection while feeding on Sheep 34. Not only did Sheep 35, on which these ticks were fed as adults, develop an IFA test titre of 1:80, but it showed a severe febrile reaction for 8 days, attaining a maximum temperature of 42 °C, 148 days after the passage level 2 ticks had attached. At this stage, 2 of the 12 male ticks were still alive and attached to Sheep 35. Passage level 3 nymphae that had engorged on sheep 36, 59 days after the attachment of the passage level 2 ticks, however only caused a mild febrile reaction in Sheep 36. No antibody was detectable in its serum 3 months later. From the 4th day of the febrile reaction Giemsa stained blood smears of Sheep 35 revealed inclusions in the monocytes. At first small colonies of organisms were seen. On subsequent days the monocytes became enlarged, their nuclei assuming bizarre shapes and the organisms scattered in small groups in their foamy cytoplasm (photo 5). At this stage the monocytes appeared to undergo necrosis.

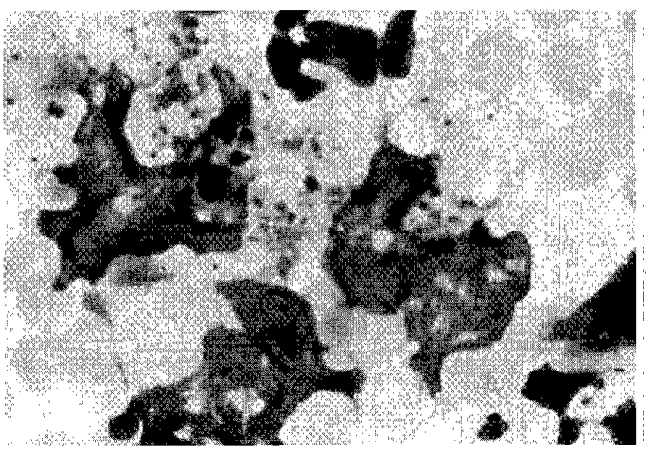

Photo 5 : Large monocytes with bizarre-shaped nuclei and organisms in their cytoplasm in the blood smear of Sheep 35 (x 5,000).

Sheep 35 showed clinical signs of inappetence and listlessness followed by nervous symptoms reminiscent of heartwater shortly before death. Salient features at autopsy were severe hydrothorax and oedema of the lungs. Only mild hydropericardium and splenomegaly were in evidence. The brain smear was typical for heartwater and numerous large to medium-sized colonies of organisms were demonstrable (photo 6).

Photo 6 : Numerous large colonies of organisms indistinguishable from C. ruminantium in the brain smear of Sheep 35 (X 5,000).

Characterization of the Vosloo agent

All 10 mice inoculated i.v. with blood from Sheep 35 developed clinical signs of a ruffled hair coat, listlessness and dyspnoea 12 days later and all 10 died during the next 48 h. At autopsy hydrothorax was consistently observed. Two out of 10 mice infected i.p. died with the same pathognomonic lesion in evidence. A smear prepared from the peritoneal cells of a 3rd mouse with similar clinical signs, revealed moderate numbers of macrophages with inclusions. These were either round and coarsely granular (photo 7) and indistinguishable from some of the inclusions regularly seen in the peritoneal macrophages of mice infected with the Kümm stock of *C. ruminantium,* or similar to the ring-form inclusions observed in the blood smear of Lamb 3, with the organisms distributed on the periphery of the inclusion (photo 8).

The febrile reaction of both oxen infected with the Vosloo agent commenced on Day 13, surpassed 41°C and lasted for 6 and 8 days. In spite of treatment, one of them died, with the brain smear positive. Apart from widespread haemorrhages in the carcass and severe lung oedema, the outstanding feature at autopsy were greatly swollen and dark red kidneys with marked perirenal oedema and haemorrhage.

The results of the cross-challenges between the Vosloo agent and several stocks of *C. ruminantium* are given in

Photo 7 : Colony of coarsely granular organisms in the cytoplasm of a mouse peritoneal macrophage (x 5,000).

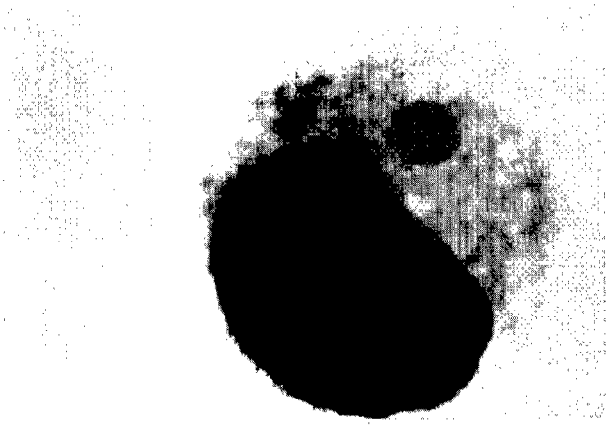

Photo 8 : Ring-like colony in the cytoplasm of a mouse peritoneal macrophage, with the organisms distributed on the periphery of the inclusion (x 5,000).

Table V. It can be seen that all 7 sheep infected with this agent reacted severely and had to be treated up to 3 times. High antibody titres were recorded in the sheep on the day of challenge. Sheep that had recovered from infection with this agent were solidly immune against challenge with the Welgevonden, Mara 87/7, Kwanyanga and Mali stocks and partially immune against the Ball 3, Germishuys and Breed stocks. In a reverse challenge, 3 sheep immune to the Ball 3 stock, were also partially immune against challenge with the Vosloo agent. Although Sheep 38, immune to the Vosloo agent, survived when it was challenged with the Kümm stock, it reacted severely and showed clinical signs of depression and inappetence.

In Table VIII the cross-immunity between the Omatjenne and Vosloo agents and 8 stocks of *C. ruminantium* are

compared. The outcome of 9 sheep/tick passage lines is summarized in Table IX.

Blood smears of sheep used in sheep/tick passaging

Apart from Sheep 35, all other blood smears prepared from sheep showing a temperature rise to 40°C or higher, were negative.

Duration of attachment of male ticks

Regular counts of the male *Amblyomma* ticks on the sheep showed that some of them remained attached for up to 6 months. Three and 2 males were *e.g.* still attached to Sheep 13 and 32, 6 months after 15 males had been placed on them. Male ticks were never seen to detach, move to another site in the bag and attach again.

TABLE VIII Comparison of the cross-immunity between the Omatjenne and Vosloo agents and 8 stocks of **C. ruminantium.**

	Challenge stock									
	Ball 3	Breed	Germishuys	Kümm	Kwanyanga	Mali	Mara 87/7	Welgevonden	Omatjenne	Vosloo
Ball 3		F-P	F	N	F-P	N	F-P	S-N	N	P
Breed	F			N				N		
Germishuys	F			N		N	S	F-P		
Kümm	P-S	N	N		P		N	S-N		
Kwanyanga	F-P	S-N		N		N	F-P	F-P		
Mali	P			S-N				S-N		
Mara 87/7	F	F-P	F		F-P	N		S-N		
Welgevonden	F-P	F-P	F-P	N	F-P	S-N	F-P		N	
Omatjenne	S	F	F	S-N	F	N	P	S-N		
Vosloo	F	F	S	N	F	F	F	F		

F = full cross-protection (Reaction index : < 4).
P = partial cross-protection (Reaction index : 5-9).
S = slight cross-protection (Reaction index : 10-20).
N = no cross-protection (Reaction index : > 20).
F-P = some of the sheep fully and others partially protected.

*TABLE IX Summary of sheep/***Amblyomma** *passages of putative ehrlichial agents.*

Passage line No.	Source of ehrlichial agent	No. of sheep/ tick passages	Conversion to *Cowdria*	Sero-conversion and increased pathogenicity	Sero-conversion only
1	*H. truncatum*	3	+ (1)		
2	*H. truncatum* (2)	4		+	
3	*H. truncatum* (2)	2			
4	*R. evertsi* pool	3			+
5	*H. truncatum* pool	2			+
6	*R. appendiculatus* pool	6		+	
7	Sero-positive Lamb 1	3			+
8	Sero-positive Lamb 2	3			+
9	Sero-positive Lamb 3	2	+ (3)		

(1) Omatjenne agent (4)
(2) Repetition of Passage line 1
(3) Vosloo agent.

DISCUSSION

In an earlier preliminary study (4) it was suggested that the behaviour of a putative ehrlichial agent changed dramatically after 3 sheep/A. *hebraeum* passages. The present study confirms this phenomenon but also shows that the change in pathogenicity is rarely as dramatic as may have been thought and that the extent of the change may vary. Out of 9 passage lines, summarized in table IX, and including the one described earlier (4), only 2 have resulted in the eventual isolation of an agent causing a disease in all respects similar to heartwater. With 2 other passage lines, the attempt to repeat the evolvement of the Omatjenne agent and in the case of the *R. appendiculatus* passage line, the development of substantial antibody titres and partial or total resistance to challenge with the Mara 90/20 stock of *C. ruminantium,* proved that here too a change in the behaviour and pathogenicity of the agent had taken place. In the case of the 4 other passage lines, there appeared to be no change. The presence of low antibody level without any resistance to challenge, nevertheless proved that the *R. evertsi* and *H. truncatum* tick pools and Lambs 1 and 2 had been infected with a putative ehrlichial agent.

The demonstration of *Ehrlichia* in blood smears and by electron microscopy in the lungs of mice infected with the *H. truncatum* homogenate from which the Omatjenne agent evolved in an earlier study (4) and with which a repetition of the phenomenon was attempted in the present study, as well as a blood smear of the lamb from which the Vosloo agent was derived, is proof of the ehrlichial nature of the agents passaged. The high prevalence of seropositivity of particularly sheep, but also of cattle, on the farms from which the ticks and lambs originated is additional indirect evidence. The extensive cross-reactions between antibodies to the ehrlichial agents and *C. ruminantium* not only in the IFA test used in this study, but also in 4 other serological tests (10), prove that the *Cowdria* antigen used in these tests and the agent responsible for the widespread seropositivity in the RSA are closely related and that the latter is in all probability *Ehrlichia*. Neither the mouse macrophage IFA(2), not the neutrophil IFA(15), nor the competitive ELISA (17) tests show cross-reactions with other rickettsial agents such as *Anaplasma marginale, Coxiella burnetti, Chlamydia* and *Rickettsia* spp. Furthermore, high antibody titres were recorded with the mouse macrophage IFA test on the sera of control dogs experimentally infected with *E. canis* (8). The cross-reactions detected with the 5 tests (10) can therefore not be considered as non-specific. This view also adds credibility to the use of the IFA test in the present study, where it was employed to follow the transmission of the ehrlichial agents over the course of the sheep/tick passages and to the discovery of the wide distribution of ehrlichiosis in the RSA.

As regards Sheep 35 that succumbed to an infection that as far as clinical signs, lesions at autopsy and the brain smear are concerned, was indistinguishable from heartwater, the question arises whether the infectious agent had changed during its persistence over 5 months in the sheep, or whether the change had occurred in one of the 2 male *Amblyomma* ticks that remained attached and alive for the same period. The former possibility seems unlikely, since Sheep 36 on which nymphae were fed that had engorged on Sheep 35 as larvae 3 months prior to the death of the latter, only showed a mild reaction, a low antibody titre and only partial resistance to challenge. The infection in Sheep 35 therefore seemed to have stabilized. The persistent agent may of course have changed shortly before the fatal reaction, but it seems more likely that the change in the behaviour of the agent occurred in one of the male ticks. In the case of the Omatjenne agent, there was more direct evidence that the transformation took place in the tick (4). In another tickborne disease, theileriosis, the buffalo-derived form of *Theileria parva,* responsible for Corridor disease of cattle, changed its behaviour to that of the cattle-associated *T. parva* causing East Coast fever after 5 tick/cattle passages, during which the parasite produced relatively high schizont parasitosis and piroplasm parasitaemia in cattle (21).

While it can be assumed that passage through *A. hebraeum* triggers the change, the question which factor(s) play a role in this phenomenon, remains unanswered. Irrespective of the developmental stage, strain or numbers of *A. hebraeum* ticks, the time lapse between the attachment of infected ticks and the application of uninfected, unengorged ticks, or the interval between the moulting of newly-infected ticks and their feeding on susceptible sheep, the majority of passages appeared to leave the agent unchanged. It is so that in the case of both the Omatjenne and the Vosloo agents, the Spesbona strain of *A. hebraeum* was involved, but this strain was also used in numerous other unsuccessful passages. Nymphae transmitted the fatal infection in the case of the Omatjenne agent, whereas an adult male appeared to have done so in the case of the Vosloo agent. The above factors were varied intentionally and on numerous occasions, but without consistent success.

In the case of both agents the change in behaviour appeared to be abrupt and not preceded by a gradual increase in pathogenicity. Likewise with the *H. truncatum* and *R. appendiculatus* passage lines, there were high antibody titres and increased resistance to challenge early on in the passages which then remained static over 4 and 6 passages, respectively. Once the change had occurred though, the Omatjenne and Vosloo agents retained their increased pathogenicity and other characteristics. It is noteworthy that the recently isolated 88/9 stock of *C. ruminantium* also remained mildly pathogenic after 5 sheep/*Amblyomma* passages (9).

The Vosloo agent, like the Omatjenne agent, is highly pathogenic to sheep and mice infected i.v. Whereas the latter is only slightly pathogenic to cattle (DU PLESSIS,

unpublished observation), 2 oxen inoculated with Vosloo agent-infected sheep blood developed severe reactions and one of them died with a positive brain smear in spite of treatment.

The cross-immunity profiles of the 2 isolates also differ. While both agents elicit immunity in sheep against the Breed, Kwanyanga and Mara 87/7 stocks of *C. ruminantium*, sheep immune to the Vosloo isolate are also immune against the Ball 3, Mali and Welgevonden stocks, whereas sheep immune to the Omatjenne isolate are not. The cross-immunity between the Vosloo isolate and the Mali stock is exceptional, since no other stock of *C. ruminantium* is known to elicit an immunity against this stock (7) and eliminates the remote possibility that any of these stocks, maintained as deep-frozen stabilates at this institute, may inadvertently have been introduced into the ticks and other material used in the experiments. It is not surprising that sheep immune to the Vosloo agent are fully susceptible to the Kümm stock.

As was argued in the case of the Omatjenne agent (4), the cross-immunity between the Vosloo agent and 6 stocks of *C. ruminantium* on one hand proves the identity of the former with the heartwater agent. On the other, the total lack of cross-immunity between the Vosloo isolate and the Kümm stock eliminates the above mentioned possibility of an accidental contamination.

Some epidemiological considerations are relevant. First, bearing in mind the varying degrees to which passage in *Amblyomma* can influence the behaviour of ehrlichial agents, it is not inconceivable that the heterogenicity of *Cowdria* stocks as far as pathogenicity, murinotropism and cross-protection are concerned, may be attributable to the influence of the tick.

Secondly, the widespread occurrence in the RSA of several species of ticks infected with ehrlichial agents *(R. evertsi, R. appendiculatus* and *H. truncatum* being implicated) and the consequent extensive seropositivity of small and large stock, questions the value of the IFA and other serological tests (10) in epidemiological surveys on heartwater. This would pose a problem only in regions where *Amblyomma* occurs, unless the very aim of the survey is to establish whether this tick does occur in a region. In a recent epidemiological study it was concluded that since not one out of 84 serologically positive cattle reacted to artificial challenge with *C. ruminantium,* interference by *Ehrlichia* was unlikely: Had some of the positive serological reactions in the challenged cattle been due to infection with *Ehrlichia,* they should have reacted to challenge, because cattle immune to *Ehrlichia* (11,19, 22) and goats immune to *Ehrlichia phagocytophila* (18) remain susceptible to challenge with *Cowdria.*

There is growing evidence that *Ehrlichia* and *Cowdria* are closely related. Apart from the findings in the present study suggesting the close relatedness between these agents, the recent observation that antibodies to *Ehrlichia* react with a *Cowdria*-specific 32kDa protein in a competitive ELISA test (17), is further support. The fact that these antibodies compete as strongly with monoclonal antibodies to a specific and dominant *Cowdria* protein as do antibodies to *C. ruminantium,* suggests that this protein is also present in *Ehrlichia.* Should future findings confirm that *Ehrlichia* and *Cowdria* are one and the same parasite, depending on whether they parasitize *Amblyomma* or one of the other tick species, the taxonomy and nomenclature of these agents should seriously be reconsidered.

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Following an earlier report that an Ehrlichia-like agent isolated from an adult *Hyalomma truncatum* female became more pathogenic and elicited a disease in sheep indistinguishable from heartwater after having been passaged through *Amblyomma hebraeum*, a similar phenomenon is herewith recorded. An ehrlichial agent demonstrated in the blood smear of a serologically positive, naturally infected lamb, changed in behaviour and assumed the characteristics of *Cowdria* after passage through *A. hebraeum*. Cross-immunity between sheep that had recovered from infection with the transformed agent and several stocks of *Cowdria ruminantium* confirmed the close relationship between the putative ehrlichial agent and *Cowdria*. Seven other sheep/tick passage lines resulted in high antibody titres and resistance to challenge with *C . ruminantium* in the sheep in the case of 2 of them, suggesting a similar change in behaviour of the agents passaged through *Amblyomma*.

Key words : Sheep - Cattle - *Ehrlichia* - *Cowdria* - *Cowdria ruminantium* - Infection - *Amblyomma hebraeum* - Tick - Antibody.

DU PLESSIS (J.L.). Relación entre *Cowdria* y *Ehrlichia* : cambios en el comportamiento de agentes de *Ehrlichia* después de pasajes por *Amblyomma hebraeum. Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 131-143

Se describe un fenómeno similar al reportado anteriormente, referente a un agente tipo Ehrlichia aislado en un adulto hembra de *Hyalomma truncatum*, el cuál, después de un pasaje por *Amblyomma hebraeum*, aumentó su patogenicidad y provocó la enfermedad en una oveja, con un cuadro idéntico al de la cowdriosis. En nuestro caso, un agente de *Ehrlichia* aislado en un frottis sanguíneo de un cordero seropositivo, infectado naturalmente, se transformó después de un pasaje por *A. hebraeum*, tomando las características de *Cowdria*. La relación entre el agente de *Ehrlichia* implicado y *Cowdria* se confirma gracias a la reacción cruzada existente entre ovinos que se han recuperado de la infección provocada por el agente transformado y varios stocks de *Cowdria ruminantium*. Se obtuvieron títulos elevados de anticuerpos mediante siete pasajes más por ovinos y garrapatas. En dos de estos pasajes se observó resistencia a la detección de *Cowdria ruminantium* en ovejas, lo que sugiere un comportamiento similar en los agentes sometidos a pasajes por *Amblyomma*.

Palabras claves : Ovino - Bovino - *Ehrlichia* - *Cowdria* - *Cowdria ruminantium* - Infección - *Amblyomma hebraeum* - Garrapata - Anticuerpo.

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The immunodominant 32-kilodalton protein of *Cowdria ruminantium* is conserved within the genus *Ehrlichia*

JONGEJAN (F.), DE VRIES (N.), NIEUWENHUIJS (J.), VAN VLIET (A.H.M.), WASSINK (L.A.). La protéine immunodominante de *Cowdria ruminantium* Cr32 conservée dans le genre *Ehrlichia*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 145-152

Les tests sérologiques pour la cowdriose sont perturbés par des réactions croisées dues à des anticorps présents chez des animaux soupçonnés d'être infectés par *Ehrlichia* spp. On a contrôlé des infections expérimentales par *Ehrlichia bovis*, *E. ovina*, *E. canis* et *E. phagocytophila*, par l'ELISA de compétition, le western blotting et l'immunofluorescence, utilisant des antigènes de cultures de cellules endothéliales infectées de *Cowdria*. Les réactions croisées par des anticorps contre *Ehrlichia* sont attribuables à leur reconnaissance d'épitopes sur la protéine immunodominante de *Cowdria*, Cr32. Ceci est surtout vrai pour *E. canis* et *E. ovina*, beaucoup moins pour *E. bovis*, mais pas du tout pour *E. phagocytophila*. De plus, une réaction croisée forte fut démontrée entre *Cowdria* et des anticorps contre E. *chaffeensis*. Ces résultats concordent avec les relations phylogénétiques trouvées récemment entre *Cowdria* et d'autres membres de la tribu des Ehrlichieae par VAN VLIET *et al.* en 1992, qui montrent que *Cowdria* est étroitement apparentée à *E. canis* et également à *E. chaffeensis*. Bien que les tests utilisés au cours de cette étude restent des outils de valeur dans les conditions du laboratoire, leur spécificité doit être améliorée. Il est proposé d'étudier des antigènes recombinants de *Cowdria* pour le développement de tests sérologiques de deuxième génération pour la maladie.

Mots clés : Protéine - *Cowdria ruminantium* - *Ehrlichia* - Technique immunologique - Test ELISA - Western blotting - Immunofluorescence - Culture de cellule - Anticorps - Antigène.

INTRODUCTION

Cowdriosis or heartwater, caused by the tick-borne rickettsia *Cowdria ruminantium*, occurs in domestic and wild ruminants and is considered one of the most important tickborne diseases in sub-Saharan Africa (17). The presence of the tick *A.variegatum*, one of the main vectors of cowdriosis, on most of the islands in the Caribbean region and the existence of the disease on at least three of these islands, constitutes a major threat for livestock on the American mainland (1).

Serodiagnosis of *Cowdria* is a possible means to assess the prevalence and present distribution of heartwater in Africa and the Caribbean in domestic and wildlife hosts (10,11,16).

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So far four types of antibody detection assays, based on endothelial cell culture antigens, have been developed : an indirect fluorescent antibody (IFA) test, (13), an indirect ELISA (14) and two tests based on recognition of the immunodominant Cr32 *Cowdria* antigen, i.e. a competitive enzyme-linked immunosorbent assay (cELISA) (7,9) and Western blots (16). A recent critical interlaboratory comparison has however indicated that cross-reactions occur in all tests currently available, probably due to *Ehrlichia* species (5).

In this study we tested antisera, obtained from experimental animals infected with different species of *Ehrlichia* (*E. bovis, E. ovina, E. canis* and *E. phagocytophila*), using immunofluorescence, competitive ELISA and Western blots to determine whether epitopes on the immunodominant Cr32 *Cowdria* antigen are also recognized by antibodies to *Ehrlichia*.

MATERIAL AND METHODS

Cultivation

Cowdria antigens for cELISA, immunofluorescence and Western blotting were prepared from rickettsiae cultivated in Bovine Umbilical Endothelial cells (BUE) as described previously (9). Briefly, monolayers of BUE cells were grown in HEPES buffered RPMI 1640 medium supplemented with antibiotics and 10 % newborn calf serum. Infection of BUE cell cultures with *Cowdria* was initiated by inoculation of BUE culture supernatant, which had been stored at -80 °C in sucrose-phosphate-glutamate (SPG) buffer (2). Infected cultures were maintained in Glasgow Minimal Essential Medium (GMEM), supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (1.25 µg/ml) HEPES buffer (20 mM; pH 7.0 to 7.2), L-glutamine (2 mM), 10 % newborn calf serum and tryptose phosphate broth (2.9 g/l).

Incubation was carried out at 37°C on a slowly rocking platform. Samples of BUE cells were scraped from the bottom of the culture flask, smeared onto a glass slide, and examined for *Cowdria* inclusions after staining with Diff-Quik (Merz & Dada AG, Dudingen, Switzerland). Cultures with virtually all BUE cells infected with reticulate bodies of *Cowdria* and containing large numbers of extracellular elementary bodies in the supernatant were used as antigen for serology.

Competitive ELISA

Infected BUE cultures were centrifuged at 4 °C for 15 min at 15,000 g. Pellets were resuspended in PBS and sonicated on ice for four periods of 15 sec with 1 min intervals (Vibracell, Sonics materials Inc., USA). The protein concentration was determined according to Bradford and sonicates were stored at -20 °C.

The cELISA was performed as described previously (9), with some modifications. Polystyrene 96-well flat-bottomed plates (Nunc) were coated overnight at 37 °C with 6 µg/ml sonicated *Cowdria* antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Plates were washed three times with tap water. Test serum (1:50 dilution) was applied, simultaneously with Mab 4F10B4 (9) (1:400), both diluted in phosphate-buffered saline (PBS) (pH 7.2), containing 3 % skimmed milk and 0.05 % Tween 20 and incubated at 37 °C for 1 h. After washing, peroxidase labeled rabbit anti-mouse immunoglobulin (Dakopatts, Denmark), at a 1:750 dilution, was added and incubated at 37 °C for 1 h. After washing, 100 µl of 0.1 M citrate buffer (pH 5.5), containing ABTS and hydrogen peroxide were added to each well. The optical density (OD) was read at 405 nm on an ELISA plate reader after 30 min incubation at room temperature in the dark.

Cowdria positive control sera and negative control sera were included on each plate in duplicate. Percentage inhibition was calculated based on the extinction obtained after incubation with monoclonal antibody without test serum.

Western blots

Endothelial cell culture sonicates, similar to the sonicates used for cELISA, were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis on a 12.5 % polyacrylamide gel. Western blotting was modified after an earlier description (9). Electrophoretic transfer was carried out at 100 V for 1 h or at 20 V overnight. Blots were quenched for 1 h in PBS/5 % skimmed milk and incubated for 3 h with test serum, positive or negative control serum or monoclonal antibody 4F10B4, diluted 1:100 in PBS containing 0.02 % Tween 20 and 5 % skimmed milk.

Bound antibodies in test sera were visualized by incubation with either rabbit anti-bovine (1:2000), rabbit anti-ovine (1:2500), rabbit anti-goat (1:5000), rabbit anti-dog (1:5000) or rabbit anti-mouse (1:500) immunoglobulins conjugated with alkaline phosphatase (Sigma) diluted in PBS containing 5 % skimmed milk. After washing, binding of conjugate was visualized by the addition of 100 mM Tris-HCl buffer (pH 9.5), supplemented with 100 mM NaCl, 5 mM MgCl2, nitroblue tetrazolium (NBT) and 5-bromo-4-dichloro-3-indocyl phosphate (BCIP). The reaction was stopped by extensive washing with a 20 mM TrisHCl buffer of pH 8.0 containing 5 mM EDTA.

Immunofluorescence

BUE cultures infected with *Cowdria* were centrifuged at 4 °C for 15 min at 15,000 g. Pellets were resuspended in PBS, spotted onto microscope slides, dried and fixed in acetone. The slides were incubated with two-fold titrations of antisera in PBS starting from 1:80 up to 1:20,480. Fluorescein isothiocyanate-labeled antibodies, *i.e.* rabbit anti-bovine, rabbit anti-sheep, rabbit anti-goat, or rabbit anti-dog immunoglobulins were used as second antibodies. Fluorescence was observed with an Olympus BH2-RFL microscope.

Experimental infections

Clinical manifestations of the experimental infections with *Ehrlichia* spp. in calves (Friesian), sheep (Tesselaar), goats (Saanen) and dogs (Beagle) are shown in table I.

***TABLE I** Clinical manifestations of experimental* **Ehrlichia** *infections.*

Animal number	Incubation period	Peak temp. (° C)	Rickettsaemia*	Treatment
dog 8765	12	41.0	(+)	oxytetracycline (1)
dog 8302	11	41.1	nps	oxytetracycline (1)
calf 456	14	40.0	(+)	–
calf 70	17	40.4	nps	–
sheep 8440	11	41.2	(+)	–
sheep 8513	9	40.5	(+)	–
goat 8769	5	41.3	++	–
goat 8727	4	41.0	++	–

* *rickettsaemia : (+) : scanty* Ehrlichia *inclusion bodies in monocytes ; ++ : large number of* Ehrlichia *inclusion bodies in granulocytes ; nps : no parasite seen.*
[1] *Engemycin®*

Ehrlichia bovis

The strain of *E. bovis*, originating from Kenya (15), caused moderate but prolonged fever in Friesian calves. In this study clinical reactions were monitored in two experimental calves (nos. 70 and 456). (Calf 456 was infected with *Cowdria* before infection with *E. bovis*).

Ehrlichia canis

A pathogenic isolate of *E. canis* was obtained from a clinical case submitted to Dr. R.J. Slappendel of the Small Animal Veterinary Clinic at the University of Utrecht, The Netherlands. Two experimental dogs were infected and monitored (nos. 8765 and 8302).

Ehrlichia ovina

E. ovina was monitored in two sheep (nos. 8440 and 8513). The isolate of *E. ovina* originated from Turkey and caused prolonged fever periods in Dutch splenectomized sheep, which eventually recovered spontaneously (G. UILENBERG, unpublished data).

Ehrlichia phagocitophila

E. phagocytophila, causal agent of tick-borne fever of ruminants, was isolated from cattle on the North Sea Island of Ameland, The Netherlands. The infection was studied in two goats (Nos. 8727 and 8769) .

Cowdria ruminantium

Two stocks of *Cowdria* were used : the Senegal isolate (6) and an isolate from Zambia (Lutale) (6). Antisera to the Senegal isolate were raised in two experimental sheep (nos. 50 and 8849) and in two goats (nos. 8737 and 8907) after intravenous inoculation of 2 ml aliquots of thawed blood stabilate. Antiserum to the Lutale isolate was raised in calf no. 57 after inoculation of 20 ml infected bovine blood. Calf 456 was infected with *Cowdria* (Lutale isolate) through feeding of infected *Amblyomma variegatum* ticks and six months later the animal was infected with *E. bovis*. Goat no. 8769 was infected with the Senegal isolate of *Cowdria* after being initially infected with *E. phagocytophila*.

Clinical reactions were treated with Terramycin LA (Pfizer). Antisera were collected 4 to 6 weeks post infection (p.i.) and stored at -20°C until further use as positive control sera.

All *Ehrlichia* and *Cowdria* isolates were stored in liquid nitrogen as infected blood stabilates, cryopreserved with 10 % dimethylsulphoxide, before being used in this study. Aliquots of 2 ml of deepfrozen blood were rapidly thawed and inoculated intravenously. The animals were monitored by daily recording of their rectal temperature and by clinical inspection. Sera were collected from all experimental animals once a week. Preinfection sera were used as negative controls.

RESULTS

Clinical manifestations of the different experimental *Ehrlichia* infections are given in table I. Only infections with *E. canis* required antibiotic treatment. Scanty ehrlichial inclusion bodies in monocytes were seen in dog 8765 and calf 456. Monocytes infected with morulae of *E.ovina* were detected on several occasions during the febrile reaction in sheep. Infections with *E. phagocytophila* in goats were characterized by large numbers of ehrlichial inclusion bodies in granulocytes during fever.

Figure 1 shows an SDS-PAGE of a *Cowdria* -infected BUE cell sonicate containing the Cr32 protein. This sonicate was used as antigen for cELISA and immunoblotting. Antibodies to *Ehrlichia canis* competed strongly with anti-Cr32 monoclonal antibody for binding sites on the Cr32 molecule in competitive ELISA. High antibody titres (5120) were also detected by immunofluorescence (fig. 2, table II). Immunodominant recognition of epitopes on Cr32 by *E.canis* antibodies is shown by immunoblotting

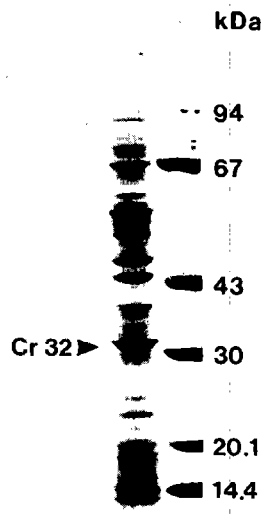

Figure 1 : SDS-PAGE of Cowdria *(Senegal) infected BUE cell culture sonicate. The Cr32 antigen is indicated.*

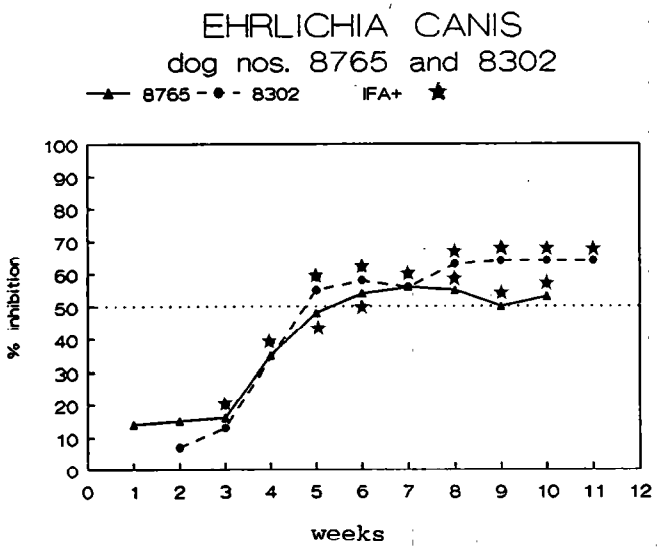

Figure 2 : Reactivity of sera from two dogs (nos. 8302 and 8765), infected with Ehrlichia canis, *with epitopes on the Cr32* Cowdria *antigen using competitive ELISA (cut-off 50% inhibition). Positive reactions determined by immunofluorescence are indicated by an asterisk (*). Both animals were infected during the first week.*

***TABLE II** Cross-reactions between* **Ehrlichia** *and* **Cowdria** *determined by IFAT*

Antigen	*Cowdria ruminantium*	*Ehrlichia canis*
antiserum		
Cowdria ruminantium	10240	5120
Ehrlichia canis	5120	20480
Ehrlichia bovis	320	nd
Ehrlichia ovina	640	nd
Ehrlichia phagocytophila	80	nd
*Ehrlichia chaffeensis**	1280	nd

nd : not determined.
** antiserum from a patient with human ehrlichiosis obtained from the Centers for Disease Control, Atlanta, Georgia, USA.*

(fig. 3, 4). Several other proteins of approximate molecular mass of 27 kDa (fig. 3, 4), 40 kDa (fig. 3) and 50 kDa (fig. 4) were also recognized.

An antibody profile similar to the one shown in fig. 2 was found in cELISA when antibodies against *E. ovina* were used (fig. 5). However, titres detected by immunofluorescence were much lower (640) with *E. ovina* antibodies than with antibodies to *E. canis* (table II). Also, in Western blots no other epitopes were recognized than those on the Cr32 protein (fig. 6, 7).

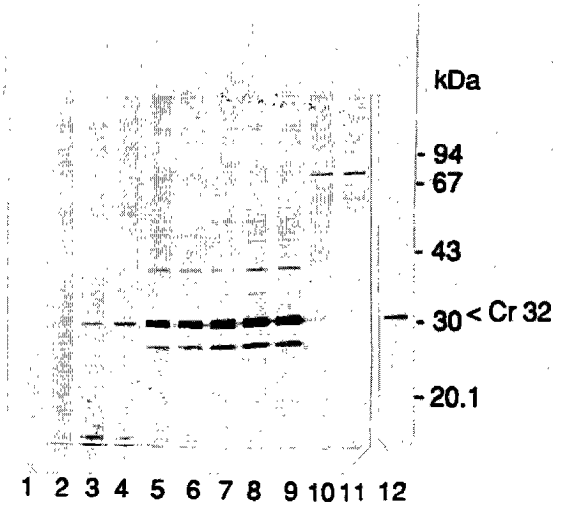

Figure 3 : Western blot analysis of sera from dog no. 8765 infected with Ehrlichia canis *on* Cowdria *antigen.*

Lane 1, pre-infection serum; lanes 2-9, sera collected respectively during week 2 and 4-10 post infection ; lane 10, non-infected control serum from dog no.138 ; lane 11, non-infected control serum from dog no. 143 ; lane 12, monoclonal antibody 4F10B4 directed against Cr32. Molecular mass markers are indicated on the right.

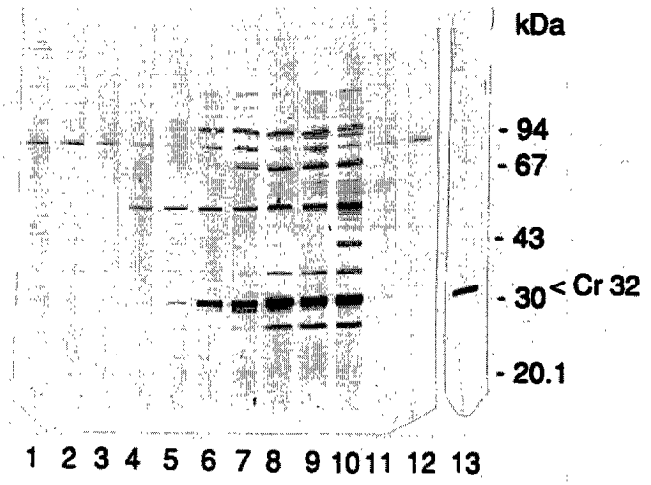

Figure 4 : Western blot analysis with sera from dog no. 8302 infected with Ehrlichia canis *on* Cowdria *antigen.*

Lane 1, pre-infection serum; lanes 2-9, sera collected respectively during week 2-10 post infection ; lane 11, non-infected control serum from dog no. 138; lane 12, non-infected control serum from dog no. 143 ; lane 13, monoclonal antibody 4F10B4 against Cr32.

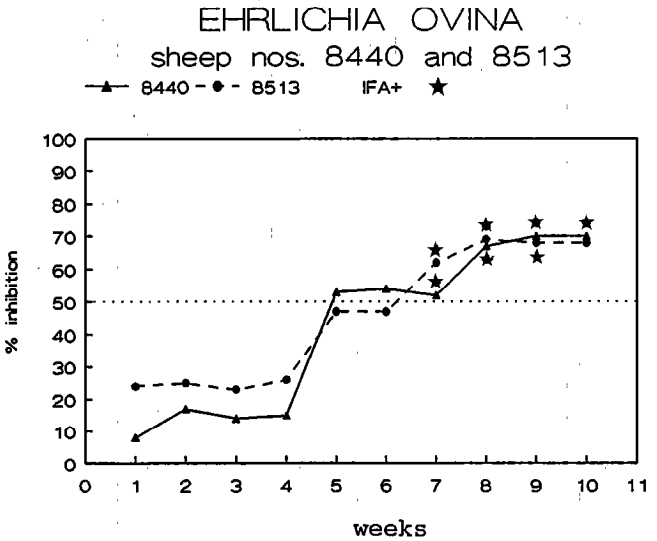

Figure 5 : Reactivity of sera from two sheep (nos. 8440 and 8513) infected with Ehrlichia ovina *with epitopes on the Cr32* Cowdria *antigen using competitive ELISA (cut-off 50 % inhibition). Positive reactions determined by immunofluorescence are indicated by an asterisk (*). Both animals were infected at week 2.*

Calf 456 was infected with *Cowdria* (Lutale) before infection with *E.bovis*. This explains the additional antibody peak in cELISA (fig. 8) and the reactivity with the Cr32 protein before the animal was infected with *E. bovis* (fig. 9). Interestingly, antibodies to *E.bovis* also recognized epitopes on an approximately 21 kDa *Cowdria* protein (fig. 9). Antibodies raised against *E. bovis* in calf no. 70

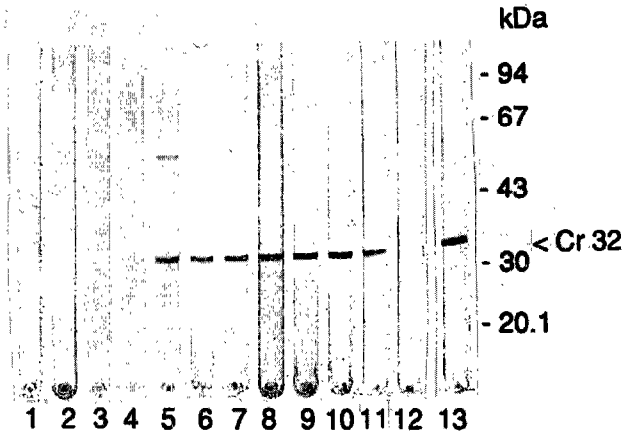

Figure 6 : Western blot analysis with sera from sheep no. 8440, infected with Ehrlichia ovina, *on* Cowdria *antigen.*

Lanes 1 and 2, pre-infection sera; lanes 3-10, sera collected during weeks 1-8 post infection, respectively ; lane 11, positive control serum from sheep no. 8849 ; lane 12, negative control serum ; lane 13, monoclonal antibody 4F10B4 to Cr32.

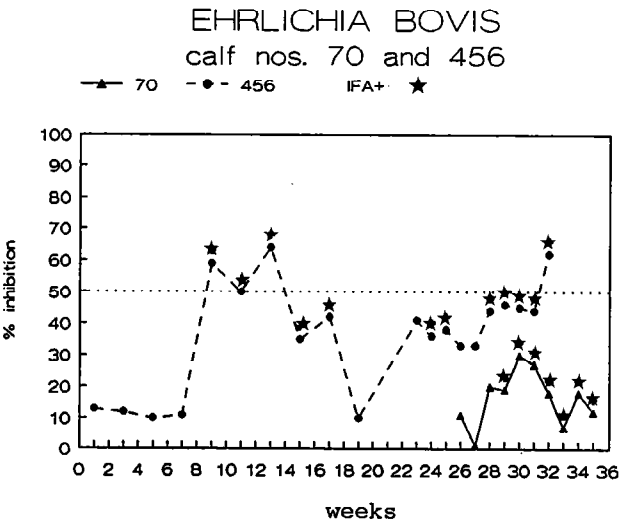

Figure 8 : Reactivity of sera from two calves (nos. 70 and 456), infected with Ehrlichia bovis, *with epitopes on the Cr32* Cowdria *antigen using competitive ELISA (cut-off 50 % inhibition). Positive reactions determined by immunofluorescence are indicated by an asterisk (*). Calf 456 was infected with* Cowdria *(Lutale isolate) in week 3 and with* E. bovis *in week 26. Calf 70 was infected at week 26.*

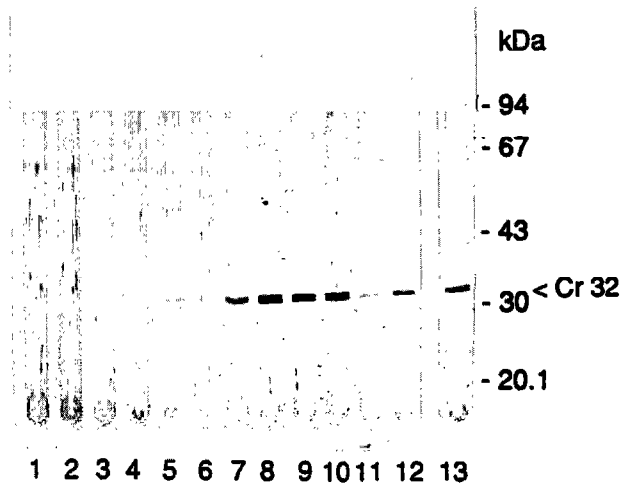

Figure 7 : Western blot analysis with sera from sheep no. 8513 infected with Ehrlichia ovina *on* Cowdria *antigen.*

Lanes 1 and 2, pre-infection sera ; lanes 3-10, sera collected during weeks 1-8 post infection ; lane 11, positive control serum from sheep no. 50 ; lane 12, positive control serum from sheep no. 8849; lane 13, monoclonal antibody 4F1OB4 against Cr32.

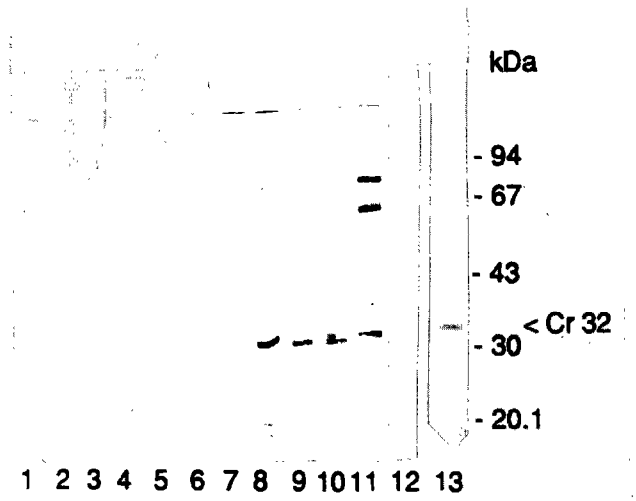

Figure 9 : Western blot analysis with sera from calf no. 456, infected with Ehrlichia bovis *on* Cowdria *antigen.*

*Lanes 1-3, sera collected during weeks 23-25 post-*Cowdria *infection, lanes 4-10 (infection with* E. bovis*) sera collected between weeks 26 and 32 (fig. 8) ; lane 11. positive control serum from calf no. 57 ; lane 12, negative control from calf no 63 : lane 13. monoclonal antibody 4F10B4 against Cr32. Calf 456 was infected with* Cowdria *(Lutale isolate) at week 3 and with* E. bovis *at week 26.*

did not recognize the Cr32 protein and antibodies of a maximum titre of only 320 were detected by immunofluorescence (fig. 10, table II).

Antibodies against *E. phagocytophila* were not detected in cELISA and Western blots (fig. 11, 12). Goat 8769 was challenged with *Cowdria* (Senegal) after the *E. phagocytophila* infection. Therefore, positive reactions in cELISA at week 4, see fig. 11, corresponding to lane 10 in fig. 12, are due to infection with *Cowdria*. Low levels of cross-reacting antibodies (80 or less) were detected by immunofluorescence (table II).

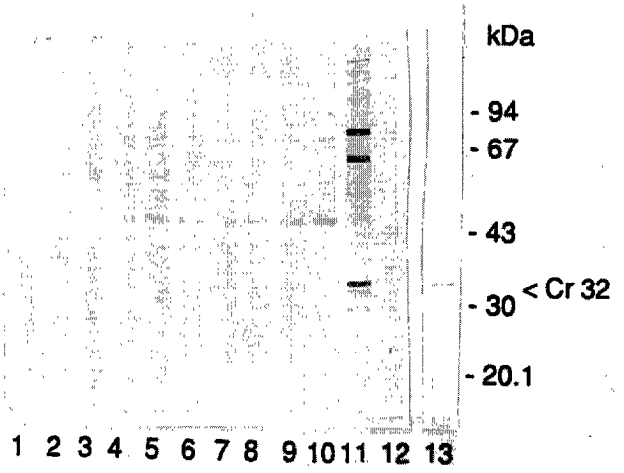

Figure 10 : Western blot analysis with sera from calf no. 70, infected with Ehrlichia bovis, *on* Cowdria *antigen.*

Lane 1, pre-infection serum ; lanes 2-10, sera collected during weeks 2 to 10 post-infection ; lane 11, positive control serum from calf no. 57 ; lane 12, negative control from calf no. 63 ; lane 13, monoclonal antibody 4F10B4 against Cr32. Molecular mass markers are indicated on the right.

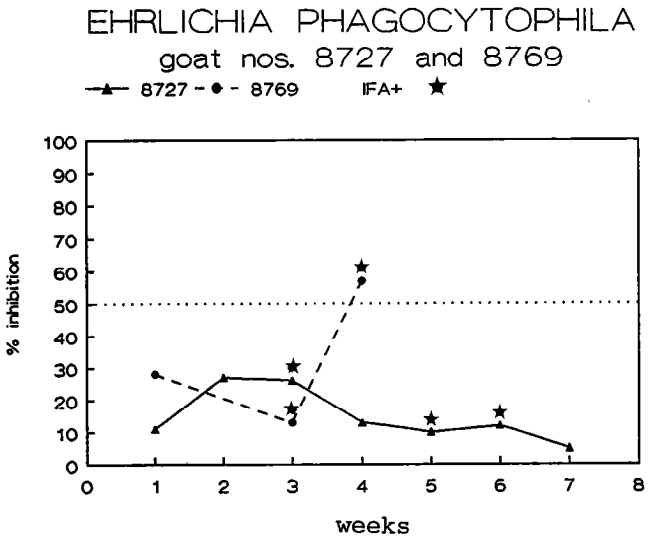

Figure 11 : Reactivity of sera from two goats (nos. 8727 and 8769), infected with Ehrlichia phagocytophila, *with epitopes on the Cr32* Cowdria *antigen using competitive ELISA (cut-off 50 % inhibition). Positive reactions determined by immunofluorescence are indicated by an asterisk (*). Goat no.8769 was infected with* Cowdria *(Senegal) after infection with phagocytophila.*

Finally, antibodies against the human pathogen *E. chaffeensis* strongly cross-reacted with *Cowdria* antigens in the IFA test (table II).

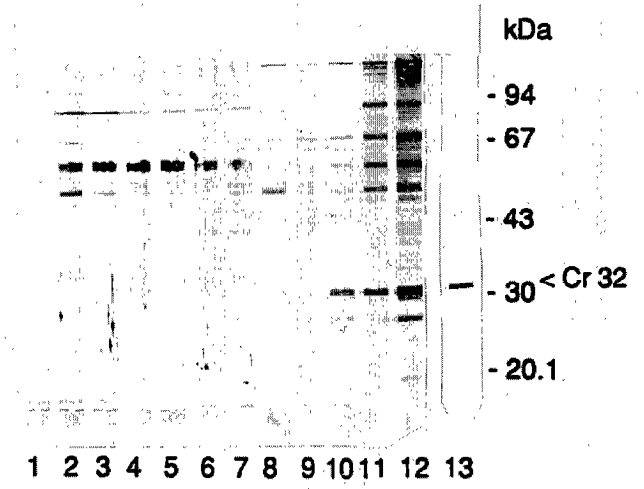

Figure 12 : Western blot analysis with sera from goats (nos. 8727 and 8769), infected with Ehrlichia phagocytophila, *on* Cowdria *antigen.*

*Lanes 1-7, sera from goat no. 8727. Lane 1, pre-infection serum ; lanes 2-7, post-infection sera ; lanes 8-10 , sera from goat no. 8769 ; lane 8, one week post-*Ehrlichia *infection ; lane 9, three weeks post-*Ehrlichia *infection ; lane 10, four weeks post-*Ehrlichia *infection and two weeks post-*Cowdria *infection ; lane 11, positive control serum from goat no. 8737 ; lane 12, positive control serum from goat no. 8907 ; lane 13, monoclonal antibody 4F10B4 against Cr32. Molecular mass markers are indicated on the right.*

DISCUSSION

Already the IFA tests based on infected neutrophils or macrophages were known to have limited specificity due to cross-reactions with *Ehrlichia* species (3, 4, 8, 12). Here we demonstrate that serological tests based on endothelial cell culture antigens are also hampered by cross-reactive epitopes shared between *Cowdria* and *Ehrlichia*. False positive serological results in competitive ELISA and Western blots due to cross-reacting antibodies against *Ehrlichia* can be attributed to the recognition of epitopes on the immunodominant Cr32 molecule. This is especially true for *E. canis* and *E. ovina*, much less so for *E. bovis* and not at all for *E. phagocytophila*.

In addition, strong cross-reactivity between *Cowdria* antigens and antibodies to *E. chaffeensis* was demonstrated for the first time (table II). Cross-reactivity between *Cowdria* and *E. canis* goes beyond the recognition of Cr32 epitopes only, since several other proteins preliminarily characterized as 27 kDa, 40 kDa and 50 kDa were shown on blots (fig. 3, 4). This was not the case for anti-*E. ovina* antibodies, which recognized Cr32 epitopes only (fig. 6, 7).

Our findings are in agreement with the recently reported phylogenetic relationships between *Cowdria* and other members of the tribe Ehrlichieae (18), wherein *Cowdria, E. canis* and *E. chaffeensis* proved to be closely related.

Antibodies to the more distantly related *E. phagocytophila* did not compete in the cELISA and only a low level of cross-reactive antibodies were detected by immunofluorescence. Although the phylogenetic position of *E. ovina* and *E. bovis* has not been studied, one could predict, based on the results obtained in this study, that both rickettsiae may have an intermediate position between the *Cowdria/E. canis/E. chaffeensis* cluster and the *E. phagocytophila/E. equi* cluster.

In conclusion, it is clear that there is a serious specificity problem in all serological tests developed for cowdriosis so far. However, the different serological tests remain valuable tools for laboratory controlled conditions and can provide useful information when used in epidemiological surveys.

Production of *Cowdria* antigens in endothelial cell cultures is relatively laborious and expensive. Moreover, the quality varies between antigen batches and this requires checkerboard titrations for every antigen batch to determine the correct test conditions. Recombinant antigens can be produced in large batches of high quality and at a low cost. It is therefore particularly relevant that we have recently cloned the gene encoding the Cr32 protein of *C. ruminantium* (19). Cloning of this gene makes it feasible to investigate whether recombinant *Cowdria* antigen can be used for second generation serological tests for cowdriosis. Expression of parts of the Cr32 protein could be used to differentiate *Cowdria* and *Ehrlichia*-specific domains by screening with polyclonal species-specific antisera. This approach is currently under investigation.

ACKNOWLEDGEMENTS

The research presented here was supported by the European Community (DG XII), STD-2 programme, contract no. TS2-0115-C entitled : "Integrated Control of Cowdriosis and Dermatophilosis of Ruminants", and under contract TS3*-CT91-0007 (STD-3 programme) entitled: "Réseau de Recherches sur la Cowdriose et ses Vecteurs". Evaluation and development of the prototype cELISA test kit for cowdriosis was carried out for the Office International des Epizooties, Paris, France, and was financially supported by the French Ministry of Cooperation and Development. Doctors J. G. OLSON and J. E. DAWSON from the Centers for Disease Control, Atlanta, Georgia, USA are thanked for providing a sample of human serum from an *Ehrlichia chaffeensis* patient.

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JONGEJAN (F.), DE VRIES (N.), NIEUWENHUIJS (J.), VAN VLIET (A.H.M.), WASSINK (L.A.). The immunodominant 32-kilodalton protein of *Cowdria ruminantium* is conserved within the genus *Ehrlichia*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 145-152

Serological tests for cowdriosis are hampered by cross-reacting antibodies from animals suspected to be infected with *Ehrlichia* species. We have monitored infections with *Ehrlichia bovis*, *E. ovina*, *E. canis* and *E. phagocytophila* in experimental animals by competitive ELISA, Western blotting and immunofluorescence using *Cowdria*-infected endothelial cell culture antigens. Cross-reactions due to *Ehrlichia* antibodies could be attributed to the recognition of epitopes on the immunodominant Cr32 *Cowdria* protein. This was especially true for *E. canis* and *E. ovina*, much less for *E. bovis*, but not at all for *E. phagocytophila*. In addition, strong cross-reactivity between *Cowdria* and antibodies to E. chaffeensis were demonstrated. These findings are in agreement with the phylogenetic relationships, recently reported by VAN VLIET *et al.* in 1992, between *Cowdria* and other members of the tribe Ehrlichieae, which showed *Cowdria* to be closely related to *E. canis* and also to *E. chaffeensis* . Although the tests used in this study remain valuable tools under laboratory conditions, their specificity requires improvement. It is suggested to study recombinant *Cowdria* antigens for the development of second generation serological tests for cowdriosis .

Key words : Protein - *Cowdria ruminantium* - *Ehrlichia* - Immunological technique - ELISA test - Western blotting - Immunofluorescence test - Cell growth - Antibody - Antigen.

JONGEJAN (F.), DE VRIES (N.), NIEUWENHUIJS (J.), VAN VLIET (A.H.M.), WASSINK (L.A.). La proteína inmunodominante 32 kilodalton de *Cowdria ruminantium* se conserva dentro del género *Ehrlichia*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 145-152

Las reacciones cruzadas con anticuerpos provenientes de animales sospechosos de *Ehrlichia* sp., perturban los tests serológicos para *Cowdria*. Se detectaron infecciones con E. bovis, *E. ovina*, *E.canis* y *E. phagocytophila* en animales experimentales mediante el ELISA, el "western blotting" y la inmunofluorescencia, gracias al uso de antígenos de cultivos celulares infectados con *Cowdria*. Las reacciones cruzadas con los anticuerpos de *Ehrlichia* podrían ser ocasionadas por el reconocimiento de epítopos en la inmunoproteína Cr32 de *Cowdria*. Esto se verificó principalmente para *E. canis* y *E. ovina*, en menor medida para *E. bovis*, pero no para *E. phagocytophila*. Se demostró tambien una fuerte reacción cruzada entre *Cowdria* y anticuerpos de *E. chaffeensis*. Estos hallazgos coinciden con los recientes reportes por VAN VLIET *et al.* en 1992 sobre las relaciones filogenéticas entre *Cowdria* y otros miembros de la familia Ehrlichieae , según los cuáles existe una relación estrecha entre *Cowdria* y *E. canis* y *E. chaffeensis*. A pesar de que los tests utilizados en este estudio representan un instrumento importante a nivel de laboratorio, es necesario mejorar la especificidad de los mismos. Se recomienda estudiar los antígenos recombinantes de *Cowdria*, con el fin de desarrollar una segunda generación de tests serológicos para la cowdriosis.

Palabras claves : Proteína - *Cowdria ruminantium* - *Ehrlichia* - Técnica inmunologica - Test ELISA - Western blotting - Inmunofluorescencia - Cultivo de célula - Anticuerpo - Antigeno.

STVM-93

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Bovine and human endothelial cell growth on collagen microspheres and their infection with the rickettsia *Cowdria ruminantium* : prospects for cells and vaccine production

TOTTÉ (Ph.), BLANKAERT (D.), MARIQUE (T.), KIRKPATRICK (C.), VAN VOOREN (J.P.), WÉRENNE (J.). Culture de cellules bovines et humaines sur des microsphères de collagène et leur infection avec la rickcttsic *Cowdria ruminantium* : perspectives pour la production des cellules et de vaccin. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 153-156

La rickettsie *Cowdria ruminantium* a été cultivée avec succès dans des lignées de cellules endothéliales bovines (ombilicales, BUEC, et de microvasculature, BMC), ainsi que dans des cultures primaires de cellules endothéliales d'aorte de bovin (BAEC), mais de manière plus surprenante, également dans des cellules endothéliales d'origine humaine : de veine ombilicale (HUVEC) et de microvasculature (HEMEC). Cette première preuve de pathogénicité de cette rickettsie bovine pour le système cellulaire humain provoque un nouvel intérêt concernant sa signification possible pour la santé humaine. Elle indique également d'autres possibilités pour l'atténuation d'isolats de *Cowdria ruminantium* et donc de nouvelles perspectives pour le développement d'un vaccin. Pour la production de vaccin, la culture en grand de cellules est essentielle. Les résultats montrent que les cellules endothéliales s'attachent de façon efficace sur des microsphères de collagène. Les BAEC se multiplient bien par lots sur ces billes, et si le processus pouvait être optimisé pour les différents types de cellules (utilisant les facteurs appropriés d'adhésion et de croissance), ces observations indiquent des perspectives intéressantes pour le développement futur d'une production de vaccin contre la cowdriose dans le réacteur à lit fluide VERAX Système un, qui donne des conditions de culture facilement contrôlables.

Mots clés : Cowdriose - Rickettsie - *Cowdria ruminantium* - Culture de cellule - Cellule endothéliale bovine - Cellule endothéliale humaine - Collagène - Vaccin.

INTRODUCTION

Cowdriosis (or heartwater) is one of the main causes of economical losses for cattle breeding in Subsaharan Africa. As the disease appeared recently in the Caribbean, the American mainland is at risk also.

Methods of vaccination against heartwater exist (e.g. deliberate infection of the animals with virulent sheep blood "vaccine" followed by tetracycline treatment at the time of hyperthermia), but their application is risky and laborious (4). The method is not applicable on a large scale either.

Other means of control are therefore needed. Before one could use an active recombinant vaccine on a large scale, there is still room for a more classical approach. The recent attenuation of one strain of *Cowdria ruminantium* by long term passage in BUEC cells (2) paved the way to this direction. Despite trials with different strains of *Cowdria ruminantium*, up to now success in attenuation has been limited. Generalization of this approach requires the possibility to attenuate different isolates of the rickettsia. As a contribution to this goal, we have undertaken a series of trials using different kinds of endothelial cells as host for the rickettsia multiplication, with the hope of extending the cell systems available to grow *Cowdria ruminantium*.

Another essential aspect in vaccine production is the development of an appropriate method for large scale cell production. Indeed the classical vaccine approach requires production of attenuated variants of the rickettsia in a convenient mass cell culture system.

The present investigation had also this prospect as a goal. As a first step towards mass cell-culture of endothelial cells in a bioreactor, we have studied adhesion properties of the collagen microspheres used routinely in the VERAX System one fluidized-bed bioreactor (5)

MATERIAL AND METHODS

Isolation and culture of endothelial cells

Bovine endothelial cells from the brain microvasculature (BMC) were received from Dr. G. TARONE (University of Torino, Department of Biology and Medical Chemistry, Torino, Italy). Bovine endothelial cells from umbilical cord arteries were obtained from Dr. F. JONGEJAN (Utrecht University, Faculty of Veterinary Medicine, Utrecht, The Netherlands) .

Human endothelial cells from the microvasculature (HEMEC) were received from Dr VAN HINSBERG (TNO, Leiden, The Netherlands). Primary cultures from human endothelial cells from the umbilical vein (HUVEC) were initiated in our laboratory as well as bovine endothelial cells from the aorta (BAEC), using essentialy the same basic method. HUVEC cells were isolated from the umbilical cord by collagenase (Boehringer Mannheim,

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Germany) digestion according to the method of Gimbrone essentially as follows. Cells pooled from three to six umbilical cords were cultured on gelatin-coated 100 mm plastic tissue culture dishes (Nunc, Denmark) in medium 199 (Gibco) supplemented with 20 % foetal calf serum (Gibco), 100 mg/ml bovine brain extract, 100 mg/ml porcine heparin (Sigma), 100 Units/ml penicillin and 100mg/ml streptomycin (M199 complete medium). Seven to eight passages could be obtained from this primary culture. For the BAEC cells, a similar method but adapted for the handling of bovine aorta was applied. In both cases, a good yield of cells was routinely obtained.

Medium BHK 21 was used for the maintenance of the cells in culture (details are described in an accompanying paper, "Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells").

For the culture on porous VERAX collagen unweighted microspheres, we adapted the standard method of the producer to the needs of endothelial cells.

Culture of the cells on collagen microspheres

The porous collagen microsphere is the basic element of the unique fluidized-bed culture system manufactured by VERAX. It is made of collagen derived from native bovine collagen using a proprietary process. Both weighted (with a density of about 1.6 for fluidized-bed technology) and unweighted (for batch procedure) forms of microspheres are available (5). The seeding conditions were as follows : 3.10^6 of endothelial cells were incubated in conical plastic flasks containing 50 ml of medium supplemented with non-essential amino-acids, penicillin and streptomycin with 10 % fetal calf serum, together with 5 ml of standard VERAX collagen microsphere suspension. To allow fixation the culture was operated first for 24 h without agitation ; further incubation was performed with shaking to allow oxygenation.

RESULTS AND DISCUSSION

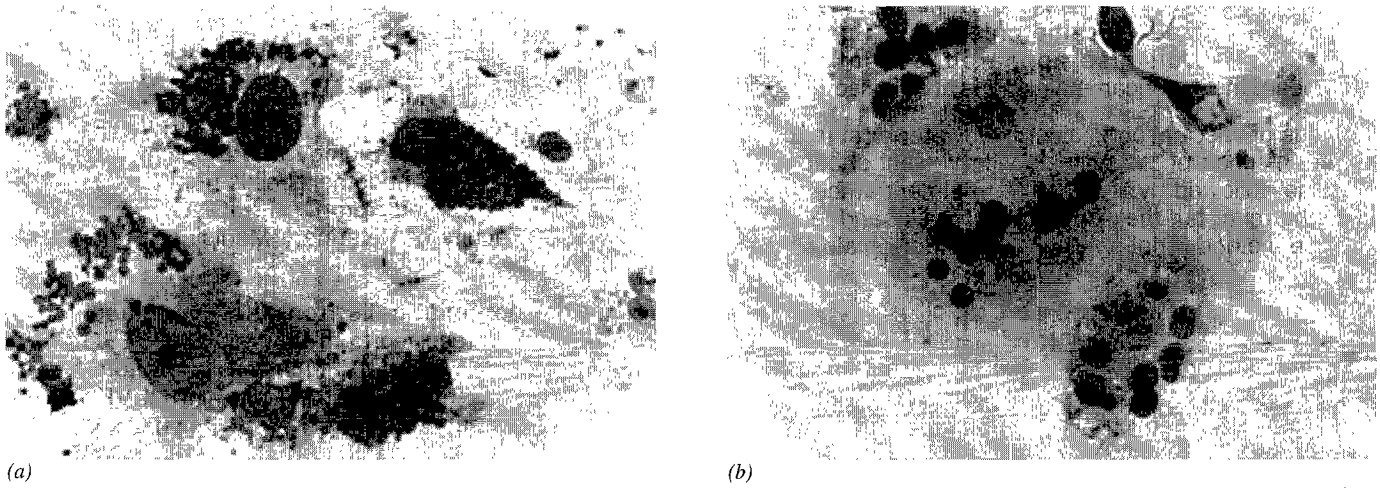
Multiplication of *Cowdria ruminantium* in bovine and human endothelial cells

In all the endothelial cells used in the present study, *Cowdria ruminantium* (Senegal), was shown to replicate efficiently, including in the human cells whether of umbilical vein or microvasculature origin (not shown).

In the different systems, the same kind of developmental pathway leading to the maturation of the rickettsia seems to occur. The morphology and development of *Cowdria* observed both in HUVEC and in BMC is similar (photo 1). Colonies of *Cowdria* were visible in the cytoplasm of the cells which finally lysed. However, the *Cowdria* morulae appeared to be less numerous per cell in the HUVEC compared to infected BMC .

Adhesion and growth of endothelial cells on porous collagen microspheres

Using unweighted porous collagen microspheres in non-agitated batch culture, a high proportion (routinely more than 70 %) of freshly trypsinized endothelial cells adhere quickly in the carriers. After a latency of about one day, in these non-agitated procedures, cell multiplication occurs first in an exponential way and then continues at



(a) (b)

Photo 1 : Diff-Quick staining of Cowdria ruminantium *in vitro in BMEC (a) and HUVEC (b), 10 days post-infection (X 735).*

a slower rate for a long period at a more or less linear fashion before reaching a plateau. The situation observed with the BAEC are presented in figure 1 as an example.

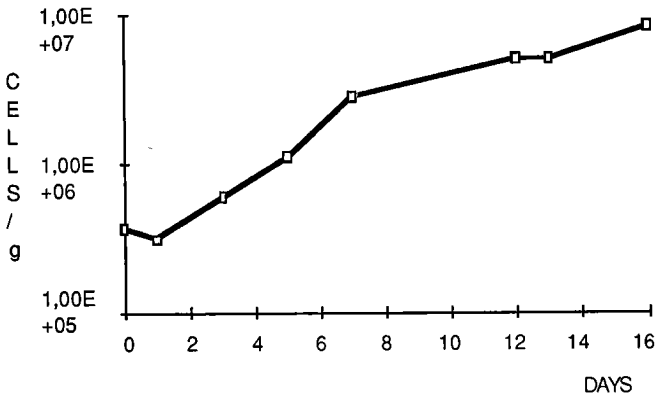


Figure 1 : BAEC growth on VERAX unweighted porous collagen microspheres.

CONCLUSION

Altogether, our results offer interesting prospects for the future development of a vaccine against cowdriosis and raise important questions concerning the possible relevance of the infection observed in human endothelial cells for human health.

The possibility to grow one isolate of *Cowdria ruminantium* in endothelial cells both of bovine and human origin that we demonstrated here, together with our previous observation that interferons and other cytokines play a role in the natural resistance against *Cowdria* (6), open new avenues to the search of attenuated variants not only for the Senegal isolate studied but for other isolates also. Systematic trials of the pathogenicity of the *Cowdria* variant obtained in cells of the heterologous species should be made.

We have also approached the question of scaling-up the production of endothelial cells in a bioreactor, and as a first step, the adhesion of BAEC on collagen microspheres is already encouraging. The differences noticed between the properties of bovine and human primary cells, indicate that specific conditions should be applied in each case and our results also indicate a need for further investigations in order to find less fragile cells for growing *Cowdria*. We are presently screening a number of different cell lines for the properties we are looking for.

The question of the relevance of the infection for human health is of particular importance in Africa since immunodeficiency conditions may affect the resistance of individuals. In AIDS related conditions, many opportunistic infections may develop that were not observed in a previous situation. In a recent survey, sera from humans exposed to vector ticks were found to be free of antibodies directed against *Cowdria ruminantium* (3). However, this does not rule out a possible immunologically silent infection already described (1), and other methods of screening should also be used in the future to adress this important question.

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TOTTÉ (Ph.), BLANKAERT (D.), MARIQUE (T.), KIRKPATRICK (C.), VAN VOOREN (J.P.), WÉRENNE (J.). Bovine and human endothelial cell growth on collagen microspheres and their infection with the rickettsia *Cowdria ruminantium* : prospects for cells and vaccine production. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 153-156

We successfully cultivated the rickesttsia *Cowdria ruminantium*, in bovine endothelial cell lines (Bovine Umbilical Endothelial Cells/BUEC and Bovine microvasculature Cells/BMC) and also in primary endothelial cells of bovine origin (Bovine Aorta Endothelial cells/BAEC) and more surprisingly in cells of human origin - Human Umbilical Vein Endothelial Cells/HUVEC - and Human Endothelial Cells from the Microvasculature/HEMEC. This first evidence of the pathogenicity of this bovine rickettsia in thehuman cell system generates new interest as regards its possible relevance for human health. It provides also further possibilities for the attenuation of *Cowdria ruminantium* isolates, and therefore brings new prospects for vaccine preparation. In vaccine production, mass cell culture is essential. Our results indicate that endothelial cells attach efficiently on collagen microspheres. As BAEC cells grow well on them in a batch mode, and if the process could be optimized for the different cell types (using appropriate adhesion and growth factors) our observations offer interesting prospects for the future development of a *Cowdria ruminantium* vaccine production in the fluidized-bed reactor VERAX System one, which provides easy control of growth conditions.

Key words : Heartwater - Rickettsia - *Cowdria ruminantium* - Cell growth - Bovine endothelial cell - Human endothelial cell - Collagen - Vaccine.

TOTTÉ (Ph.), BLANKAERT (D.), MARIQUE (T.), KIRKPATRICK (C.), VAN VOOREN (J.P.), WÉRENNE (J.). Crecimiento de células endoteliales bovinas y humanas en microplacas de colágenos y su infección con la rickettsia *Cowdria ruminantium* : perspectivas para la producción de células y de vacunas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 153-156

La rickettsia *Cowdria ruminantium* se cultivó, con éxito, en líneas celulares de endotelio bovino (células de endotelio umbilical bovino/BUEC y células microvasculares bovinas/BMC), así como en células de endotelio primario de origen bovino (células de endotelio aórtico bovino/BAEC) y células de origen humano (células humanas de endotelio de la vena umbilical/HUVEC y células de endotelio microvascular humano/HEMEC). Esta primera evidencia de la patogenicidad de la rickettsia bovina sobre el sistema celular humano, presenta un nuevo interés en cuanto a su posible importancia para la salud humana. También provee otras posibilidades para la atenuación de los ailamientos de *Cowdria ruminantium* y nuevas perspectivas para la preparación de vacunas. El cultivo celular en masa es esencial para la producción de vacunas. Nuestros resultados indican que las células endoteliales atacan en forma eficiente en las microesferas de colágeno. En vista de que las células BAEC crecen correctamente en grupos en esta materia y siempre y cuando el proceso sea mejorado para los diferentes tipos celulares (mediante el uso apropiado de factores de crecimiento y de adhesión), nuestras observaciones representan una perspectiva interesante para el desarrollo futuro de la producción de la vacuna contra *Cowdria ruminantium*, en una capa fluida del agente reactor del Sistema uno VERAX, el cual presenta facilidades en las condiciones de crecimiento.

Palabras claves : Cowdriosis - Rickettsia - *Cowdria ruminantium* - Cultivo de célula - Célula endotelial bovina - Célula endotelial humana - Colágeno - Vacuna.

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Isolation and characterization of antigenic proteins of *Cowdria ruminantium*

VAN KLEEF (M.), NEITZ (A.W.H.), DE WAAL (D.T.). Isolement et caractérisation de protéines antigéniques de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 157-164

Deux protéines conservées antigéniquement, une immunodominante de 31 kilodalton et une mineure de 27 kDa, ont été caractérisées. Les protéines de 31 kDa et de 27 kDa sont des chaînes polypeptides simples. La protéine de 31 kDa ne contient pas de glycoconjugués et la séquence interne des acides aminés a été partiellement déterminée. La nature acide de cette protéine, déduite de sa composition en acides aminés, a été confirmée par la détermination du point isoélectrique (5,7). Des antisérums ont été préparés contre les protéines de 27 et de 31 kDa et les résultats indiquent qu'elles sont immunogènes et partagent des épitopes communs.

Mots clés : *Cowdria ruminantium* - Protéine antigénique - Isolement - Acide aminé - Point isoélectrique - Antisérum - Immunisation.

INTRODUCTION

Two antigenically conserved proteins, an immunodominant 31 kDa and a minor 27 kDa protein, were identified in our laboratories which are common amongst 9 stocks of *C. ruminantium*, differing in virulence, pathogenicity and origin (15). These proteins may be suitable for the development of nucleic acid probes, diagnostic assays and vaccines. In 1989, JONGEJAN and THIELEMANS identified a 32 kDa immunodominant, antigenically conserved *C. ruminantium* protein (7).

Amino acid analysis and partial sequencing of these proteins should make it possible to develop appropriate oligonucleotide probes either for screening *C. ruminantium* genomic libraries or in a diagnostic assay of the disease. Furthermore the development of a monospecific antisera against the 27 or 31 kDa proteins, would allow a means for identification of specific DNA clones coding for these proteins.

Determination of whether the 27 or 31 kDa are glycoproteins or not is of importance since prokaryotes are not capable of glycosylating proteins. Their presence would therefore imply that these organisms incorporate host cell material.

In this article the purification and certain characteristics of the 27 kDa and specifically the 31 kDa proteins of the *C. ruminantium* are described.

MATERIALS AND METHODS

In vitro cultivation of *C. ruminantium*

The Welgevonden stock (3) of *C. ruminantium* was cultured in a calf endothelial cell line (E_5 cell line) as described previously (1). Crude Welgevonden stock infected and uninfected extracts were prepared from cell cultures as previously described (15).

SDS-PAGE with or without reducing agent

Crude Welgevonden stock infected and uninfected cell cultures were dissolved in buffer containing 0.06 M Tris-HCl (pH 6.8), 16 % glycerol, 2 % sodium dodecyl sulphate (SDS) and 0.001 % bromophenol blue, with or without 2.5 % dithiothreitol (DTT), by heating at 100 °C for 10 min. The samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis SDS-PAGE and the gels either stained with Coomassie or immunoblotted with anti-Welgevonden stock bovine serum as described previously (15).

Production of monospecific anti-serum

Preparative SDS-PAGE was performed with crude Welgevonden stock infected culture extracts. An amount of 1900 µg crude protein was loaded per 1.5 x 120 x 160 mm gel, corresponding to approximately 133 µg of the 27 kDa protein and 114 µg of the 31 kDa protein. The amount of protein was estimated from a standard curve obtained after scanning a Coomassie stained SDS-PAGE gel of Welgevonden stock uninfected and infected crude cell cultures and known amounts of bovine serum albumin and chymotrypsinogen A. After preparative SDS-PAGE the gel was stained with 0.3 M $CuCl_2$ as described previously (8). The 27 and 31 kDa protein bands were excised and a volume of PBS added to the excised bands of one preparative gel, giving a final volume of 2.5 ml. The gel was then fragmented by passing back and forth

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between 2 syringes first connected by a 19 G needle followed by a 21 G needle, and stored at -70 °C until required for immunization.

Four rabbits, 2 goats and a sheep were immunized with either the 27 kDa protein or the 31 kDa protein according to table I. The serum collected at intervals indicated in table I were evaluated and titers determined by immunoblotting using crude Welgevonden stock infected and uninfected culture extracts as antigen in the western blots. Immunoblotting was performed as described earlier (15).

Determination of whether the 31 kDa protein of *C. ruminantium* is protective towards infection with heartwater

Goats A and B and sheep A were immunized as described in table I. Goat C and sheep B were immunized once with Welgevonden stock infected blood stabilate (12) and treated as previously described (15). All these animals including a heartwater naive goat and sheep were challenged by inoculation with 5 ml Welgevonden stock blood stabilate as described in table II. Daily rectal temperatures of the goats and sheep were monitored. They were not treated when heartwater symptoms developed after challenge and the cause of death was determined by post mortem investigations.

Amino acid analysis

Welgevonden stock infected crude cell culture extracts were subjected to SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membranes and stained with Coomassie as described earlier (15).

Amino acid analysis by HCl hydrolysis, standard phenylisothiocyanate derivatization and reverse phase high performance liquid chromatography (HPLC) analysis were performed according to the PICO-TAG method of Millipore. Tryptophan analysis by methane sulphonic acid (MSA) hydrolysis and cysteine analysis by performic acid oxidation were performed on duplicate samples according to the Millipore instruction manual. The data obtained was statistically analysed according to a Parameters of Varieties program written by Dr VAN ARK, Computer Section, OVI, Onderstepoort.

TABLE I Immunoblot titers obtained from monospecific anti-serum produced in rabbits, goats and a sheep. Only serum from one of the duplicate rabbits were tested. All inoculations were administered subcutaneously and intramuscularly.

Day	Inoculum bled	Reciprocal of immunoblot titer									
		Rabbit 27 kDa*		Rabbit 31 kDa#		Goat A		Goat B		Sheep A	
						31 kDa@		31 kDa@			
		27 kDa	31 kDa	27 kDa	31 kDa	27 kDa	31 kDa	27 kDa	31 kDa	27 kDa	31 kDa
0	Antigen/ FCA (1 : 1)	√		√		√		√		√	
14	Bled	neg	neg	neg	neg	1 000	125 000	200	5 000	1 000	2 500
28	Antigen/ FIA (1 : 1)	√		√		√		√		√	
42	Bled	neg	40	200	1 000	5 000	125 000	2 500	625 000	5 000	125 000
56	Antigen	√		√		√		nd		nd	
70	Bled	40	200	200	1 000	5 000	125 000	nd	nd	nd	nd
84	Antigen	√		√		√		nd		nd	
98	Bled	200	1 000	200	1 000	1 000	125 000	nd	nd	nd	nd

** inoculated with 133 µg of the 27 kDa protein/immunization.*
 # inoculated with 114 µg of the 31 kDa protein/immunization.
@ inoculated with 266 µg of the 31 kDa protein/immunization.
√ immunized.
nd not done.
FCA Freunds complete adjuvant.
FIA Freunds incomplete adjuvant.

TABLE II Determination of whether immunization with the 31 kDa protein of **C. ruminantium** *is protective towards infection with heartwater.*

Animal	Immunized	Reciprocal immunoblot titer prior to challenge	Week challenged# after first immunization	Outcome after challenge
Goat A	31 kDa*	< 500	48	febrile reaction, died
Goat B	31 kDa*	625 000	8	febrile reaction, died
Goat C	Welgevonden stock infected blood stabilate	< 500	45	no febrile reaction, survived
Goat D	nd	neg	0	febrile reaction, died
Sheep A	31 kDa*	125 000	8	febrile reaction, died
Sheep B	Welgevonden stock infected blood stabilate	neg	90	no febrile reaction, survived
Sheep C	nd	neg	0	febrile reaction, died

** immunized as described in Table I.*
 # inoculated with 5 ml of the Welgevonden infected blood stabilate.
nd : not done.
neg : negative.

Amino acid sequencing

Automated amino acid sequencing was performed on the 31 kDa protein electroeluted from SDS-PAGE gels and a CNBr peptide fragment of this protein. The electrophoresis was performed with the pH 7.28 MZE 3328.IV buffer system as described previously (11). The gels were stained for 60 min with 0.1 % Coomassie, 10 % methanol and 0.5 % acetic acid and destained in 10 % methanol. The 31 kDa protein band was excised from the Coomassie stained SDS-PAGE gel, cut into small pieces and soaked in 0.5 % (w/v) N-cetyl-N,N,N,-trimethylammonium bromid (C-TAB) containing 10 % 2-merkapto-ethylammonium and 0.45 M acetic acid for 60 min at room temperature. The equilibrated gel piece was placed into the elution chamber of the Bio-Trap, containing C-TAB buffer. The buffer chamber of the Bio-Trap was filled with 0.45 M acetic acid and electroelution performed at 200 V for 120 min at room temperature. The eluted sample was freeze dried and a volume of 200 µl methanol added followed by 800 µl cold acetone. This solution was incubated at - 20 °C for 30 min and centrifuged at 11 000 x g for 6 min. The supernatant was removed and the pellet washed with 800 µl cold acetone and centrifuged at 10 000 x g for 6 min. The resulting pellet was dried under vacuum and its purity determined by SDS-PAGE before amino acid sequencing or CNBr cleavage.

CNBr cleavage was performed as described previously (10). A volume of 50 µl 70 % formic acid was added to the electroeluted and dried 31 kDa protein, thereafter a 600 molar excess of CNBr in 70 % formic acid was added. After incubating overnight at room temperature the samples were dried under N_2 gas. An aliquot was investigated by SDS-PAGE and the rest subjected to HPLC.

The sample was dissolved in 100 µl trifluoroacetic acid TFA and HPLC was performed, using a narrow-bore VIDAC C4 column 2.5 x 400 mm, with the following buffer system : 10 min isocratic with 0.1 % TFA in water and 60 min 0-100 % gradient of 0.08 % TFA in 70 % acetonitrile. The peptides were detected by monitoring at 229 nm. The peptide containing fractions were collected manually into Eppendorf tubes, freeze dried and stored at -20 °C.

Amino acid sequencing was performed on selected peptides in a gas phase sequencer constructed as previously outlined (6), and modified (2). The converted phenylthiohydantoin amino acids were identified by isocratic HPLC employing a 3 x 250 mm 3µ Lichrospher C_{18} (Bishoff) column as previously described (9).

Glycan assay

Welgevonden stock infected and uninfected crude cell cultures were subjected to SDS-PAGE, western blotted onto PVDF membranes and assayed for carbohydrates by an enzyme immunoassay according to the protocol described in the Glycan kit (Boehringer Mannheim). As reference the western blotted PVDF membranes were also stained for 10 min with Coomassie or immunostained with goat anti-Welgevonden stock serum as described earlier (15) .

Isoelectric focusing

The 31 kDa protein was eluted from electroblotted PVDF membranes as described previously (16). The protein was precipitated by adding 4 times the volume of acetone and incubating overnight at -20 °C. After centrifuging at 20 000 x g for 20 min the pellet was stored at -20 °C.

A portion of the pellet was checked for purity by SDS-PAGE. The gel was stained with Coomassie or western

blotted onto PVDF membranes and immunostained with goat anti-Welgevonden serum prepared as described earlier (15) .

Denaturing isoelectric focusing (IEF)

Crude Welgevonden stock infected and uninfected cell cultures, PVDF membrane eluted 31 kDa protein and standard proteins (Pharmacia) with known isoelectric points were dissolved 1:1 in a sample buffer containing 15 % (v/v) glycerol, 2 % (v/v) Triton X-100, 8 M urea, 15 mM DTT and 2.4 % (w/v) ampholyte pH 3-10 (Bio-Rad). Gels of dimensions 0.75 x 80 x 80 mm, containing 5.5 % (w/v) acrylamide, 0,15 % N,N-methylene bisacrylamide, 10 % (v/v) glycerol, 2 % (v/v) Triton X-100, 8 M urea, 2.4 % (w/v) ampholyte pH 3-10, 0.1 % (v/v) tetramethylenediamine (TEMED) and 0.04 % (w/v) ammonium persulphate were cast at 37 °C. Prefocusing was performed using 0.02 M NaOH at the cathode and 0.02 M CH_3COOH at the anode, at 200 V for 15 min followed by 300 V for 30 min and 400 V for 30 min, at 10 °C. Electrophoresis was carried out at 400 V for 16 h followed by 800 V for 1 h. The gel was either stained with Coomassie (5) or western blotted onto PVDF membranes using CAPS buffer, pH 9 and immunostained with goat anti-*C. ruminantium* 31 kDa protein monospecific serum diluted 1:5000 (15).

Native IEF

The PVDF membrane eluted 31 kDa protein as well as protein standards with known isoelectric points were dissolved in distilled water and applied at the anodal, cathodal or middle position. IEF and Coomassie staining was performed in a PhastSystem™ using PhastGel IEF 3-9, as described by the Pharmacia instruction manual (Pharmacia).

RESULTS

SDS-PAGE with or without reducing agent

The 27 kDa and 31 kDa proteins of *C. ruminantium* were detected in the Coomassie stained gel and immunoblots irrespective of whether DTT was present or not (fig. 1).

Production of monospecific anti-serum

Serum from one of two rabbits inoculated with the 27 kDa protein remained negative even after three inoculations and only recognized the 31 kDa (and not the 27 kDa) protein after the fourth immunization (results not shown). Serum from the second rabbit identified the 31 kDa (and not the 27 kDa) protein of *C. ruminantium* after the second administration. After the third immunization the 27 kDa and the 31 kDa proteins were recognized (table I, fig. 2).

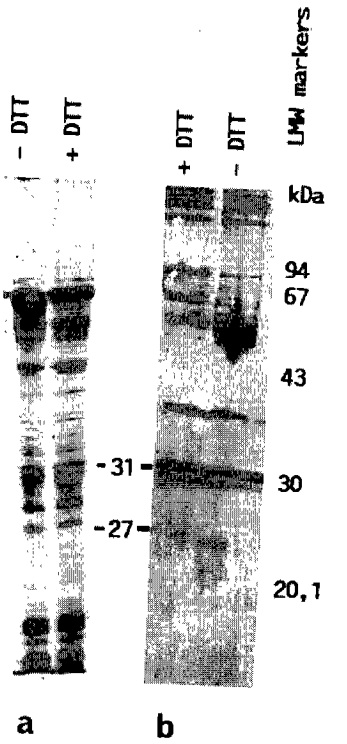

Figure 1 : SDS-PAGE protein patterns of Welgevonden stock infected crude culture extracts with or without reducing agent. a) Coomassie stained gel and b) western blot probed with bovine anti-Welgevonden serum.

Serum obtained from two rabbits inoculated with the 31 kDa protein recognized the 27 and the 31 kDa proteins after the second, third and fourth inoculation (table I, fig. 2).

The serum of goat A inoculated with the 31 kDa protein recognized the 31 kDa protein and cross reacted with the 27 kDa protein at low serum dilutions after the first, second, third and fourth inoculation (table I, fig. 3). Other *C. ruminantium* proteins were also detected by the serum and an E_5 protein in the region just above 31 kDa was recognized at a dilution of ≤1:200.

Determination of whether the 31 kDa protein of *C. ruminantium* is protective towards infection with heartwater

The goats and sheep, immunized with the 31 kDa protein of *C. ruminantium* as well as the naive controls all developed a febrile response after challenge which lasted bet-

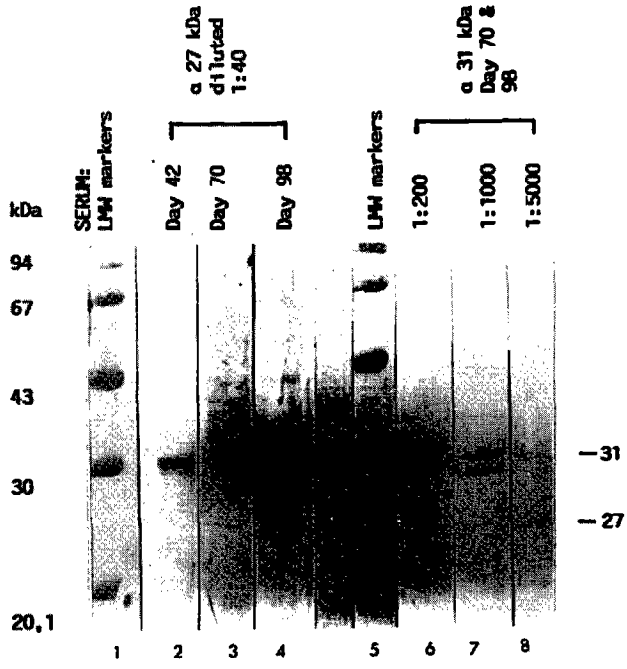

Figure 2 : Western blot analysis of crude Welgevonden stock infected culture extracts probed with rabbit anti-serum raised against the 27 kDa or 31 kDa proteins of C. ruminantium. *Lanes 6, 7 and 8, representative of days 70 and 98 anti-31 kDa serum.*

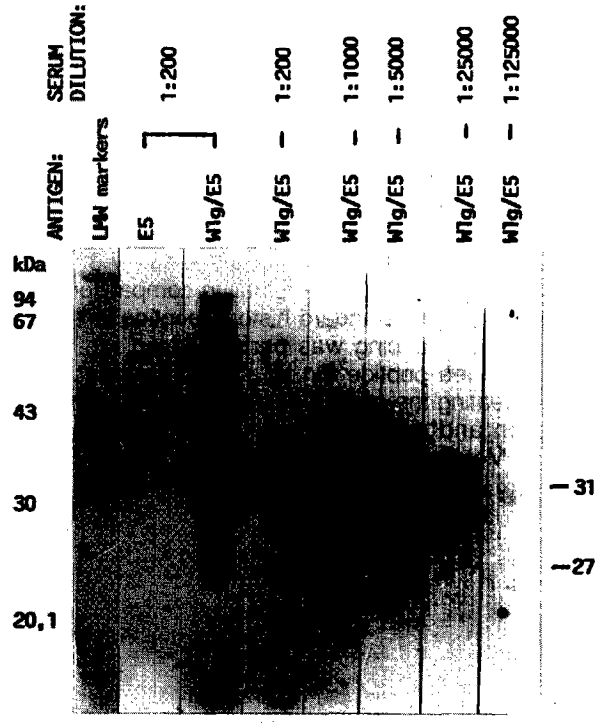

Figure 3 : Western blot analysis of crude Welgevonden stock infected and uninfected culture extracts probed with goat anti-serum raised against the 31 kDa protein of C. ruminantium.

ween 5 - 6 days before death (table II). Post mortem examinations confirmed heartwater as the cause of death. The goat and sheep previously immunized with Welgevonden stock blood stabilate did not develop a febrile response after challenge and were considered immune to heartwater (table II).

Amino acid analysis

The amino acid composition of western blotted 31 kDa protein of *C. ruminantium* is shown in table III. From this it was calculated that the 31 kDa protein contains 23 % acidic and 12 % basic amino acids. It appears that the 31 kDa protein does not contain tryptophan or cysteine since these amino acids were absent following MSA hydrolysis and performic acid oxidation, respectively.

Amino acid sequencing

Automated amino acid sequencing was done on approximately 2000 pmol of the 31 kDa protein, eluted from Coomassie stained SDS-PAGE gels. No clear amino acid sequence could be obtained, therefore it was concluded that the protein was N-terminally blocked.

TABLE III Amino acid composition (residues per mole) of the 31 kDa protein of* C. ruminantium *western blotted onto PVDF membranes.

Amino acid component	Average no of residues ± SD n = 5
asp	41 ± 4
glu	28 ± 3
ser	33 ± 3
gly	29 ± 2
his	7 ± 1
arg	11 ± 2
thr	22 ± 2
ala	24 ± 1
pro	9 ± 3
tyr	15 ± 1
val	14 ± 1
met	5 ± 1
ile	17 ± 3
leu	17 ± 1
phe	13 ± 2
lys	19 ± 1
cys	0
trp	0

SD: standard deviation.
n : number of variables.

Amino acid analysis revealed that there are 5 methionine residues per mole of the 31 kDa protein. Therefore the 31 kDa protein was cleaved with CNBr to yield peptide fragments not N-terminally blocked. Approximately 1600 pmol of the 31 kDa protein was eluted from SDS-PAGE gels. Re-electrophoresis of the electroeluted protein showed a single polypeptide in the region of 31 kDa. Purification of the resultant CNBr peptides by HPLC showed several peaks (fig. 4). The amount of material cleaved with CNBr appeared to be in the region of approximately 3 µg as the size of the HPLC peaks were small in comparison to peak heights of 10 µg for standard histone peptides. Automated amino acid sequencing was performed on the indicated selected purified peptide (fig. 4). The sequence was verified by repeating the protein purification, cleavage, peptide purification and analysis. The following sequence was obtained : Met-Pro-Ile-Ala-Glu-Asp-Phe-Gly-Asp-Thr.

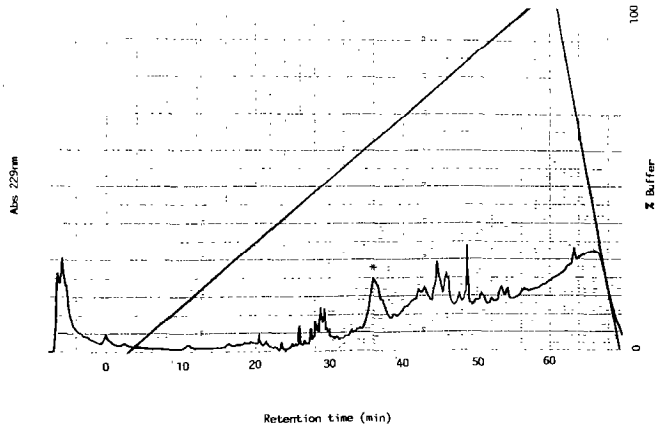

Figure 4 : HPLC chromatogram of peptides released after CNBr cleavage of the 31 kDa protein of C. ruminantium. ** - peptide sequenced. Buffer = 0,08 % TFA in 70 % acetonitrile.*

Glycan assay

The glycan enzyme immunoassay revealed that the 29 and 31 kDa proteins of *C. ruminantium* are not glycoconjugated proteins (fig. 5). No conclusion could be made as to whether the 27 kDa or other proteins of *C. ruminantium* are glycoproteins or not as the patterns of infected and uninfected cell cultures were identical.

Isoelectric focusing

The isoelectric point of the 31 kDa protein was determined to be 5.7 under denaturing conditions (fig. 6).

The pI could not be determined under native conditions because the protein precipitated regardless of the position of application.

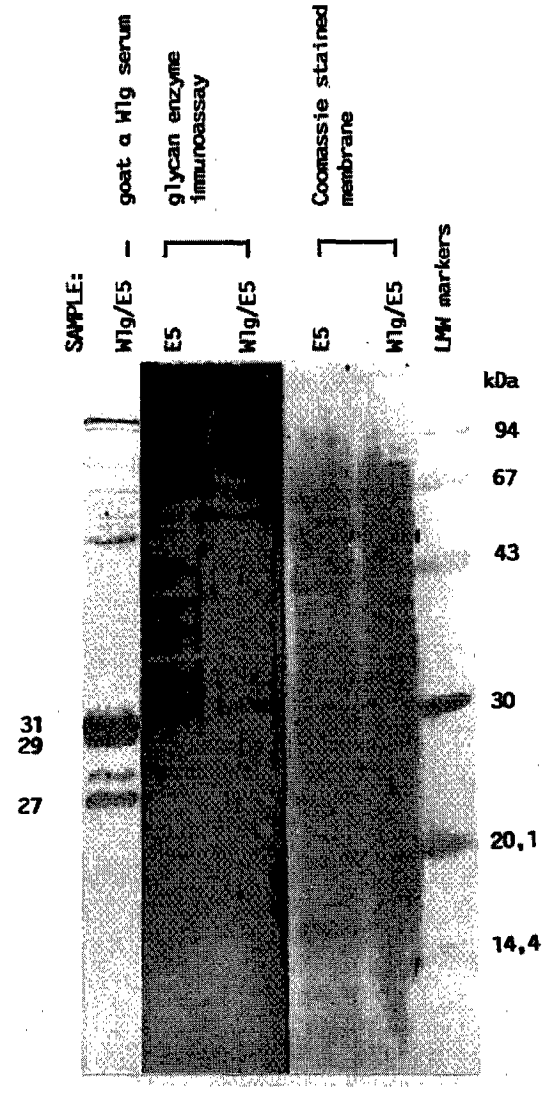

Figure 5 : Glycan enzyme immunoassay, immunostaining and Coomassie staining of crude, Welgevonden stock infected and uninfected cell cultures.

DISCUSSION

Although antibodies have become useful reagents for identification, localization and purification of proteins, their usefulness depends on their specificity. The results obtained when monospecific antisera were prepared against the 27 and 31 kDa proteins, suggest that the 27 and 31 kDa proteins share common epitopes and that the epitopes on the 31 kDa protein are immunologically and antigenically dominant in comparison to the 27 kDa protein. The antibodies that are produced and directed towards these epitopes are therefore termed heteroclitic antibodies (13). Although the isolated 27 and 31 kDa proteins were in a denatured state they retained their immunogenicity which was also unaltered by the staining procedures.

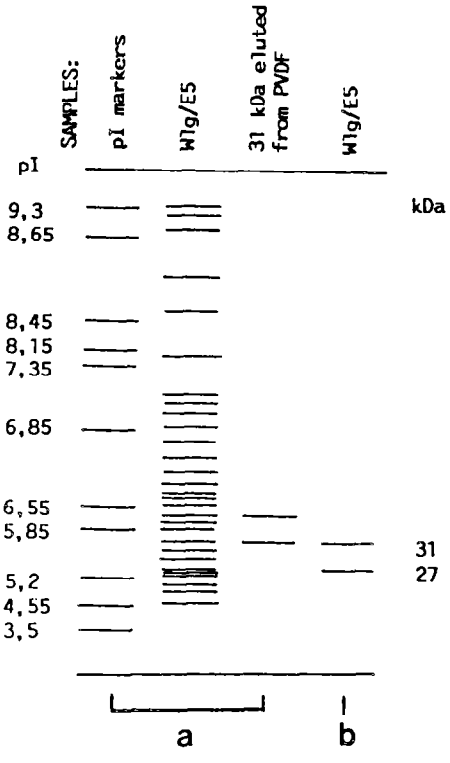

Figure 6 : Representation of analytical denaturing isoelectric focusing of the 31 kDa protein of C. ruminantium. *a) Coomassie stained gel and b) Western blot probed with goat anti-31 kDa protein serum.*

The monospecific anti-serum prepared against the 31 kDa protein appears unspecific at low serum dilutions in the immunoblot and the specificity increased as the dilution of the serum increased. However, the anti-27 kDa and anti-31 kDa sera do not react with cell culture proteins at high serum dilutions and are therefore specific for *C. ruminantium* proteins.

The acidic nature of the 31 kDa protein as determined from the amino acid composition correlates with the pI of 5.7 that was obtained by IEF. Knowledge of the pI of a protein is important for the proper use of several purification techniques such as disc electrophoresis, isotachophoresis, IEF, ion-exchange chromatography and even ammonium sulphate fractionation (14).

Results of amino acid analysis must be evaluated carefully and critically due to the fact that the quantification of several amino acids is biased because of artefacts caused by contamination (affecting gly, glu, ser), the hydrolysis procedure (affecting cys, ser, thr, trp, met, tyr), the derivatization procedure (affecting lys) or chromatography (affecting his) (4).

Considering that complete, partial or no cross-protection is observed between various stocks of *C. ruminantium* in vitro, it seemed unlikely that the common 27 and/or the 31 kDa proteins play a role in cross-protection. The animals that were immunized with the 31 kDa protein failed to survive a challenge with heartwater infective blood. This should be further investigated with respect to the titer at the time of challenge, the dose of antigen and immunization strategy before any conclusions may be made regarding the protection by this immunogen towards heartwater infection.

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VAN KLEEF (M.), NEITZ (A.W.H.), DE WAAL (D.T.). Isolation and characterization of antigenic proteins of *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 157-164

Two antigenically conserved *Cowdria ruminantium* proteins, an immunodominant 31 kDa and a minor 27 kDa protein, were characterized. The 31 kDa and 27 kDa proteins are single polypeptide chains. The 31 kDa protein contains no glycoconjugates and the partial, internal amino acid sequence was determined. The acidic nature of this protein, deduced from the amino acid composition, was confirmed by IEF (pI 5.7). Monospecific antiserum was prepared against the 27 and 31 kDa proteins and results indicate that they are immunogenic and share common epitopes.

Key words : *Cowdria ruminantium* - Antigenic protein - Isolation - Amino acid - Isoelectric point - Antiserum - Immunization.

VAN KLEEF (M.), NEITZ (A.W.H.), DE WAAL (D.T.). Aislamiento y caracterización de las proteínas antigénicas de *Cowdria ruminantium*.. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 157-164

Se caracterizaron dos proteínas conservadas antigenicamente para *Cowdria ruminantium*, una inmunodominante de 31 kilodalton y una menor de 27 kDa. Ambas estructuras son cadenas simples de polipéptidos. La proteína de 31 kDa no contiene glicoconjugados y se determinó la secuencia parcial del amino ácido interno. La naturaleza ácida de esta proteína, deducida a partir de la composición del amino ácido, se confirmó mediante IEF (pI 5,7). Se preparó un anti suero, específico contra las proteínas de 31 kDa y de 27 kDa y los resultados indican que son inmunogénicas y que comparten epítopos comunes.

Palabras claves : *Cowdria ruminantium* - Proteina antigenica - Aislamiento - Amino ácido - Punto isoelectrico - Anti suero - Inmunización.

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Sequence, high level expression and purification of a recombinant 21 kDa protein of *Cowdria ruminantium* from *Escherichia coli* *

BARBET (A.F.), McGUIRE (T.C.), MAHAN (S.M.). Séquence, expression élevée et purification d'une protéine recombinante de 21 kDa de *Cowdria ruminantium,* à partir d' *Escherichia coli. Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 165

Un des buts de notre projet est de fournir une source commode, économique et pure de protéine pour être testée dans des réactions diagnostiques et dans des vaccins contre la cowdriose. L'insertion d'ADN dans *E. coli* recombinante de la colonie F5.2, exprimant une protéine immunodominante de *Cowdria ruminantium*, a été séquencée. L'ADN était riche en A et T (74 p. 100), et hybridait avec tous les isolats de *C. ruminantium* testés et non pas avec de l'ADN bovin ou d'*Anaplasma marginale*. Il contient deux long cadres ouverts de lecture (COL) de 627 et 831 paires de bases. Les COL étaient plus riches en G et C, comparés à la composition globale en bases et les deux COL codaient potentiellement pour des protéines contenant un peptide N-terminal. Des expériences de délétion suivies par des tests d'expression suggéraient que le COL de 627 paires de bases codait pour la protéine immunodominante de *C. ruminantium* reconnue par des sérums d'animaux infectés. Le COL pour ce polypeptide mature a été amplifié par réaction en chaîne de la polymérase et inséré dans un vecteur d'expression élevée où il fut exprimé comme protéine de fusion. Le peptide de fusion attaché, de 9 acides aminés, a permis une purification rapide de la protéine recombinante de *C. ruminantium*.

BARBET (A.F.), McGUIRE (T.C.), MAHAN (S.M.). Sequence, high level expression and purification of a recombinant 21 kDa protein of *Cowdria ruminantium* from *Escherichia coli. Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 165

One goal of our project is to provide a convenient, inexpensive and pure source of protein for testing in diagnostic assays and vaccines against heartwater. The DNA insert from recombinant *E. coli* colony F5.2, expressing an immunodominant *Cowdria ruminantium* protein, was sequenced. The DNA was AT rich (74 %), hybridized to all isolates of *C. ruminantium* tested and not to bovine or *Anaplasma marginale* DNA, and contained two long open reading frames (ORFs) of 627 and 831 base pairs. The ORFs were of enriched GC content compared to the overall base composition and both ORFs potentially encoded proteins containing an N-terminal signal peptide. Deletion experiments followed by expression assays suggested that the 627 bp ORF encoded the immunodominant *C. ruminantium* protein recognized by infection sera. The ORF for this mature polypeptide was amplified using the polymerase chain reaction and inserted into a high level expression vector where it was expressed as a fusion protein. The attached 9 amino acid fusion peptide enabled rapid purification of the recombinant *C. ruminantium* protein.

BARBET (A.F.), McGUIRE (T.C.), MAHAN (S.M.). Secuencia, expresión de alto nivel y purificación de una proteína recombinante de 21 kDa de *Cowdria ruminantium* a partir de *Escherichia coli. Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 165

El objetivo de nuestro proyecto es el de proveer una fuente proteíca conveniente, barata y pura, para ser utilizada en vacunas y ensayos dianósticos de *Cowdria ruminantium*. Se llevó a cabo una secuencia de ADN de inserción, proveniente de una colonia de *E. coli* F5.2 recombinante, con expresion de una proteína inmunodominante de *Cowdria ruminantium*. Este ADN era rico en AT (74 p. 100), híbrido para todos los aislamientos de *C. ruminantium* que se probaron, pero no para ADN bovino o de *Anaplasma marginale* y era portador de dos marcos de lectura prolongada (ORFs) de dos pares de base de 627 y 831. Los ORFs fueron enriquecidos con GC (en comparación con la composición de base general) y ambos codificaron potencialmente proteínas portadoras de un péptido de señal N-terminal. Los experimentos de supresion, seguidos de ensayos de expresion, sugieren que el ORF de 627 codifica la proteína inmunodominante de *C. ruminantium* reconocida en los sueros infectados. Se amplificó el ORF de este polipéptido maduro y se unió a un vector de expresion de alto nivel, en el cual se expresó como una proteína de fusion. El péptido de fusion, resultado de la unión de 9 amino ácidos, permitió una rápida purificación de la proteína recombinante de *C. ruminantium*.

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* *Seuls les résumés de cette communication sont publiés dans ce volume.*

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Cloning and partial characterization of the Cr32 gene of *Cowdria ruminantium*

VAN VLIET (A.H.M.), JONGEJAN (F.), VAN KLEEF (M.), VAN DER ZEIJST (B.A.M.). Clonage et caractérisation partielle du gène codant pour la protéine Cr32 de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 167-170

Les *Cowdria* ont été purifiées par centrifugation en gradient de densité. L'ADN a été utilisé pour la construction de banques génomiques d'expression. La protéine immunodominante Cr32 a été purifiée et la séquence N-terminale d'acides aminés déterminée. Les banques génomiques d'expression ont été criblées avec des anticorps monoclonaux spécifiques de la protéine Cr32, mais aucun n'a réagi. Une partie du gène codant pour la Cr32 a ensuite été amplifiée utilisant des amorces provenant de la séquence N-terminale et d'une autre séquence d'acides aminés interne. La séquence amplifiée a servi de sonde pour détecter le fragment d'ADN génomique codant pour la protéine Cr32. Ce fragment, provenant du stock Sénégal de *Cowdria ruminantium*, a alors été cloné. Une partie du gène, représentant les deux tiers de la longueur totale, a été exprimée dans le vecteur pGEX2T. Le produit d'expression obtenu est reconnu par les anticorps monoclonaux spécifiques de la Cr32.

Mots clés : *Cowdria ruminantium* - Gène - Protéine - Clonage moléculaire - ADN - Anticorps monoclonal.

INTRODUCTION

Molecular characterization of *Cowdria ruminantium*, the causative agent of heartwater (cowdriosis), depends on the availability of cloned genes. A problem in obtaining *Cowdria*-DNA is the contamination of the DNA-samples with bovine DNA originating from the cells used to cultivate the organism. With *Ehrlichia*- and *Rickettsia*-species this problem has been solved by purifying the rickettsiae using density gradient centrifugation (2, 12).

The Cr32 protein of *Cowdria ruminantium* is immunodominant, conserved in all *Cowdria* strains tested so far, and monoclonal antibodies directed at epitopes of this protein have been raised (3). One of these monoclonal antibodies is used in a competitive ELISA to detect antibodies against *Cowdria* in serum (3). The Cr32-antigen used in this system is derived from cultured *Cowdria*-organisms. Preparation of this antigen is time consuming and expensive. Each batch of new antigen must be standardized for optimal perforrnance. Recombinant Cr32-antigen would allow the preparation of large batches of optimized antigen, at low cost.

Therefore we directed our attention at cloning and expression of the gene coding for the Cr32-protein of *Cowdria*.

MATERIALS AND METHODS

Cowdria ruminantium (Senegal, Umm Banein and Welgevonden stocks) was cultivated in BUE9 cells and isolated from these cells as described previously (3). Unless specified otherwise, the Senegal stock was used. Rickettsial suspensions were treated with DNAse I (5 µg/ml) for 15 min at 37 °C to reduce the background of bovine DNA. Half of the DNAse-treated rickettsiae were purified by discontinuous Renografin density gradient centrifugation (2, 12). The band containing *Cowdria* organisms was collected, centrifuged, washed and resuspended in sucrose-phosphate-glutamate (SPG) buffer (1). A part (10 %) of the purified rickettsiae was centrifuged nd resuspended in Laemmli buffer, boiled for 5 min and subjected to sodium odecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (6). The proteins in this gel were blotted onto an Immobilon-filter, and the N-terminal amino acid sequence of the Cr32 protein was determined and interpreted at EuroSequence (Groningen, the Netherlands) as described previously (4). Sequencing of an internal part of the 31 kilodalton protein of the Welgevonden stock of *Cowdria ruminantium* is described elsewhere (10).

Genomic DNA from purified and non-purified organisms was extracted using standard methods (11). Genomic expression libraries were constructed by ligating *Sau*3AI-digested genomic DNA with *Bam*HI digested, dephosphorylated pEX11, pEX12 and pEX13 vectors (5) using T4 DNA ligase. Transformation to *Escherichia coli* strain pop2136, growth and screening were performed as described previously (8). All other recombinant DNA techniques were performed as described previously (7).

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RESULTS

Genomic expression libraries were constructed in the vectors pEX11, 12 and 13, so that fusion products with ß-galactosidase could be formed in all three reading frames. Screening with four anti-Cr32 monoclonal antibodies (2) did not result in positive clones. However, a fifth monoclonal antibody (reacting with a *Cowdria*-protein of > 100 kDa) gave three positive clones. All inserts were *Cowdria*-specific, but the expression products did not react with polyclonal sera. Therefore these clones were not characterized further.

As this approach did not yield Cr32-related clones another approach was chosen. Using Cr32-protein derived from gradient purified rickettsiae the N-terminal amino acid sequence of the Cr32-protein was determined. The sequence is (in one-letter code) : (N-terminus) D V I Q E E N N P V G S V Y I S A K Y M P T... (C-terminus). An internal amino acid sequence was derived from the 31 kDa protein of the Welgevonden stock after cyanogen bromide cleavage (10). This 31 kDa protein is expected to be identical to the Cr32 protein. Oligonucleotide primers for use in the polymerase chain reaction (PCR) were deduced from both protein sequences. Using the PCR, a fragment of 100 basepairs was amplified from *Cowdria*-DNA as template, whereas this fragment was not seen when bovine DNA was used. The PCR-fragment was cloned and the nucleotide sequence was determined. Translation of the sequence between the primers yielded the rest of the N-terminal amino acid sequence. Using the PCR-fragment as a probe, a 1100 base pair fragment was detected in genomic DNA ; this fragment was cloned (pCRS18). The insert hybridized with genomic DNA of *Cowdria* but not with bovine DNA. The nucleotide sequence of this fragment has been determined and translation of this sequence showed an open reading frame (ORF) of 660 base pairs with no stop codon, which means that the rest of the ORF should be found on another, overlapping clone. A fragment containing the noncharacterized part of the Cr32-gene has been cloned from genomic DNA. It is indicated in figure 1 (pCRS23) ; figure 1 also shows the clones that were made and the position of the open reading frame. The insert of plasmid pCRS18 was used as a probe in a Southern hybridization experiment with a blot containing digestions of genomic DNA of three *Cowdria*-strains isolated in three different geographical regions (Senegal (Senegal), Umm Banein (Sudan) and Welgevonden (South Africa) stocks). This Southern blot is given in figure 2 : it shows this part of the gene is present in these three *Cowdria*-strains.

The part of the Cr32-gene found on plasmid pCRS18 has been amplified using PCR. It was cloned in the expression vector pGEX2T (8) as a fusion product with glutathion transferase, a protein with molecular weight of 27 kDa. The fusion product, with a size of 49 kDa was

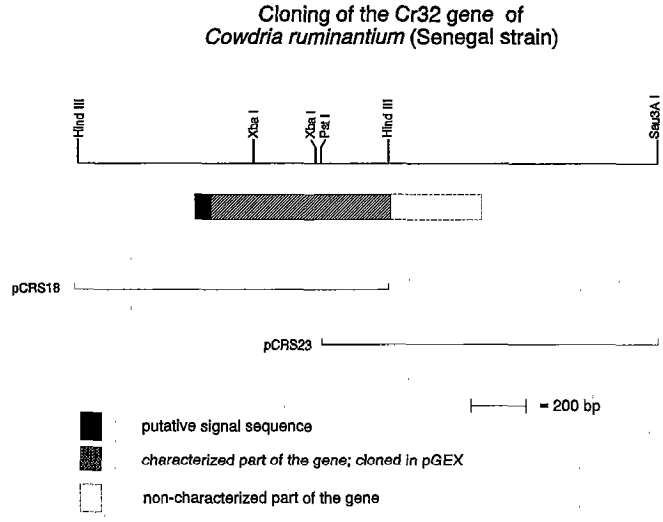

Figure 1 : Genomic restriction map of the region containing the Cr32-gene of Cowdria ruminantium *(Senegal stock). The position of the Cr32-gene and constructed clones are indicated.*

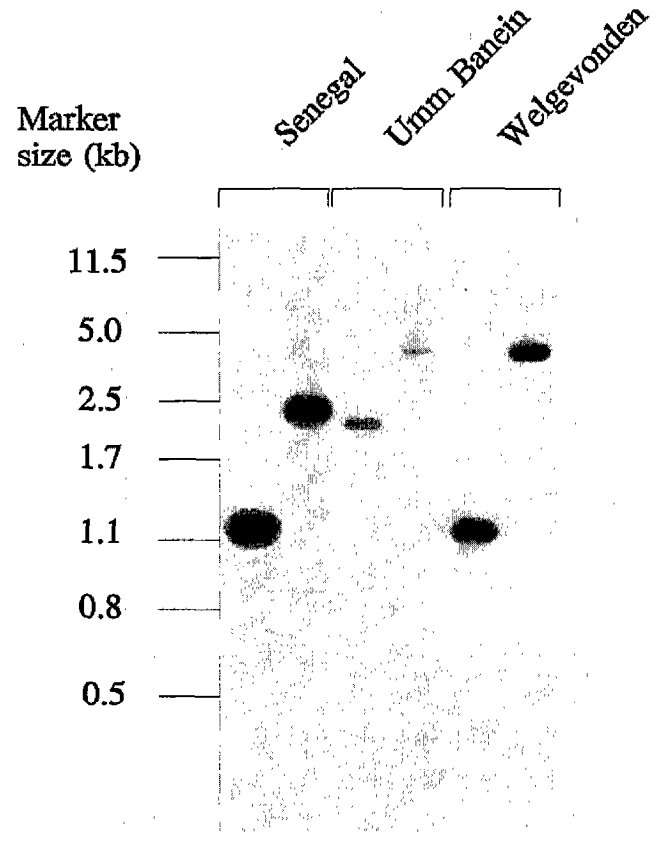

Figure 2 : Southern blot of HindIII and Sau3AI digested genomic DNA derived from Cowdria *stocks isolated in different geographical areas: Senegal (Senegal), Umm Banein (Sudan) and Welgevonden (South Africa). The probe used was the insert of plasmid pCRS18 (see fig. 1).*

subjected to SDS-PAGE, transferred to nitrocellulose and incubated with a mixture of the four Cr32-specific monoclonal antibodies. This Western blot is shown in figure 3 : positive signals were obtained with *Cowdria* antigen and the fusion protein, though not with bovine antigen and the pGEX protein. The additional bands under the 49 kDa fusion protein are degradation products of the fusion protein.

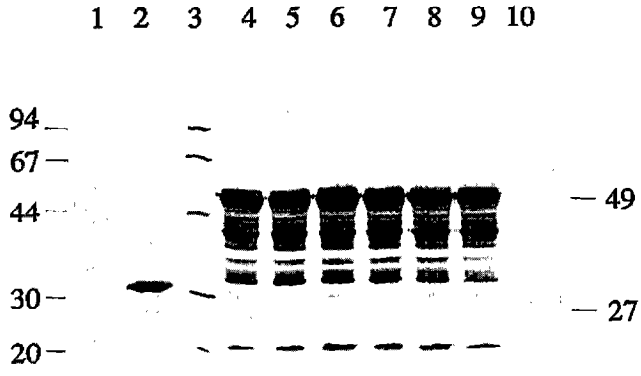

Figure 3 : Western blot of pGEX-fusion proteins expressing a part of the Cr32-gene (pGEX-Cr32(S)) reacted with Cr32-specific monoclonal antibodies. Lane 1 : BUE9 antigen, lane 2 : Cowdria ruminantium *antigen, lane 3 : the molecular weight marker, lane 4-9 : pGEX-Cr32(S) clones and lane 10 : pGEX-protein encoded by the empty vector. Marker sizes are indicated on the left, the sizes of fusion protein (49 kDa) and pGEX-protein (27 kDa) are indicated on the right.*

DISCUSSION

The gene coding for the Cr32 protein of *Cowdria ruminantium* has been cloned from the Senegal stock and has partially been characterized. Clones to complete the sequence of the Cr32-gene have been obtained. Cloning of the first 21 kDa of the mature protein in the pGEX vector yielded a fusion protein which reacted with Cr32-specific monoclonal antibodies. After completion of the sequence, the search for *Cowdria*-specific epitopes can start. A specific epitope will exclude cross-reactivity with *Ehrlichia*-species, which hampers serological tests at present.

ACKNOWLEDGEMENTS

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VAN VLIET (A.H.M.), JONGEJAN (F.), VAN KLEEF (M.), VAN DER ZEIJST (B.A.M.). Cloning and partial characterization of the Cr32 gene of *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 167-170

Cowdria **organisms were purified by density gradient centrifugation. The DNA was used to construct expression libraries. The immunodominant Cr32 protein was purified and its N-terminal amino acid sequence was determined. The expression libraries were screened with Cr32-specific monoclonal antibodies, but did not yield Cr32-positive clones. Therefore a part of the Cr32-gene was amplified using primers derived from the N-terminal and an internal amino acid sequence. This DNA was used as a probe to detect the genomic DNA fragment encoding the Cr32 protein. This fragment was cloned, using genomic DNA of the Senegal strain of *Cowdria ruminantium*. A part of the gene comprising two third of its total length has been expressed in vector pGEX2T. This expression product is recognized by Cr32-specific monoclonal antibodies.**

Key words : *Cowdria ruminantium* - Gene - Protein - Molecular cloning - DNA - Monoclonal antibody.

VAN VLIET (A.H.M.), JONGEJAN (F.), VAN KLEEF (M.), VAN DER ZEIJST (B.A.M.). Caracterización parcial y clonaje del gen Cr32 de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 167-170

Se purificaron organismos de *Cowdria* mediante centrifugación de gradiente de densidad. Para la construcción de los libreros de expresión se uso ADN. La proteína inmunodominante Cr32 se purificó y se determinó la secuencia del amino ácido N terminal. La expresión de los libreros se monitoreó con anticuerpos monoclonales específicos para Cr32, pero no se produjeron clones Cr32-positivos. Sin embargo, una parte del gen Cr32 se amplificó, gracias al uso de "primers" derivados de la secuencia del amino ácido N terminal y del amino ácido interno. Este ADN se utilizó como agente probador para la detección del fragmento codificador de ADN del genoma de la proteína Cr32. Este fragmento se sometió a un clonaje, mediante el uso de ADN genómico de la cepa Senegal de *Cowdria ruminantium*. Una parte del gen, que comprende dos terceras partes de su longitud total se exprimió en el vector pGEX2T. Este producto es reconocido mediante anticuerpos monoclonales específicos para Cr32.

Palabras claves : Cowdria ruminantium - Gene - Proteína - Clonación molecular - ADN - Anticuerpo monoclonal.

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Seroconversion to *Cowdria ruminantium* of Malawi zebu calves, reared under different tick control strategies

SOLDAN (A.W.), NORMAN (T.L.), MASAKA (S.), PAXTON (E.A.), EDELSTEN (R.M.), SUMPTION (K.J.). Séroconversion à *Cowdria ruminantium* de veaux zébus au Malawi, soumis à des stratégies différentes de lutte contre les tiques. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 171-177

L'ELISA indirect a été utilisé au Malawi pour comparer la séroconversion *à Cowdria ruminantium* jusqu'à l'âge d'un an de 66 veaux zébus locaux nés dans des groupes passés au bain détiqueur 17 fois par an, à celle de 32 veaux nés dans des groupes non détiqués. Le nombre d'*Amblyomma variegatum* et les cas cliniques de la maladie ont été enregistrés dans chaque groupe de bovins. Aucun cas de cowdriose n'a été observé chez les veaux des 2 groupes pendant les 22 premiers mois de leur vie. Un seul cas fut enregistré chez une vache de 8 ans chez les 1 800 bovins suivis de façon intensive pendant la même période. Presque tous les veaux non détiqués étaient devenus séropositifs à l'âge d'un an et 50 p. 100 des séroconversions ont été attribuées à des infections par des nymphes. Par ailleurs, seulement 41 p. 100 des veaux dans les groupes passés au bain étaient devenus positifs à l'âge d'un an. Ce régime de détiquage a donc diminué le taux de séroconversion de façon significative chez ces veaux. Soixante quatorze pour cent des veaux avaient des anticorps maternels contre *C. ruminantium* pendant les quatre premières semaines de leur vie. Chez tous les veaux des groupes détiqués, le taux de ces anticorps était tombé sous le seuil considéré comme positif dans l'ELISA à l'âge de 8 à 12 semaines et il n'y avait pas de conversion de séronégatif à séropositif pendant cette période chez ces veaux. La proportion de séropositifs détectés par un seul test ELISA à l'âge de 12 mois n'était pas significativement différente de celle déterminée par les résultats cumulatifs des 9 mois précédents et semble donc utilisable comme indication de l'état de l'immunité du troupeau. Les auteurs concluent qu'une stabilité enzootique *à C. ruminantium* existe chez les zébus locaux non détiqués, caractérisée par une résistance à l'infection, innée et élevée, et une séroconversion entre 3 et 9 mois d'âge de la plupart des veaux nés en mai-juin.

Mots-clés : Zébu - Cowdriose - *Cowdria ruminantium* - *Amblyomma variegatum* - Tique - Lutte antiacarien - Infestation - Test ELISA - Anticorps - Malawi.

INTRODUCTION

The vector of *Cowdria ruminantium* in Malawi is considered to be *Amblyomma variegatum*, which has a widespread distribution in the country, except for lowland areas of southern Malawi such as the Lower Shire Valley (2). Heartwater has been frequently reported in unprotected *Bos taurus* cattle, but the disease is seldom reported in indigenous Malawi Zebu cattle *(Bos indicus)*. The number of taurine cattle in Malawi is very low (less than 20,000) in comparison with numbers of Malawi Zebu in the national herd of 800,000 (10). The status of traditionally managed Malawi Zebu to *Cowdria ruminantium* is not known, although a state of enzootic stability and/or genetic resistance in indigenous ruminants to the infection, is considered to be present in *Amblyomma variegatum* infested areas. The occurrence and timing of seroconversion to *C. ruminantium* in relation to *Amblyomma* infestations in cattle is important to understanding the nature of infection in both undipped and dipped cattle populations.

Dipping to control tick-borne disease was first made compulsory in certain areas of Malawi in the early 1920's (DE MAZA, 1925, cited in MARES (8)). Although current legislation provides for weekly dipping of cattle in arsenic trioxide it is estimated that only 20-40 % are dipped regularly. A survey of cases presented to veterinary assistants at diptanks in north and central regions showed that ECF morbidity varied between 0.5 and 1.8 % (all ages) ; clinical cases of babesiosis and anaplasmosis were rare and heartwater was not recorded. The dipping attendance over the year was only 50 % and therefore the low ECF morbidity was not attributed to the suppression of ticks through dipping (5).

A three year trial commenced in 1990, which undertook to investigate the effect of reduced intensity dipping and non-dipping in traditionally managed cattle, upon morbidity, mortality, productivity and economic indicators. Seroconversion to *C. ruminantium* in cohorts of calves born during this trial into study herds at six diptanks is reported here.

An indirect ELISA test was used to test serum samples for antibodies to detergent soluble antigens extracted from the purified elementary body of *C. ruminantium* (SUMPTION, MASAKA and PAXTON, unpublished results). The significance of positive test results in immunofluorescent antibody (IFA) tests upon sera from animals from areas where *Amblyomma* ticks are present is unclear, because positive reactions have been observed in IFA tests with sera from some *Amblyomma* free areas (3). The latter results are presumed to be the result of cross-reactions caused by antibodies to *Ehrlichia* species, because serological cross-reactions in IFA tests have been observed between antisera raised to various *Ehrlichia* species and *C. ruminantium* antigens in infected mouse macrophages (3) or neutrophils (6, 7). The

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indirect ELISA used to test sera from Malawi utilized detergent soluble elementary body antigens, because a number of cross-reactive antigens to *E. phagocytophila* and *E. ondiri* were removed during the detergent extraction process ; the ELISA test consequently has a low level of detection of antibodies to these pathogens in comparison with IFA using *Cowdria* infected goat neutrophils or infected endothelial cells (SUMPTION and PAXTON, unpublished). The presence of *E. ondiri* or *E. bovis* has not been demonstrated in Malawi, and therefore antibody reactions in this study are assumed to be to *C. ruminantium.*

MATERIALS AND METHODS

Location of study area

Malawi is located between 9° - 17° South and 33° - 36° East in Central Africa. As part of a larger study, six diptanks in the same ecological zone were chosen in the Lilongwe area. This area is on the Central African Plateau with an undulating, almost flat topography about 1,100 m above sea level. Four of the tanks chosen were in good repair and had been using arsenic trioxide up to the start of the study in November 1991. Cattle at two diptanks which were to act as non-dipped controls had effectively not been regularly dipped as a result of tank disrepair, or because there was a large group of farmers who did not dip their cattle.

Organization of study

Approximately 300 animals at each of the six tanks were tagged in November 1990. Each of the cattle were Malawi-Zebu and belonged to smallholders, and were communally grazed. No alteration in management was instituted and no prophylactic treatments were given during the trial. Dipping was carried out in four tanks at 2 weekly intervals in the rainy season (December 1990 to March 1991 and December 1991 to March 1992) and at 4 weekly intervals through the dry season (April 1991 to November 1991). Dipping at two tanks was in chlorfenvinphos (Supona 30, Shell Chemicals Ltd.) and at the other two in amitraz (Triatix TR, Coopers Animal Health).

Acaricide concentrations and replenishment were as recommended by the manufacturers, and the total replacement method was used for amitraz. Dipping of cattle was not carried out at the two control tanks. An active disease monitoring system was set up with the aim of identifying the specific cause of each case of death or disease in cattle at each dip tank in the trial. The routine samples collected from dead animals were faeces, blood, lymph node, spleen, and brain crush smears. These were examined by staff of the protozoology section of the Central Veterinary Laboratory and a project veterinary officer. The veterinary assistant associated with each diptank visited owners with tagged cattle every week and project staff visited every 2 weeks throughout the study period.

Calf cohort study

The peak calving season in the Lilongwe area occurs between May and July each year. As part of a productivity study 15 calves born in May/June 1991 had serum samples collected at 8 week intervals with the first sample being collected at the first visit after birth. Samples were frozen at -20 °C and aliquots for *Cowdria* serology were forwarded on ice by airfreight to the Centre for Tropical Veterinary Medicine, Edinburgh, where they were tested by indirect ELISA. Chi squared tests were used to compare proportions.

Indirect ELISA for the detection of antibodies to *Cowdria ruminantium*

An indirect ELISA developed at the CTVM was used to test sera at a dilution of 1 in 50. The ELISA uses soluble antigens extracted from the elementary body (EB) stage of *C. ruminantium* (Welgevonden stock) following release from cell cultures, and has an extremely low reactivity to antibodies present in antisera raised to *Ehrlichia phagocytophila,* in comparison with immuno-fluorescent antibody tests (IFA) using Welgevonden infected neutrophils or infected endothelial cells. It also has an excellent sensitivity in the detection of experimentally infected animals (SUMPTION and PAXTON, unpublished results). Cross-reactions with *Ehrlichia* spp. present considerable difficulties in the interpretation of IFA tests for heartwater (3, 4). The indirect ELISA was developed using detergent soluble fractions of the *Cowdria* elementary body because these fractions have a reduced number of antigens with cross-reactivity to *E. phagocytophila* and *E. ondiri* sera, than is found in whole EB or infected cell preparations (SUMPTION and PAXTON, manuscript in preparation). Soluble antigen is prepared from EB's semipurified from culture medium by centrifugation for 20 min at 1,000 g for the pelleting of endothelial cells, followed by centrifugation at 10,000 g for the pelleting of EB's. Pellets were washed in sterile phosphate buffered saline (PBS, pH 7.4), and recentrifuged at 10,000 g for 20 min. The procedure was repeated two times, followed by detergent lysis of the elementary body in 0.5 % nonidet NP40 and 0.5 % sodium deoxycholate in 50 mM Tris-HCL (pH 8.0), 2 mM EDTA, 150 mM sodium chloride and 1 mM phenylmethylsulfonylfluoride for 2 min at room temperature followed by rapid passage through a 26 g needle to disaggregate elementary bodies, and incubation at 37 °C for 30 min. After a further round of needle passage, insoluble antigen was removed by centrifugation at 4 °C for 30 min at 16,000 g. Soluble antigen extracts were then stored at -20 °C until

used in ELISA. Antigen was diluted in 0.05M carbonate-bicarbonate buffer (pH 9.6) and 100 µl added to each well of 9 well immunoplates (Immulon II, Dynatech Laboratories) and incubated overnight at 4 °C. Plates were then washed 5 times in PBS diluted 1:4 in distilled, de-ionized water which contained 0.05 % Tween 20. Serum samples were diluted to 1 in 50 in PBS containing 0.05 % Tween 20 (PBST) and 100 µl added to duplicate wells and incubated for 60 min at 37 °C in a shaking incubator. Plates were then washed as before and 100 µl of rabbit anti-bovine IgG horse radish peroxide conjugate (Sigma Chemical Company) added to each well and incubated for 60 min at 37 °C. Plates were then washed and 100 µl orthophenylene diamine (OPD, 0.4 mg/ml) and hydrogen peroxide (0.015 %) added to each well and incubated for 6 min at 20 °C. 100 µl of IM sulphuric acid was then added to each well to stop the reaction and the optical densities recorded at 492 nm in a Titertek Multiscan Spectrophotometer. Four positive controls, 2 negative controls and two blank wells (no serum) were used per plate.

Tick counts

Half body counts of *Amblyomma variegatum* adults and nymphs were conducted every 4 weeks at each diptank on 5 animals between the ages of 6 and 12 months. Individual animals were not counted more than once in the study period and each animal, on any one date, came from different farmers. In dipped cattle, tick counts represent maximal burdens as they were carried out immediately prior to dipping.

RESULTS

Disease

A laboratory confirmation of diagnosis was reached in over 80 % of deaths occurring in tagged cattle during this study. East Coast Fever was the only tick borne disease observed in both dipped and undipped cattle. No cases of heartwater were observed between May 91 and June 92. A significant difference was not observed in disease mortality and tick numbers between cattle in herds in which a regime of chlorfenvinphos or amitraz was used. Dipped cattle are therefore compared to non-dipped cattle populations for the comparison of seroconversion.

Dipping percentages of cattle

For tagged cattle the average dipping percentage was 83 % and for untagged cattle attending the same tanks it was 41 %. Tagged cattle made up between 10 and 22 % of the cattle population for each tank.

Tick counts

Figure 1 shows the relationship between *A. variegatum* adults and nymphs in undipped cattle. Infestations of cattle showed a highly seasonal distribution, with peak numbers of nymphs in August and September, followed by peak numbers of adults in November and December. Nymphal activity was from March to November with adults present from October to February. Table I shows mean adult and nymph *A. variegatum* half body counts for various periods of the study.

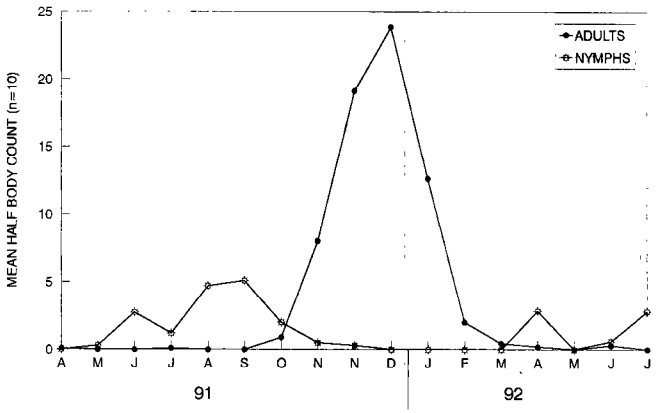

Figure 1 : Amblyomma variegatum *adults and nymphs undipped cattle.*

TABLE I **Amblyomma variegatum** ***counts on 6 to 12 month calves for three periods od the year.***

	May-October 1991		November 1991-February 1992		March-June 1992	
	non-dipped	dipped	non-dipped	dipped	non-dipped	dipped
Adults	0.2	0.1	13.1	3.3	0.2	0.0
Nymphs	2.7	1.0	0.2	0.1	0.9	0.9

Figures are mean half body tick counts for the three periods indicated, for 20 calves in the four dipped groups and 10 calves in the two non-dipped groups.

Determination of cut-off values for ELISA

Sera collected from calves in October-November 1991 and May-June 1992, from dipped and non-dipped groups, were tested in ELISA at a dilution of 1/50 and the frequency distributions of OD values was plotted (figures 2, 3, 4, 5). The dipped calves in October-

November had OD values which were characterized by a bimodal frequency distribution with a group of values less than 0.25 OD units, and a small number of sera with values greater than this value. A similar distribution was also observed for the same calves in May-June 1992 (figure 4), whereas non-dipped calves in October-November (figure 3) had a comparatively high

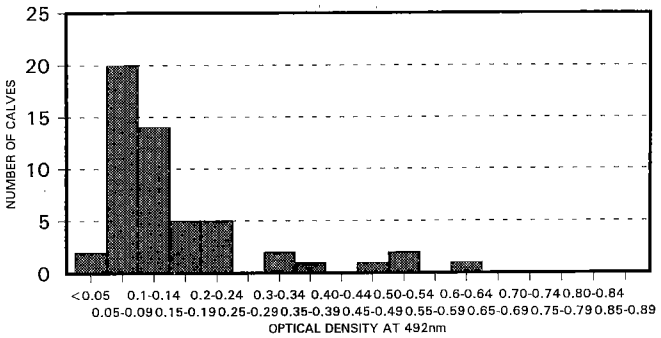

Figure 2 : Indirect ELISA for antibodies to Cowdria *OD values frequency distribution dipped calves samples October or November 1991.*

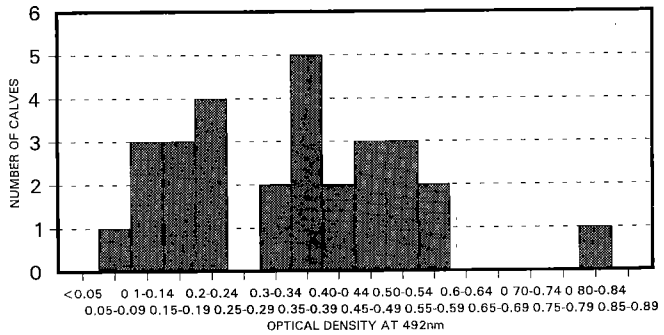

Figure 3 : Indirect ELISA for antibodies to Cowdria *OD values frequency distribution non-dipped calves October or November 1991.*

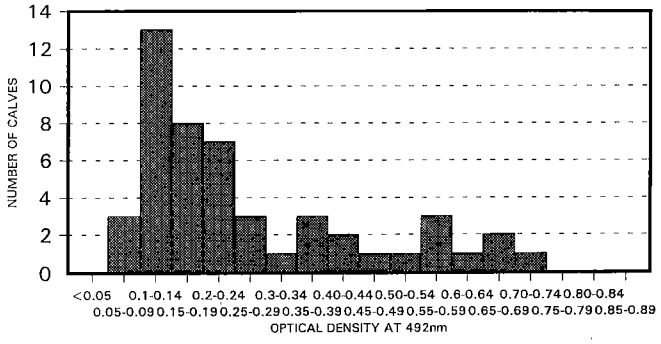

Figure 4 : Indirect ELISA for antibodies to Cowdria *OD values frequency distribution dipped calves samples May or June 1992.*

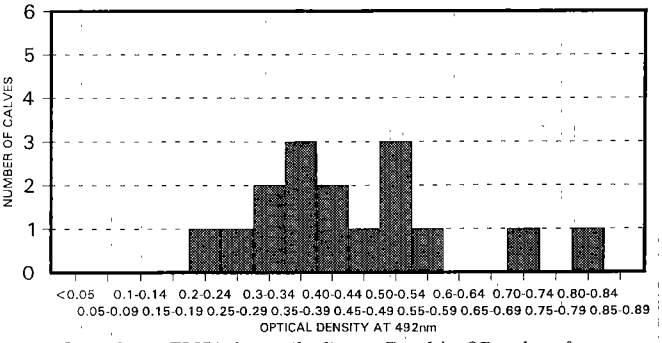

Figure 5 : Indirect ELISA for antibodies to Cowdria *OD values frequency distribution non-dipped calves May/June 1992.*

proportion of values (18/29) greater than 0.25 OD and a single peak of values for sera collected in May-June (figure 5) of which a high proportion (15/16) were greater than 0.25 OD units. The low values (less than 0.25 OD units) were assumed to represent a population of calves which had not seroconverted to heartwater, because dipped calves had very low tick counts from birth to the time of sampling in October-November. The presumed negative population had an expected skewed distribution which was characterized by a tail of high OD values ; the latter was also observed for sera from heartwater free areas of Europe and the Caribbean (unpublished results). The chosen cut-off value of 0.25 was similar to that determined for sera from other parts of the world, and was chosen on the basis of OD values which separated the high and low OD value distributions, and from the baseline values observed following the decline in maternal derived antibody in dip trial calves.

Maternally derived antibody levels in calves

Twenty-nine of the forty calves (72.5 %) which were sampled in the first four weeks of life were seropositive (table II). This was considered to be the result of maternal antibody. The number of calves with detectable maternal antibody was slightly higher in the undipped group (10/12, 80 %) than in the dipped group (19/30, 63 %) but the difference was not significant ($P > 0.05$). No calves in dipped herds had a positive result in samples taken when they were aged between 8 and 12 weeks. The chosen cut-off value of 0.25 was higher than the maximum value (0.247) observed (median = 0.113, n = 42) in calves 8 to 12 weeks of age in dipped groups. Evidence of seroconversion was therefore considered to be the finding of an OD value greater than 0.25 in calves over 12 weeks of age.

TABLE II Proportion of seropositive serum samples from cohort calves less than 16 weeks of age at the six diptanks in the trial.

Diptank	Regime	0 to 4 weeks	4 to 8 weeks	8 to 12 weeks	12 to 16 weeks
Likuni	non-dipped	1/2	3/5	2/10	3/4
Tonde	non-dipped	9/10	6/6	0/1	3/6
	Total non-dipped	10/12	9/11	2/11	6/10
Namaguya	dipped	2/3	1/13	0/8	0/11
Dickson	dipped	8/11	0/7	0/11	1/14
Mbabzi	dipped	6/9	2/15	0/5	1/10
Sinyala	dipped	3/5	2/12	0/3	1/12
	Total dipped	19/28	5/47	0/27	3/47
	Total all tanks	29/40 (73 %)	14/58 (24 %)	2/38 (5 %)	*9/57 (16 %)

Serum considered seropositive if the OD value exceeded 0.25 OD units relative to a positive control (Nyaga 2) at 1.053 OD units.
** 8 of the positives were from probable seroconversions.*

Seroconversion to *C. ruminantium* in cohort calves

The proportion of calves considered seropositive by ELISA in samples collected in October 1991, and February and June 1992 (or in samples collected in the preceding month if no sample was taken in that month) is shown in table III. There was a significant difference ($P < 0.01$) between seroconversion in dipped and undipped calves at all three periods of the year. After 12 months of the trial 96 % (23/24) of undipped calves had seroconverted compared to only 41 % (18/44) of dipped calves. Tagged cattle at these dip tanks were monitored for a further year but clinical cases of heartwater were not observed in cohort calves or other tagged cattle at the diptank except for a single confirmed case in an 8 year old cow.

TABLE III Seroconversion to **Cowdria ruminantium** *in calves born May/June 1991.*

	October 1991		February 1992		June 1992	
	Spot	Cumulative	Spot	Cumulative	Spot	Cumulative
Dipped	3/55 (5 %)	5/56 (9 %)	17/49 (35 %)	19/51 (37 %)	15/42 (36 %)	18/44 (41 %)
Non-dipped	15/29 (52 %)	15/29 (52 %)	21/26 (81 %)	23/26 (88 %)	17/18 (94 %)	23/24 (96 %)

Results are given of single sample tests (spot tests) and cumulative seroconversion of the groups (including samples collected in the month for which results are stated).

The seroconversion of cohort calves was also determined from the results of the serial collection of serum samples, and the results are given as a cumulative seroconversion for the cohort (table III). The proportion of calves considered seropositive was very similar by both methods ($P > 0.05$).

The highest OD value observed for serum samples from calves which seroconverted varied from 0.261 to 0.993 OD units, with a median value for 58 calves of 0.501. Optical density values for serum samples from ten of the 58 calves declined to below the cut-off value, between 8 and 24 weeks after seroconversion. However, a decline to below the cut-off value was observed in only 3 of the 31 calves which seroconverted in the non-dipped groups which had received a higher tick challenge.

DISCUSSION

The undipped calves in this study had an almost continuous *Amblyomma* challenge through the first year of life (figure 1). For the first 5 months this was almost entirely of nymphae (table I), and approximately 50 % of the calves seroconverted to *C. ruminantium* in this period (table II). The remaining calves seroconverted during the months of adult *Amblyomma* activity. At the end of one year of life almost 100 % of undipped calves had seroconverted to *C. ruminantium.* In contrast the dipped calves had a significantly reduced level of seroconversion throughout the study period, and therefore the reduced intensity dipping had significantly affected exposure of calves to the agent of heartwater. Dipped cattle carried significantly lower tick burdens than undipped cattle and this was reflected in significantly lower seroconversion rates in dipped calves, which reached only 41 % at 12 months of age. In the subsequent 10 months (June 1992-April 1993), heartwater was not observed in any of the dipped calves which had not seroconverted by June 1992, despite a suspension in dipping from April 1992 to November 1992. Seroconversion in the absence of clinical cases of heartwater was observed among the calves aged between 5 and 12 months in the non-dipped groups, between October 1991 and June 1992. Approximately 50 % of the non-dipped calves seroconverted in this period. The results therefore suggest that Malawi Zebu are resistant to heartwater until at least one year of age. The observation of a case of heartwater in an 8 year old cow is surprising, because of the high seroconversion in non-dipped herds and the low dip attendance prior to the onset of the dip trial which would be expected to result in a very high herd immunity of animals older than 2 or 3 years. Cases of heartwater in this age group have been attributed to relapse as a result of stress (9). In addition, an increased susceptibility to immunization with the Ball3 vaccine stock in animals older than 8 years has been observed (11). The apparently high innate resistan-

ce of Malawi Zebu calves to the development of clinical heartwater suggests that a combination of factors may have been associated with clinical heartwater on this occasion.

The suppression of *Babesia bovis* seroconversion in the same calves by dipping during the dry season was considered to create an enzootically unstable situation and therefore at the end of the rains in March 1992, dipping was discontinued in favour of a strategy of non-dipping in the dry season. This strategy may be expected to increase nymphal *Amblyomma* infestations and seroconversion to *C. ruminantium* at 6 months of age in calves born in May-June 1992.

The finding that there was no significant difference between the proportion of dipped and undipped calves which had maternal antibodies is probably the result of similar exposure of cattle in these groups to *Amblyomma* ticks despite the statutory requirements which existed for dipping. This suggests that the dipping in arsenic trioxide which occurred before the trial in 4 of the groups resulted in a similar proportion of seropositive cows to the non-dipped groups; this may have occurred because of insufficient dip attendance or acaracide activity. The observation that maternal antibody to *C. ruminantium* was not detected after 8-12 weeks of age is similar to that of DU PLESSIS *et al.* (4) who found that in 18 out of 21 calves born to naturally exposed dams, maternal derived antibody was not detected after 12 weeks of age, in IFA tests. Comparison of spot seroconversion rates and cumulative seroconversion rates (table II) showed that there was no difference between the two methods. This suggests that this ELISA test may be of value in herd studies for the investigation of comparative seroconversion rates in the field, with serum samples collected at a single point in time. In this study, seropositive status continued for at least 8-10 months after seroconversion in the majority of calves which seroconverted, in the presence of intermittent or continuous tick challenge, and only 3 out of 31 calves exposed to high tick numbers in the non-dipped groups underwent a temporary reversion to a seronegative status.

CONCLUSION

In undipped Malawi Zebu cattle in the study area, *C. ruminantium* appears to be in a state of enzootic stability with high seroconversion rates in cattle and the absence of clinical disease. Malawi Zebu seem to have some degree of innate resistance to heartwater because calves not challenged in their first year showed no clinical disease under tick challenge in their second. The ELISA proved useful in determining comparative seroconversion rates in herds kept under different management regimes, and results of single sampling were not significantly different from serial sampling in the determination of seroconversion rates at a given point in time.

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SOLDAN (A.W.), NORMAN (T.L.), MASAKA (S.), PAXTON (E.A.), EDELSTEN (R.M.), SUMPTION (K.J.). Seroconversion to *Cowdria ruminantium* of Malawi zebu calves, reared under different tick control strategies. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 171-177

The seroconversion by indirect ELISA to *Cowdria ruminantium* over the first year of life of sixty-six Malawi zebu calves born into groups which were dipped 17 times per year was compared to seroconversion of 32 calves born into non-dipped groups. *Amblyomma variegatum* tick counts and clinical disease in each group of cattle were monitored throughout the study period. No cases of heartwater were seen in either group of calves over the first 22 months of life. Only one case of heartwater was observed, in an 8 year old cow, in the 1,800 intensively monitored cattle over the same period. By 12 months of age almost all undipped calves had seroconverted and 50 % of seroconversions were attributed to nymphal challenge. In contrast, only 41 % of calves had become seropositive by 12 months of age in the dipped groups. The dipping regime used therefore significantly decreased seroconversion rates to *C. ruminantium* in these calves. 73 % of calves had detectable levels of maternal antibodies to *C. ruminantium* in the first 4 weeks of life. Antibody levels in each of the calves in dipped groups had waned to below the cut off point for the ELISA by 8-12 weeks. Seroconversion did not occur in the first 8-12 weeks of life in dipped herds. The indirect ELISA test results were not significantly different in the proportion positive in single tests at 12 months of age, or by cumulative test results of the previous 9 months, and therefore the test may be of value as a test of herd immunity. It is concluded that a state of enzootic stability exists to *C. ruminantium* in undipped Malawi zebu cattle in the study area, which is characterized by a high innate resistance to the infection and seroconversion of the majority of the calves born in May-June to the agent between 3 and 9 months of age.

Key words : Zebu - Cattle - Heartwater - *Cowdria ruminantium* - *Amblyomma variegatum* -Tick - Tick control - Reinfection - Elisa test - Antibody - Malawi.

SOLDAN (A.W.), NORMAN (T.L.), MASAKA (S.), PAXTON (E.A.), EDELSTEN (R.M.), SUMPTION (K.J.). Seroconversión a *Cowdria ruminantium* en terneros cebú Malawi, criados con diferentes estrategias de control de garrapatas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 171-177

Mediante el ELISA indirecto, se compara la seroconversión a *Cowdria ruminantium*, durante el primer año de vida, de sesenta y seis terneros cebú Malawi, nacidos de grupos bañados 17 veces al año, contra la seroconversión de 32 terneros nacidos en grupos no tratados. El número de *Amblyomma variegatum* y la enfermedad clínica de cada grupo fueron seguidos durante el estudio. Durante los primeros 22 meses de vida, no se observaron casos de cowdriosis en ninguno de los grupos de terneros. De los 1 800 animales seguidos durante el período de estudio solamente se observó un caso de cowdriosis, en una vaca de 8 años. La seroconversión se demostró a los 12 meses de edad, en casi todos los animales no tratados. Cincuenta por ciento de las seroconversiones se atribuyen a los estadios ninfales. Solamente 41 p. 100 de los terneros en el grupo tratado mostró una seropositividad a los 12 meses de edad. El sistema de baños disminuyó consecuentemente la tasa de conversión a *C. ruminantium* en estos terneros. Setenta y tres por ciento de los terneros mostraron niveles detectables de anticuerpos maternos contra *C. ruminantium* durante las primeras 4 semanas de vida. Hacia 8 o 12 semanas, los títulos de anticuerpos en los terneros tratados bajaron más allá de los niveles detectables mediante el ELISA. En las primeras 8 a 12 semanas de vida no hubo seroconversión en los terneros tratados. El resultado del ELISA indirecto entre los tests individuales a 12 meses y los resultados de los tests acumulados de los 9 meses anteriores, no fue significativamente diferente, lo que sugiere que el test podría ser de utilidad como examen de la inmunidad del hato. Se concluye que un estado de estabilidad enzoótica de *C. ruminantium* existe en los hatos no tratados de ganado cebú Malawi, caracterizado por una resistencia innata a la infección y una seroconversión de la mayoría de los terneros nacidos entre mayo y junio, entre 3 y 9 meses de edad.

Palabras claves : Cebú - Cowdriosis - *Cowdria ruminantium* - *Amblyomma variegatum* - Garrapata - Control de garrapata - Infestación - Test ELISA - Anticuerpo - Malawi

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Cowdria ruminantium identified in *Amblyomma gemma* using a DNA probe pCS20

WESONGA (F.D.), MUKOLWE (S.W.), FRED RURANGIRWA. Identification de *Cowdria ruminantium* dans la tique *Amblyomma gemma* par une sonde ADN, pCS 20. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46**, (1-2) : 179-181

Des tiques de l'espèce *Amblyomma gemma* ont été récoltées sur des animaux sauvages dans un ranch de 10 000 hectares, dans une région endémique pour la cowdriose au Kenya, proche de Nairobi. *A. variegatum* est le vecteur principal de la cowdriose au Kenya. E.A. LEWIS a incriminé *A. gemma* comme vecteur de la cowdriose, dans un rapport publié en 1947, sans donner de détails. Des *A.gemma* adultes ont été récoltés sur girafe (*Giraffa camelopardis*), bubale (*Alcephalus busephalus*), antilope canna (*Taurotragus oryx*) et autruche (*Struthio camelus*). Les tiques non gorgées prélevées sur girafe ont été nourries sur 3 moutons Dorper sensibles, qui ont été examinés quotidiennement pour des signes cliniques de la cowdriose. Toutes les tiques, y compris celles nourries sur les moutons, ont été disséquées et les intestins ont été testés sur la présence de *Cowdria ruminantium* à l'aide d'une sonde ADN, la pCS20. Aucun des moutons sur lesquels les tiques ont été nourries n'a montré de symptômes de la cowdriose pendant les 60 jours d'observation après la fixation des tiques. La sonde ADN a identifié *C.ruminantium* dans les tiques prélevées sur antilope et girafe.

Mots clés : Antilope - Girafe - Ovin - Cowdriose - *Cowdria ruminantium* - Isolement - Tique - *Amblyomma gemma* - Sonde ADN.

INTRODUCTION

Amblyomma gemma is the commonest species of *Amblyomma* on the Hopcraft ranch(4) located 20 km South East of Nairobi.This tick species has been reported to be capable of transmitting heartwater in the laboratory (15) and to be one of the vectors of heartwater in Kenya (6); however this was a single statement report and no details were given.

The main objective of the study was to establish if *A. gemma* is a vector of the disease on the ranch or if it simply acts as a reservoir of *Cowdria ruminantium* but playing little role in the actual transmission (of the disease). NEITZ (9) reported that infection in *Amblyomma* species can survive in a single tick generation for over 3 years.

Two methods were used to determine if the ticks were infected : feeding the ticks on susceptible sheep and probing the midguts of the adult ticks using a cloned DNA probe, the pCS20.

Because of its sensitivity and the large number of samples involved, a cloned DNA probe is suitable for detecting *Cowdria* in tick vectors (14). *C. ruminantium* has been detected in *A.variegatum* (14) using a cloned DNA probe, pCS20 derived from a strain of the parasite isolated from Crystal Springs (Zimbabwe).This probe is recommended for the detection of *C. ruminantium* as it has a high level of specificity and sensitivity (compared to another clone, the pCS9 from the Kiswani strain of Kenya).

MATERIALS AND METHODS

The study was conducted over a 3 month period, late October to January. This is the period of the short rains and there is a relative increase in the number of most tick species (including *A. gemma)* on the ranch (12).

Feeding of the ticks on sheep

Adult *A. gemma* ticks were collected from 4 species of wildlife eland, giraffe, ostrich and hartebeest, during the weekly cropping exercises.

The unengorged ticks were attached to sheep within 24 hours of collecting them from the wild animals. Three Dorper sheep were used in the experiment. A total of 104 unengorged ticks, all from the giraffe (very few ticks were obtained from the other 3 wild animal species) were attached on the sheep with the help of earbags. The earbags were opened after 9-12 days. By this time, some of the female ticks were fully engorged and dropping off. The earbags were left on the sheep up to 70 days to allow the males to engorge and drop off.

During the entire period of tick feeding, the sheep were monitored by daily rectal temperature records and clinical inspection.

1. Kenya Agricultural Research Institute, P.O. Box 274, Uthiru, Nairobi, Kenya.

2. Small Ruminant Collaborative Research Support program, Kabete, Kenya.

DNA separation from the mid-guts of *A. gemma* and probing for *C. ruminantium*

The separation of the DNA material from the tick mid-guts and the subsequent DNA probing for *C. ruminantium* was done as described by WAGHELA *et al.* (14) the only difference being that the mid guts were pooled in groups or batches of 10 according to the animal species from which they were collected.The exception to this group size was for the ostrich and hartebeest where only 5 and 2 ticks respectively were collected.

RESULTS

Feeding of the ticks on sheep

None of the sheep on which *(A. gemma)* ticks were fed showed heartwater symptoms for up to 70 days after tick attachment. One of the sheep died of pneumonia 48 days after tick attachment. A brain crush smear was made and on observation there were no *Cowdria* colonies.

Detection of *C. ruminantium* DNA in the ticks

The pCS20 probe detected *C. ruminantium* in 2 out of the 3 tick batches collected from the giraffe and in the batch (one) from the eland. All the ticks from the ostrich and hartebeest were negative.

DISCUSSION

Whereas it is known that A. *gemma* can become infected and transmit *C. ruminantium* under laboratory conditions, the results of this experiment do indicate that there is indeed a good chance of this tick species acquiring the infection in an endemic area and subsequently transmitting the infection to susceptible livestock under natural conditions.

However, the efficiency of this tick species as vector compared to *A. variegatum* is not known. Further experiments need to be done to ascertain this.

There is little information on the host range of the immature stages of *A. gemma*. Since transmission of infection is transstadial, it is important from the epidemiological point of view to know the hosts on which these immature stages feed and thus acquire infection. The next phase of this study will focus on this.This will be useful in providing information regarding the control strategies to be adapted in cases of outbreaks especially in the non-endemic areas. *A. gemma* appears to play a bigger role in the epidemiology of heartwater in Kenya than it is thought.

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WESONGA (F.D.), MUKOLWE (S.W.), FRED RURANGIRWA.
Cowdria ruminantium identified in *Amblyomma gemma* using a DNA probe pCS20. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46**, (1-2) : 179-181

***Amblyomma gemma* ticks were collected from wild animals on a 20,000 acre game ranch in a heartwater endemic area in Kenya, close to Nairobi. *A. variegatum* is the main vector of heartwater in Kenya. E.A. LEWIS, 1947, in a one sentence report has implicated *A.gemma* to be a vector of heartwater without giving any details. Adult *A. gemma* were collected from giraffe *Giraffa camelopardalis,* hartebeest *Alcephalus busephalus* , Eland *Taurotragus oryx* and ostrich *Struthio camelus* during cropping exercises. The unengorged ticks were fed on 3 susceptible Dorper sheep which were monitored daily for the clinical symptoms of heartwater. All the ticks, including those that were fed on sheep were dissected and the guts probed for the presence of *Cowdria ruminantium* using a cloned DNA probe, the pCS20. None of the sheep on which the ticks were fed showed heartwater symptoms up to 60 days from the attachment of the ticks. The DNA probe identified *Cowdria ruminantium* in the ticks collected from eland and giraffe.**

Key words : Eland - Giraffe - Sheep - Heartwater - *Cowdria ruminantium* - Isolation - Tick - *Amblyomma gemma* - DNA probe.

WESONGA (F.D.), MUKOLWE (S.W.), FRED RURANGIRWA.
Identificación de *Cowdria ruminantium* en *Amblyomma gemma* mediante el uso de ADN probador pCS20. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46**, (1-2) : 179-181

Se recolectaron garrapatas de *Amblyomma gemma* en reses bravías, en un rancho de 10 000 hectáreas, en una zona endémica para la cowdriosis en Kenia, cerca de Nairobi. *A. variegatum* es el principal vector de la cowdriosis en Kenia. En un reporte de 1947, E. A. LEWIS, menciona *A. gemma* como vector de la cowdriosis, pero no proporciona mayor detalle. Los adultos de *A. gemma* se colectaron de jirafas (*Giraffa camelopardalis*), búbalos (*Alcephalus busephalus*), antílope (*Taurotragus oryx*) y avestruces (*Struthio camelus*) durante diversas prácticas de cultivo. Las garrapatas, no ingurgitadas, fueron alimentadas sobre tres ovejas Dorper susceptibles, en las cuáles se controlaron diariamente los síntomas clínicos de la cowdriosis. Todas las garrapatas, incluyendo aquellas alimentadas en ovejas, fueron disecadas y se examinaron los intestinos para la presencia de *Cowdria ruminantium* , mediante la utilización de clones probadores de ADN, el pCS20. Sesenta días después del inicio de la alimentación de las garrapatas, ninguna de las ovejas mostró síntomas de cowdriosis. El probador de ADN identificó *Cowdria ruminantium* en las garrapatas colectadas de antílopes y jirafas.

Palabras calves : Antílope - Jirafa - Ovino - Cowdriosis - *Cowdria ruminantium* - Aislamiento - Garrapata - *Amblyomma gemma* - Sonda de ADN.

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Development and transmission of *Cowdria ruminantium* by *Amblyomma* males transferred from infected to susceptible sheep

KOCAN (K.M.), NORVAL (R.A.I.), DONOVAN (P.L.). Développement et transmission de *Cowdria ruminantium* par des mâles d'*Amblyomma* transférés de moutons infectés à des moutons sensibles. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 183-188

Des mâles d'*Amblyomma* ont été testés comme vecteurs de *Cowdria ruminantium*, agent causal de la cowdriose. Les mâles ont été nourris sur des moutons infectés expérimentalement avec *C. ruminantium* et ont ensuite été transférés à des moutons sensibles. Dans une première expérience, *A. hebraeum* et le stock Palm River de *C. ruminantium* ont été utilisés, une deuxième expérience a été faite avec le stock de *Cowdria* Kiswani et *A. variegatum*. Des tiques ont été récoltées quotidiennement pendant toute la durée de chaque expérience, coupées en deux et préparées pour examen par microscopie classique et électronique, afin d'étudier le développement de *C. ruminantium* dans leurs tissus. Dans les deux expériences les tiques ont transmis *Cowdria* à un mouton sur deux. A l'examen microscopique quelques colonies ont été trouvées dans des cellules intestinales, mais aucune n'a été observée dans les glandes salivaires. Les deux espèces de tiques étaient infectées par *Rickettsia conorii*, comme en témoignait l'existence de rickettsies dans les noyaux et le cytoplasme de cellules des glandes salivaires.

Mots clés : Ovin - Cowdriose - *Cowdria ruminantium* - Tique - *Amblyomma hebraeum* - *Amblyomma variegatum* - *Rickettsia conorii* - Transmission des maladies.

INTRODUCTION

Cowdria ruminantium is the tick-borne agent of heartwater disease in cattle. This organism is one of several rickettsias that live and multiply only within membrane-bound colonies within host cell cytoplasm. Other rickettsias that develop within colonies include *Anaplasma, Ehrlichia* and *Coxiella* (22). *Cowdria* can be transmitted to cattle or sheep transstadially by nymphal and adult ticks (3). Transovarial transmission has been demonstrated once experimentally (4), but does not appear to occur readily. Although LOUNSBURY (14) was unable to demonstrate intrastadial transmission of *C. ruminantium* by male *A. variegatum* infected as adults, NORVAL et al. (1990) demonstrated intrastadial transmission using *Amblyomma hebraeum* males (16). Transmission of heartwater occurred when small numbers of males were transferred from infected to susceptible hosts, and the same males transmitted *C. ruminantium* repeatedly to successive, susceptible hosts.

Colonies of *C. ruminantium* were first described by COWDRY in 1925 in *A. hebraeum* ticks (5) and were later confirmed in this tick species in 1985 (2) and 1991 (6). KOCAN *et al.* (1987) described similar colonies of *C. ruminantium* in *A. variegatum* (9). In these studies, colonies of the rickettsia were seen within midgut epithelial cells and occasionally within the gut lumen. Recently, colonies of *C. ruminantium* were also described in salivary glands of *A. hebraeum* nymphae that were experimentally infected with the rickettsia as larvae (10). The morphology of *C. ruminantium* in tick gut and salivary glands was similar.

The importance of intrastadial infection and transmission of a similar rickettsia, *Anaplasma marginale,* by male *Dermacentor andersoni* was described recently (18, 19). Highest infection rates were demonstrated in males exposed as adults ; the ticks remained infected throughout their life and were able to reattach and transmit *A. marginale* to successive, susceptible cattle (11, 12, 20). Most notable in these studies was the demonstration of *A. marginale* in salivary glands of male ticks exposed as adults. In contrast, when male ticks were exposed to *A. marginale* as nymphs, colonies were not evident with microscopy, even though homogenates of salivary glands were infective for cattle and were proven to contain *A. marginale* DNA using a *Anaplasma*-specific DNA probe (13).

The experimental design from the *Anaplasma* studies was applied to the present experiments on *C. ruminantium* to confirm intrastadial transmission of *C. ruminantium* to sheep by male *Amblyomma* sp. exposed as adults. A primary objective of this study was to determine whether intrastadial infection of male ticks enhanced development of *C. ruminantium* in tick salivary glands.

MATERIALS AND METHODS

Agent

Two isolates of *Cowdria ruminantium* were used for these studies : the Palm River stock from Zimbabwe and the Kiswani isolate from Kenya. The inoculum used for infection of sheep was *Cowdria*-infected endothelial cell cultures.

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K.M. Kocan R.A.I. Norval P.L. Donovan

Tick propagation

Amblyomma hebraeum and *A. variegatum* were raised in colony for several generations at the University of Florida/USAID Heartwater Project, Harare, Zimbabwe. *Amblyomma variegatum* were originally collected from the Trafalgar Farm, Zimbabwe. Larval and nymphal ticks were fed on rabbits, collected after engorgement and allowed to molt in a humidity chamber until the male ticks were used for these studies.

Sheep

Twelve 6-month old sheep, previously unexposed to *C. ruminantium*, were used for these studies. Four sheep were experimentally infected with 2 ml *C. ruminantium*-infected tissue culture supernatant and used for feeding-exposure of male ticks. A brain smear was prepared after death from sheep inoculated with infected cell cultures and examined with light microscopy for colonies of *C. ruminantium to* confirm infection. Four sheep were used for the second tick feeding to test for tick transmission of heartwater. Two susceptible sheep were inoculated with 2 ml uninfected tissue culture supernatant and used for feeding of uninfected (control) ticks. Two susceptible sheep were used for the second feeding of these control ticks.

Exposure of ticks

Amblyomma hebraeum or *A. variegatum* males (250 per sheep) were placed in cotton cells glued to the sheep 4 days after the sheep was inoculated with infected and control cultures. The ticks were allowed to feed for 5 days after which they were removed and placed in a humidity chamber.

Experimental design

These experiments were done in two trials. Trial 1 tested transmission of the Palm River stock of *C. ruminantium* by *A. hebraeum* males. In Trial 2, transmission of the Kiswani stock was tested by *A. variegatum.* In each trial, two sheep were infected with *C. ruminantium* for exposure of ticks to the agent and two susceptible sheep were used for the second feeding of these exposed ticks (fig. 1). Two additional sheep were used for the first and second feeding of control (uninfected) ticks. The ticks were allowed to feed for 5 days on *Cowdria*-infected sheep, held for 5 days in a humidity chamber and then were allowed to feed on susceptible sheep for 10 days. Feeding periods of control ticks on susceptible sheep were the same as those for the feeding of infected ticks.

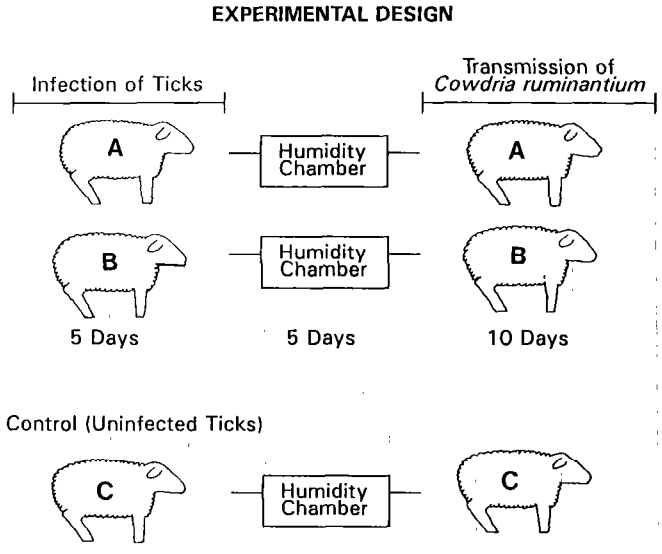

Figure 1 : Experimental design for study of intrastadial transmission of Cowdria ruminantium *by male ticks to sheep.*

Collection of ticks for electron microscopy

Five infected and three control ticks were collected and fixed for light and electron microscopy (LM and EM) before placement of ticks on sheep, on each day the ticks fed on the infected or control sheep, while held in the humidity chamber for 5 days and throughout the 10 days of the second tick feeding on susceptible sheep (total, 21 days). Each tick was cut in half with a razor blade, separating the right and left sides, placed in a 1.5 ml Eppendorf tube filled with 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer and processed according to the procedures of KOCAN *et al.* (8). Thick sections (1 µm) were prepared

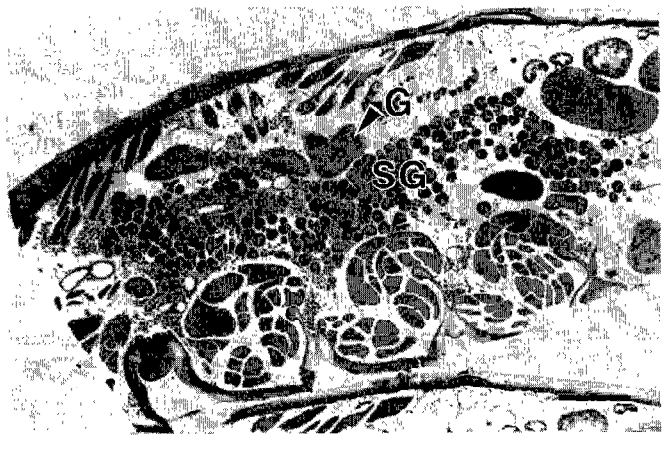

Photo 1 : A 1 µm plastic cross section of a half tick that includes gut (G) and salivary glands (SG). Bar = 460 µm.

from the tick halves, stained with Mallory's stain (17) for 2 min at 60° C and were examined by LM for colonies of *C. ruminantium*. Cross sections of entire tick halves enabled examination of all tick tissues (photo 1). Just prior to fine sectioning, a 1 µm section was prepared for LM photography. Ultrathin (silver-gold reflective) sections were cut with a Sorvall MT-5000 ultramicrotome and a Diatome diamond knife. The sections were collected on 300-mesh copper grids, stained with uranyl acetate and lead citrate (21) and observed and photographed with a JOEL 100 CX electron microscope operated at 80 kV.

RESULTS

Infection of ticks and transmission of C. *ruminantium*

The four sheep used for infection of ticks died from heartwater disease 7-12 days post-inoculation (table I). In both trials, ticks transferred from sheep A died and therefore failed to transmit *C. ruminantium* to susceptible sheep. The ticks that fed on sheep B reattached and transmitted *C. ruminantium* to susceptible sheep during a second feeding (table I), causing fatal heartwater disease.

TABLE 1 Transmission of Cowdria ruminantium *(Palm River and Kiswani stock) by* Amblyomma *spp. males transferred from infected to susceptible sheep.*

Ticks	Tick feeding	
	Exposure	Transmission
Trial 1 :	Transmission of the Palm River stock of *C. ruminantium* by *A. hebraeum* male ticks	
Group A*	Sheep died 9 days PI	—
Group B	Sheep died 10 days PI	Sheep died 18 days PI
Trial 2 :	Transmission of the Kiswani stock of *C. ruminantium* by *A. variegatum* male ticks	
Group A*	Sheep died 12 days PI	—
Group B	Sheep died 7 days PI	Sheep died 10 days PI

* *Ticks died before infestation of the second sheep.*

Morphology of C. *ruminantium* tick tissues

Small numbers of colonies of *C. ruminantium* were seen with LM and EM in midgut epithelial cells of *A. hebraeum* and *A. variegatum* (photos 2, 3, 4). The morphology of organisms within colonies was similar to that described previously. *Cowdria* colonies were not seen in salivary glands with LM or EM. Organisms that appeared to be *Rickettsia conorii* were seen within the cytoplasm and nucleus of cells surrounding the collecting

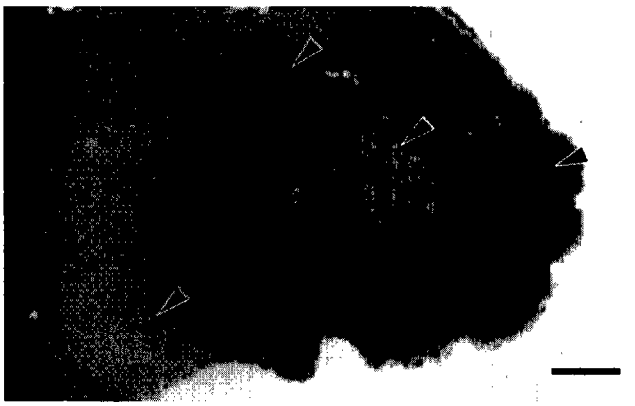

Photo 2 : Colonies of Cowdria ruminantium *(arrows) in midgut cells of* Amblyomma hebraeum *in a 1 µm plastic section stained with Mallory stain. Bar = 4,5 µm.*

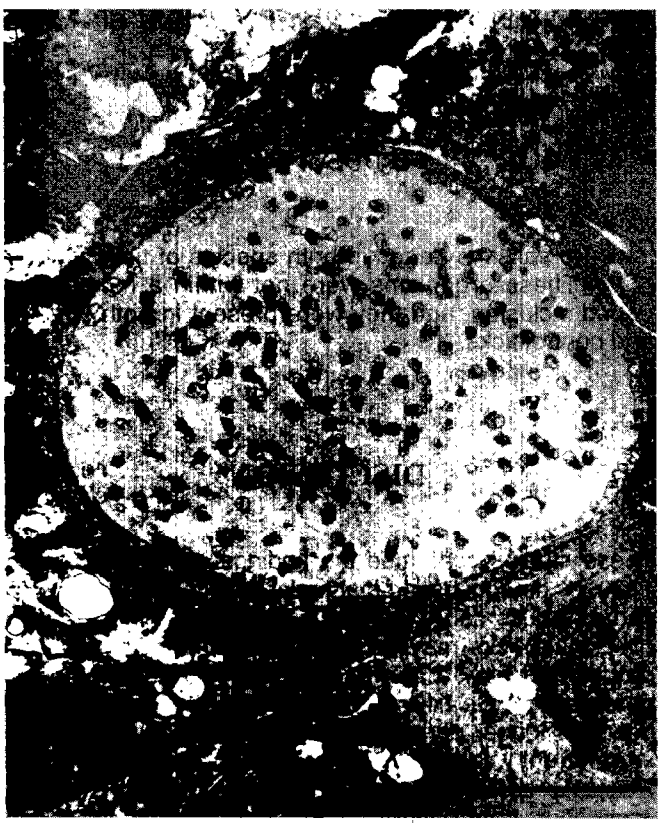

Photo 3 : An electron micrograph of a colony of Cowdria ruminantium *in a midgut epithelial cell of* Amblyomma hebraeum. *Bar = 9,25 µm.*

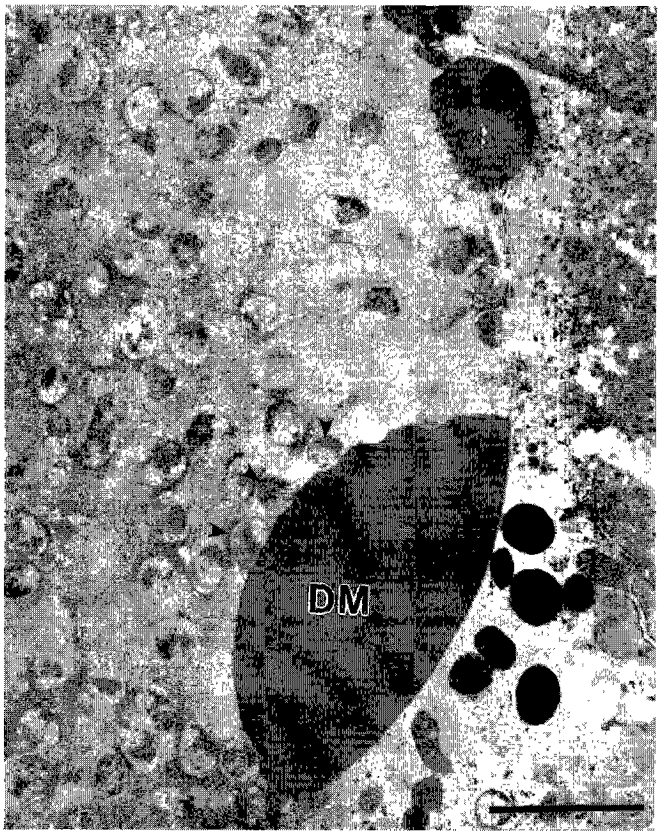

Photo 4 : A higher magnification of a colony of Cowdria ruminantium *in a midgut cell of* Amblyomma hebraeum. *The colony contains dense material (DM) to which some organisms are attached (small arrows). Bar = 18,5 µm.*

Photo 5 : Rickettsia conorii *(R) within the cytoplasm of a cell associated with salivary gland collecting ducts. Bar = 1,85 µm.*

ducts of salivary glands in both species of ticks (photos 5, 6). These organisms were not within a membrane-bound inclusion, and they were present in both infected and control ticks.

DISCUSSION

These studies confirmed the findings of NORVAL *et al.* 1990, by demonstrating that male *Amblyomma* spp. exposed as adults can transmit *C. ruminantium* intrastadially (16). Male ticks may be efficient vectors of *Cowdria* in nature and play a central role in the epidemiology of heartwater. Male ticks remain attached to their ruminant hosts for longer periods (up to eight months) where they intermittently feed and mate. The males attain sexual maturity after feeding approximately one week. Thereafter, they emit an aggregation-attachment pheromone (AAP) which can be detected by unfed adults and nymphs, contributing to the tick's selection of hosts and attachment sites (15). Males thus accumulate on suitable hosts, where they occur in clusters on the parts of the ruminants that are groomed least effectively.

Small numbers of *C. ruminantium* were seen in gut cells in *Amblyomma* ticks exposed to either *Cowdria* stock and none were seen in salivary glands. Although COWDRY (5) did not observe *C. ruminantium* in salivary glands, BEZUIDENHOUT (1) found homogenates of salivary glands of prefed adults exposed as nymphae to be infective for sheep. Furthermore, colonies of *C. ruminantium* were described with LM and EM in approximately 15 % of the nymphal ticks studied that were exposed to *C. ruminantium* as larvae. YUNKER *et al.* (1993), using a *Cowdria*-specific DNA probe, demonstrated that, although salivary glands contained *Cowdria* DNA, the gut was the main site of replication of *C. ruminantium* in ticks (23). However, infected salivary glands were more frequently demonstrated in male ticks. Because intrastadial infection of male ticks allowed for amplification of *A. marginale* in salivary glands of *Dermacentor* sp. ticks, we applied the same experimental design to *Cowdria* in *Amblyomma* males. However, this method of exposure was not effective in increasing salivary gland infections ; colonies were not seen with LM or EM. It appears that the major site of infection of *Cowdria* in ticks is in midgut cells.

Photo 6 : Rickettsia conorii *(R) within the nucleus (Nu) of a cell associated with salivary gland collecting ducts. Bar = 1,85 µm.*

The *Amblyomma* ticks in this study were also infected with *Rickettsia conorii,* causative agent of boutonneuse fever (7). This organism is easily differentiated from *Cowdria* because it occurs free in the cell cytoplasm rather than in membrane-bound inclusions. Furthermore, *R. conorii* is one of the few rickettsias reported to occur within the cell nucleus. This rickettsia is transmitted from one tick generation to the next via the egg, thus resulting in persistent infection of ticks, even those reared in the laboratory for many generations.

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KOCAN (K.M.), NORVAL (R.A.I.), DONOVAN (P.L.). Development and transmission of *Cowdria ruminantium* by *Amblyomma* males transferred from infected to susceptible sheep. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 183-188

Male *Amblyomma* sp. were tested as vectors of *Cowdria ruminantium*, causative agent of heartwater disease. The males were allowed to feed on sheep experimentally infected with *C. ruminantium* and then were transferred to susceptible sheep to test for transmission of the rickettsia. The experiments were done in two trials. In the first trial, *A. hebraeum* were exposed to the Palm River stock of C. *ruminantium*, while in the second trial the Kiswani stock of *Cowdria* was tested with *A. variegatum*. Ticks were collected daily throughout each experiment, cut in half, and processed for light and electron microscopy to study development of *C. ruminantium* in tick tissues. In both trials, the male ticks transmitted *Cowdria* to one of two susceptible sheep. When ticks were examined with microscopy, a few colonies were found in gut cells while none were seen in salivary glands. Both species of ticks were infected with *Rickettsia conorii*, as evidenced by the occurrence of rickettsiae in the nucleus and cytoplasm of salivary gland cells.

Key words : Sheep - Heartwater - *Cowdria ruminantium* - Tick - *Amblyomma hebraeum* - *Amblyomma variegatum* - *Rickettsia conorii* - Disease transmission.

KOCAN (K.M.), NORVAL (R.A.I.), DONOVAN (P.L.). Desarrollo y transmisión de *Cowdria ruminantium* mediante *Amblyomma* : transferencia de machos de ovinos infectados hacia ovinos susceptibles. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 183-188

Se probaron los machos de *Amblyomma* sp. como vectores de *Cowdria ruminantium*, agente causal de la cowdriosis ("heartwater disease"). Los ácaros machos fueron alimentados a partir de ovejas infectadas experimentalmente con *C. ruminantium* y transportados a ovejas susceptibles con el fin de probar la transmisión de la rickettsia. Los experimentos se llevaron a cabo en dos etapas. La primera consistió en exponer *A. heabraeum* al stock de *C. ruminantium* de Palm River y el segundo ensayo examinó el stock Kiswani de *Cowdria* con *A. variegatum*. En los dos casos las garrapatas se recolectaron diariamente, se cortaron en mitades y se procesaron para el estudio en microscopio de luz y electrónico, con el fin de estudiar el desarrollo de *C. ruminantium* en los tejidos del ácaro. En ambos ensayos, el ácaro macho transmitió *Cowdria* a uno de los dos ovinos susceptibles. Cuando las garrapatas se examinaron con el microscopio, se encontraron algunas colonias en las células intestinales, pero no se observó ninguna en las glándulas salivales. Ambas especies de garrapatas se infectaron con *Rickettsia conorii*, como lo demostró la aparición de rickettsias en el núcleo y el citoplasma de las células de las glándulas salivales.

Palabras claves : Ovino - Cowdriosis - *Cowdria ruminantium* - Garrapata - *Amblyomma hebraeum* - *Amblyomma variegatum* - *Rickettsia conorii* - Transmisión de enfermedades.

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Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells

TOTTÉ (Ph.), BLANKAERT (D.), ZILIMWABAGABO (P.), WÉRENNE (J.). Inhibition de l'infectiosité de *Cowdria ruminantium* dans des cellules endothéliales par les interférons alpha et gamma. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 189-194

Une corrélation positive entre la résistance de bovins à l'infection par *Cowdria* et la production précoce d'IFN a été démontrée auparavant. Les études *in vitro* rapportées ici ont montré une activité de rBoIFNα2C et de rBoIFNγ contre *Cowdria* dans des cellules endothéliales bovines de la microvasculature cérébrale (BMEC). Le rBoIFNγ est beaucoup plus actif que le rBoIFNα2C. Ces résultats suggèrent un rôle des IFNs dans la résistance contre la maladie. Étonnamment, dans les mêmes conditions le rBoIFNα2C n'a pas d'effet sur la production de *Cowdria* infectieuses dans des cellules endothéliales bovines originaires de l'artère ombilicale (BUEC). Il a déjà été démontré que le HuIFNα n'a pas d'effet sur la multiplication de *Cowdria* dans des cellules endothéliales ombilicales humaines. Nous n'avons pas trouvé de différence dans la capacité de cellules BUE et BME à fixer le rBoIFNα2C. Ceci pourrait indiquer une véritable différence entre les capillaires et les grands vaisseaux sanguins.

Mots clés : Bovin - *Cowdria ruminantium* - Résistance aux maladies - Culture de cellule - Cellule endothéliale bovine - Interféron.

INTRODUCTION

Cytokines are a family of proteins which are synthesized by the cells of vertebrates in response to a wide variety of stimuli. They have pleiotropic effects and act both in an autocrine and paracrine way (3). There are more than 40 different cytokines known so far which interact in a very complex and still obscure manner to orchestrate the body's immune response. The understanding and mastering of the cytokine network would enable us to control immunity and that is one of the great challenges of the future. Of course, there is still much work to do but we are beginning to get a glimpse at this very fine equilibrium between cytokines which makes the immune response beneficial or detrimental for the host. In this perspective, the study of the role played by cytokines in a new model, e.g. *Cowdria ruminantium* infections, is of considerable fundamental interest.

From a more practical point of view, cytokines may help to explain the mechanisms involved in the protective immune response against *Cowdria* and in the pathogenesis of cowdriosis which in turn would be of help in the search for a safe, easy to use and efficient vaccine against this disease. Our group has started an extensive study on the involvement of interferons (IFN α and γ), interleukins (IL-1 and IL-6) and tumor necrosis factor (TNF) in *Cowdria ruminantium* infections. We have shown (11) that cattle that resisted experimental infection with the rickettsia produced significant level of circulating IFN whereas animals that died did not. IFNs were first known for their antiviral activity but have been shown both *in vitro* and *in vivo* to have antirickettsia (2) and antichlamydia (8) properties. In this report we demonstrate for the first time that IFN a and g are capable of inducing, *in vitro* an anticowdria state in the cells treated with subsequent reduction of the yield of infectious *Cowdria* organisms. The possible role of IFNs in the resistance of cattle against *Cowdria* infection is discussed.

MATERIAL AND METHODS

Isolation and culture of cells

Bovine endothelial cells from the brain microvasculature (BMEC) were a kind gift of Dr. G. TARONE (University of Torino, Department of Biology and Medical Chemistry, Italy). Bovine endothelial cells from umbilical cord arteries were a kind gift of Dr. F. JONGEJAN (The Netherlands). These cells were grown in BHK-21 (Glasgow modification) supplemented with 10 % foetal calf serum, penicillin (100 U/ml), streptomycin (100 µg), fungizone and L-Glutamine (2 mM). BMEC and BUEC at passage 20 were used in this study. Primary cultures of human endothelial cells from the umbilical vein (HUVEC) were initiated in our laboratory (for further details see accompanying paper "Bovine and human endothelial cells growth on collagen microspheres and their infection with the rickettsia *Cowdria ruminantium*", p. 153). HUVEC at passage 3 to 7 were used in this study.

Cowdria cultivation *in vitro*

The Senegal stock of *Cowdria* was given to us as sucrose-phosphate-glutamate (SPG) cryopreserved stabilates by Dr. F. JONGEJAN. The cultivation of *Cowdria* was identical for all endothelial cells and was done as previously described for BUE cells (4). Briefly, the cells were

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grown to confluency in 80 cm² tissue culture flasks (Nunc). The cells were infected using SPG cryopreserved stabilates of *Cowdria* diluted in complete growth medium (see isolation and culture of cells) supplemented (subsequently reported as "infection medium") with tryptose phosphate broth (Gibco) at 2.9 g/l and hepes buffer (20 mM) (pH 7.0-7.2). The cells were incubated in a 5 % CO_2 - 37 °C incubator (no rocking platform was used). When more than 70 % of the cells were lysed, the culture supernatant was centrifuged for 15 min at 1,500 g; the pellet obtained from 2 ml of supernatant was resuspended in 1 ml SPG buffer before snap freezing in liquid nitrogen. Stabilates of *Cowdria* at passage 9 to 11 (with an incubation period of about 10 days between each passage) were used in this study.

IFNs, IFN assay, anti-IFN antibodies

Recombinant Bovine Interferon alpha C (rBoIFNαC, specific activity 2.10^8 U/mg) was cloned and expressed in *E. coli* (provided by Dr. A. SHAFFERMAN, Israel Institute of Biological Research, Ness-Ziona). Recombinant Bovine Interferon gamma (rBoIFNγ, specific activity 2.5 10^6 U/mg) and neutralizing antibodies to BoIFN α and γ were kindly given to us by Dr. R. STEIGER from CIBA-GEIGY. Recombinant human interferon alpha 2 (rHuIFNα2, specific activity 10^8 IU/mg) was a gift of Dr. C. WEISSMANN from Zürich University. The antiviral activity of all IFNs was estimated before use on Madin Darby bovine kidney cells (MDBK) using the classical test of reduction of the cytopathogenicity of Vesicular Stomatitis Virus (10). The antiviral activity is expressed in laboratory units, no international standards being available as reference for bovine IFNs. One unit of antiviral activity is defined as the reciprocal of the dilution that gives 50 % protection against viral challenge under standard conditions.

Anti-*Cowdria* activity of interferons *in vitro*

We have studied the effect of IFNs on the infectious yield of *Cowdria ruminantium*. It was done by titrating the infectivity of supernatants collected from *Cowdria*-infected cells (in the presence or absence of IFN) using a Tissue Culture Lethal dose 50 % (TCLD50) test we have established (see below). Supernatant freshly collected from an 80 cm² flask, showing 70 % lysis, or more, due to *Cowdria* infection, was used as inoculum. The infected supernatant was centrifuged 5 min at 300 g to remove cell debris and diluted 2-fold in "infection medium" (see *Cowdria* culture *in vitro*). 500 µl of the diluted supernatant was added to confluent BME cells in a 24-well plate at 37 °C-5 % $C0_2$. After 24 hours incubation the medium was renewed. At day five post-infection 500 µl of fresh medium was added to the wells. IFNs were added at different times (see results) and the medium was replaced 24 h after every treatment. Control cells, IFN-treated but not infected, and infected but not treated, were included in each experiment. In order to demonstrate the involvement of BoIFN α and γ, these IFNs were incubated together with their respective neutralising antibodies (20 min at 37 °C) before addition to the cells and compared to IFN alone. The progress of the infection was followed daily by light microscopy. Supernatants were collected in all wells at day 9 post-infection. Each supernatant was centrifuged at 15,000 g for 15 min and the pellet was resuspended in 100 µl SPG before snap freezing in liquid nitrogen. The infectivity of these supernatants was measured using the tissue culture lethal dose 50 % (TCLD50) assay described below.

TCLD50 assay for *Cowdria ruminantium* titration

BME cells were grown to confluency in 96-well plates (Nunc). *Cowdria* samples to be tested and cryopreserved in SPG were thawed at 37°C, serially diluted 2-fold in "infection medium" with a final volume of 100 µl/well and added to the cells. The medium was replaced 24 h after infection and from there on one half of it was replaced every four days. The plates were incubated 15 days in a 37 °C-5 % CO_2 incubator. After incubation the cells were fixed in formaldehyde for 30 min and then stained with crystal violet. The end point (50 % lysis) can be determined using a light microscope or by measuring the absorbance (A_{540}). The TCLD50 of the sample is the reciprocal of the dilution that gives 50 % lysis of the cells after 15 days of incubation. The TCLD50 test measures the infectivity of the samples which depends on the concentration in *Cowdria* elementary bodies. An example of *Cowdria* titration is shown in fig. 1.

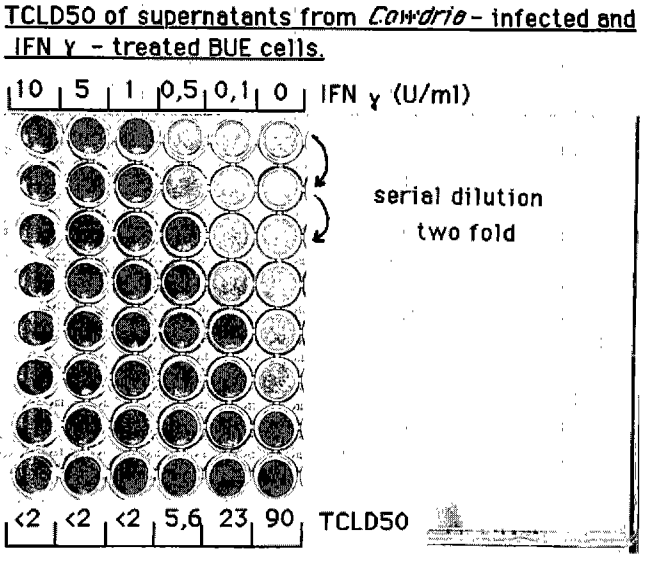

Figure 1 : Illustration of a Cowdria *titration using the TCLD50 method. BUE cells were IFN treated at day 0 and 1. Supernatants were collected at day 9 post-infection.*

2-5A synthetase assay

2-5A synthetase activity was assayed in the cytoplasmic fraction of the cells according to a method described elsewhere (see accompanying paper "Role of Interferons in infectious diseases in the bovine species").

RESULTS

Kinetics of *Cowdria* yield as measured by the TCLD50 method

The development of *Cowdria* (followed by light microscopy) in BME cells in the absence of IFN was similar to observations previously reported with BUE cells (5). Non-fusing colonies (morulae) of *Cowdria* were detected in the cytoplasm of the cells as soon as day four after infection. Cell lysis (with release of infectious organisms) occurred at day five and progressively increased until complete destruction of the monolayer by day 10 - 11 post-infection. The infectious yield of *Cowdria* was not detected by the TCLD50 assay before day 7 post-infection (fig. 2). Thereafter the yield rapidly increased and reached a peak at day 9, which corresponded to more or less 80 % cell lysis, and then slowly decreased (fig. 2).

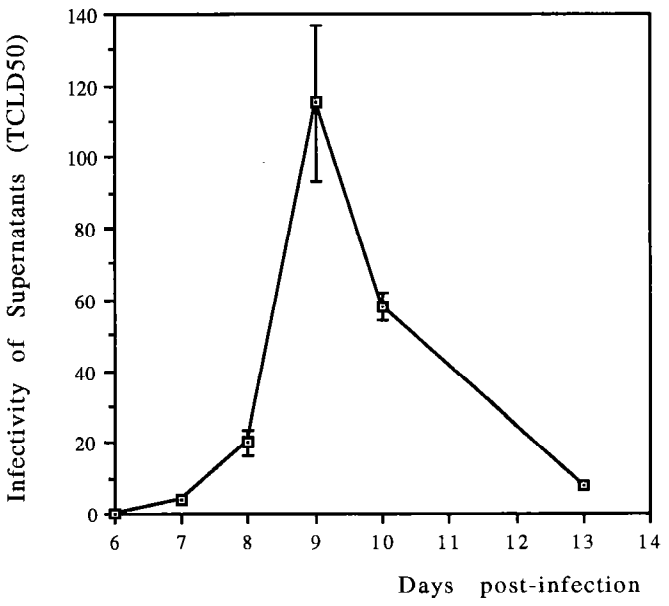

Figure 2 : Kinetics of Cowdria *yield as measured by the TCLD50 assay in the supernatants of infected BME cells. Each point represents the mean value of three different wells in the same experiment, bars indicate standard deviation .*

Effect of IFNs α and IFN γ on the infectious yield of *Cowdria* in endothelial cells

We found that the infectivity of supernatant collected from *Cowdria*-infected BME cells was significantly reduced when rBoIFNαC was added to the medium. The inhibitory effect was dependent on the dose of rBoIFNαC and completely blocked by anti-rBoIFNα antibodies (fig. 3). The number of colonies was significantly reduced in the IFN-treated cells (not shown) but a few morulae were still visible even at the highest IFN concentration and ultimately lead to a complete destruction of the monolayer. rBoIFNαC was not cytotoxic for the cells in the presence or absence of *Cowdria*.

Strangely enough, in the same experimental conditions, rBoIFNα C had no anti-*Cowdria* activity on BUE cells nor did rHuIFNα2 on HUVEC (fig. 3).

rBoIFNγ was found to strongly reduce the infectious yield of *Cowdria* in both BME and BUE cells (fig. 4). The inhibitory effect was completely reversed by anti-rBoIFNγ. In contrast with rBoIFNαC, complete protection of the cells was easily achieved with rBoIFNγ . When the cells received 10 U/ml of rBoIFNγ at day 0 and 1 no colonies were observed in these cells for up to 30 days post-infection (reinfection was not tested). Cytotoxicity of rBoIFNγ for uninfected BME and BUE cells was observed but only when 50 U/ml or more were added to the medium for three consecutive days.

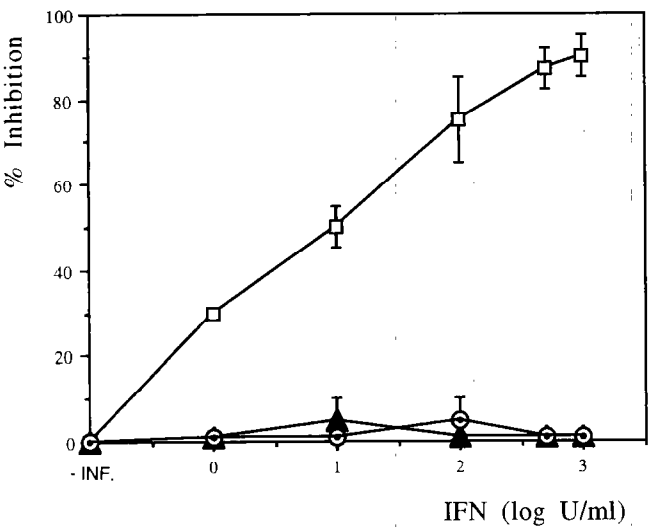

Figure 3 : Inhibitory effect of IFNα on the yield of Cowdria. *BME (□), BUE (O) and HUVEC (Δ) cells were treated with IFN of the homologous species at day 0, 1 and 2. A control with anti-IFN alpha antibodies (•) was included for BME cells. Each point represents the mean value of three different experiments, bars indicate standard deviation.*

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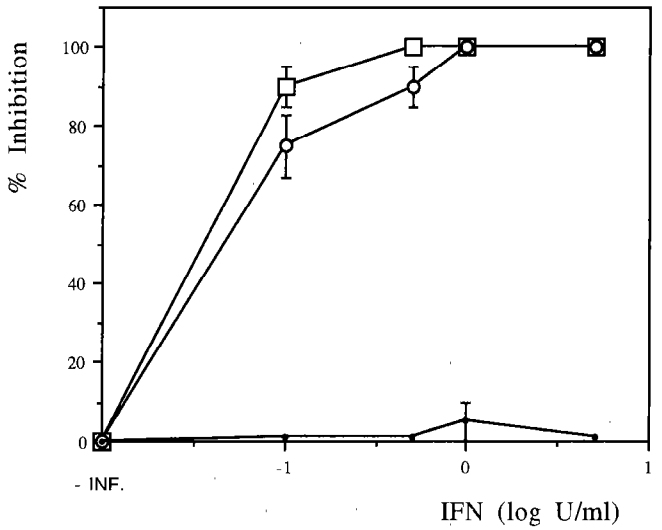

Figure 4 : Inhibitory effect of IFNγ on the yield of Cowdria. *BME (□) and BUE (O) cells were IFN treated at day 0 and day 1. A control with anti-IFN gamma antibodies (•) was included for BME cells. The experiment was run in triplicate, bars indicate standard deviation .*

Comparison of Antiviral, 2-5A synthetase and cowdriacidal activity of rBoIFNαC in BME and BUE cells

In order to investigate the possibility that IFN-induced activities other than the cowdriacidal activity could also differ from one cell type to another, we compared the antiviral and 2-5A synthetase (an enzyme of which synthesis is induced by IFNs, 10) activities of rBoIFNαC in BME and BUE cells. We found (table I) that these cells have the same sensitivity to the antiviral effect of rBoIFNαC and that the small difference observed in the 2-5A synthetase activity can not explain the difference in the activity against *Cowdria*.

TABLE I Antiviral, 2-5 A synthetase and anticowdria activity of rBoIFNα in BME and BUE cells.

Cell type	Antiviral activity (U/ml)	2-5A synthetase activity (a)	% reduction of *Cowdria* yield
BUEC	100 500	219 (± 9) 242 (± 16)	0 0
BMEC	100 500	157 (± 27) 288 (± 16)	75 (± 10) 90 (± 5)

(a) : pmoles ATP polymerized/ µ g protein/h. (standard deviation)

Kinetics of rBoIFN (α and γ) induction of an anticowdria state in BME cells

The inhibitory effect was highest (fig. 5) when both IFNs were present on day one (when *Cowdria* organisms have been removed from the medium) which demonstrates that IFNs act through the host cells and not directly on the free organisms. The anticowdria state induced by rBoIFNαC in BME cells has a very short life time compared to that of rBoIFNγ. Pretreatment of the cells with rBoIFNαC did not affect the yield of *Cowdria*. In contrast, an anticowdria state could be induced in BME cells by addition of rBoIFNγ as soon as two days before infection.

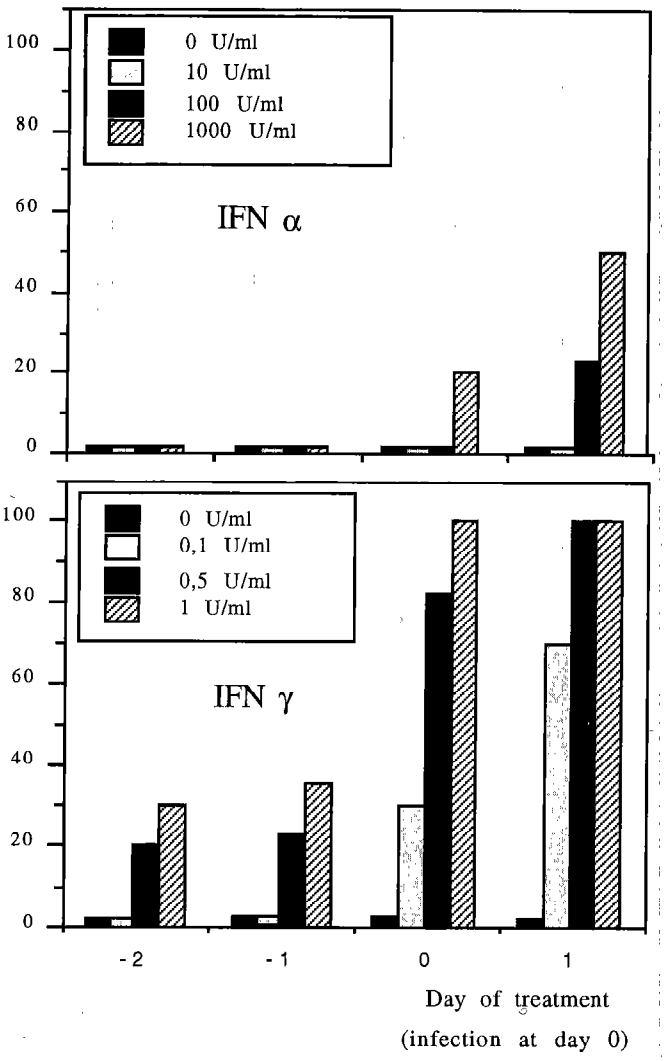

Figure 5 : Kinetics of rBoIFN (α and γ) induction of an anticowdria state in BME cells. IFNs were added to the cells at various times relative to infection (as indicated on the figure). Cowdria *yield (TCLD50) was determined at day 9 after infection (one-step-growth-yield assay). Two repetitions of this experiment yielded similar results.*

When both IFNs anticowdria activities are compared in experimental conditions that give maximal inhibition (e.g. day 1) rBoIFNγ appears 10,000 times more efficient than rBoIFNαC.

DISCUSSION AND CONCLUSION

We have shown that rBoIFNαC has the property to induce *in vitro* an anticowdria state in BME cells. These results, together with previous data showing that IFN was produced in the plasma of animals that resisted an experimental infection with the rickettsiale (11), suggest that IFNα plays a role in the resistance of cattle to cowdriosis. However, rBoIFNαC has no prophylactic effect *in vitro*. Moreover, complete inhibition of *Cowdria* growth in BME cells was never achieved even at high IFN concentration. Therefore, IFNα may be very useful *in vivo* to slow down the infection allowing other mechanisms to take place in order to ensure survival of the infected animals. One possibility is that IFNγ is also produced in response to the infection. We have shown here that rBoIFNγ is a very powerful anticowdria agent *in vitro* but it remains to be demonstrated that it is also produced *in vivo*.

The mechanisms underlaying the IFNs-induced anticowdria activity *in vitro* are not known. The anticowdria activity of rBoIFNαC is undoubtedly dissociated from its antiviral and 2-5A synthetase inducing activities. We have shown that IFNs act on the cells to render them unsuitable for *Cowdria* growth but only electronic microscopy will tell us at which stage of the *Cowdria* replication cycle (fixation, transformation of elementary bodies to reticulate bodies, metabolism of reticulate bodies, etc.) IFNs actually intervene. It has been shown that degradation of tryptophan in the case of *Chlamydia trachomatis* (6) and metabolism of L-arginine in the case of *Ehrlichia risticii* (9) are among the possible pathways involved in the *in vitro* effect of IFNγ. The possible role played by amino acids in our model is under study.

We have found that, in contrast to BMEC, BUE cells were not sensitive to the anticowdria effect of rBoIFNαC. Differences in receptor to BoIFNα are unlikely to be the cause here since in both type of cells rBoIFNαC has similar antiviral and 2-5A synthetase activities. This may reflect a true difference in cell type between capillary and large blood vessels. Endothelial cells isolated from large vessels and capillaries have indeed been shown to differ in the concentration of insulin receptors (1) and in their collagen secretory phenotypes (7). On the other hand, the difference we observed may result from cell isolation and initial culture conditions, in which case, we should not observe the same phenomenom in primary cultures. We know already that HUVEC is insensitive to IFNα- mediated anticowdria activity. We have now undertaken a study in order to compare the anticowdria activity of IFNα in primary cultures of human endothelial cells from the macrovasculature (HUVEC) and the microvasculature (endothelial cells of the human foreskin-HEMEC). In both cell types *Cowdria ruminantium* multiplies efficiently (unpublished data). However, endothelial cells from capillaries and from large blood vessels may very well respond differently to *Cowdria* infection in a way which may be relevant to the immunity and pathogenesis of cowdriosis.

ACKNOWLEDGEMENTS

Special thank to Dr. F. JONGEJAN who provided us with *Cowdria ruminantium* stabilates and useful advice. J. WÉRENNE and Ph. TOTTÉ wish to dedicate this paper to the late Pr. I. SAROV with whom they started a collaboration a few years ago.

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TOTTÉ (Ph.), BLANKAERT (D.), ZILIMWABAGABO (P.), WÉRENNE (J.). Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 189-194

We have shown before that there is a positive correlation between resistance of cattle against *Cowdria* infection and early IFN production. Our *in vitro* studies demonstrated an activity of rBoIFNα2C and rBoIFNγ against *Cowdria* in bovine endothelial cells of brain microvasculature (BMEC). rBoIFNγ is much more active in this respect than rBoIFNα2C. These results suggest a role of IFNs in the resistance against the disease. Strikingly, in the same conditions rBoIFNα2C has no effect on the yield of *Cowdria* from infected bovine endothelial cells of umbilical artery origin (BUEC). Similarly we showed that HuIFNa had no effect on the multiplication of *Cowdria* in human vein umbilical endothelial cells (HUVEC). We found no differences in the capacity of BUE and BME cells to bind rBoIFNα2C. This may reflect a true difference between capillary and large blood vessels.

Key words : Cattle - *Cowdria ruminantium* - Disease resistance - Cell culture - Bovin endothelial cell - Interferon.

TOTTÉ (Ph.), BLANKAERT (D.), ZILIMWABAGABO (P.), WÉRENNE (J.). Inhibición de las infecciones por *Cowdria ruminantium* mediante el interferón alfa y gama en células endoteliales. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 189-194

Anteriormente se demostró la existencia de una correlación positiva entre la resistencia del ganado contra la infección por *Cowdria* y la producción temprana de IFN. Nuestros estudios *in vitro* demuestran una actividad de rBoIFNα2C y rBoIFNg contra *Cowdria* en las células endoteliales bovinas de los microcapilares cerebrales (BMEC). El rBoIFNγ es mucho más activo que rBoIFNα2C. Estos resultados sugieren un posible papel del IFNs en la resistencia contra la enfermedad. Sorprendentemente, bajo las mismas condiciones, el rBoIFNα2C no actuó sobre *Cowdria* en las células de endotelio bovino infectado, provenientes de la arteria umbilical (BUEC). Así mismo, se desmostró que el HuIFNα no actúa en la multiplicación de *Cowdria* en las células de endotelio de lavena umbilical humana (HUVEC). No se encontraron diferencias en cuanto a la capacidad de unión de BUE y de BME con rBoIFNα2C, lo cual podría reflejar una diferencia entre los grandes vasos sanguíneos y los capilares.

Palabras claves : Bovino - *Cowdria ruminantium* - Resistancia a la enfermedad - Cultivo de celulas - Célular endotelial bovina - Interferón.

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STVM-93

Interleukin 6 expression upon infection by *Cowdria ruminantium* of bovine brain endothelial cells *

BENSAID (A.), BOURDOULOUS (S.), LERHUN (D.), CALVEZ (D.), DROOGMAN (L.), MARTINEZ (D.), COURAUD (P.O.). Expression d'interleukine 6 après infection par *Cowdria ruminantium* de cellules endothéliales de cerveau bovin. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 195

Les interleukines sont des médiateurs protéiques solubles qui peuvent déclencher une activation cellulaire. D'abord impliquée dans l'activation des cellules T dans la production d'immunoglobulines par les cellules B, l'interleukine 6 (IL6) provoque aussi l'induction de protéines de la phase aiguë par les hépatocytes. Ainsi, l'IL6 est impliquée dans les processus inflammatoires qui sont en grande partie responsables de la pathogénie de la cowdriose. Originellement produit par des macrophages et des cellules endothéliales activées, l'IL6 agit comme un stimulant de la réponse immunitaire. Néanmoins, quand elle est produite constamment et en grandes quantités, l'IL6 provoque des réactions inflammatoires non contrôlées. Afin de tester si l'IL6 est impliquée dans la cowdriose, une culture primaire de cellules endothéliales de cerveau bovin (BBEC) a été infectée *in vitro* par *C. ruminantium*. Les cellules infectées ont été récoltées tous les jours et ce jusqu'au sixième jour où toutes les cellules sont lysées. La même expérience a été effectuée sur des BBEC qui ont été simultanément infectées et traitées avec de l'INFγ. De l'ARN a été purifié à partir de ces cellules et après électrophorèse sur gel d'agarose transféré sur un filtre de nylon. Ce filtre a été sondé avec un ADNc radiomarqué codant pour l'IL6 bovine. Des signaux à 1 kb ont été détectés seulement sur les ARN de cellules après le quatrième jour de l'infection, que celles-ci aient été traitées ou non par l'INFγ. Tous les autres échantillons, incluant l'ARN de cellules qui n'ont pas été infectées mais traitées à l'INFγ, se sont révélés négatifs pour l'expression d'IL6. Ainsi, après infection par *C. ruminantium*, l'expression d'IL6 est induite peu avant que l'effet cytopathogène n'apparaisse. A l'heure actuelle, des surnageants de milieu de culture de ces cellules sont testés pour leur capacité à induire des réponses prolifératives des cellules T.

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* *Seuls les résumés de cette communication sont publiés dans ce volume.*

BENSAID (A.), BOURDOULOUS (S.), LERHUN (D.), CALVEZ (D.), DROOGMAN (L.), MARTINEZ (D.), COURAUD (P.O.). Interleukin 6 expression upon infection by *Cowdria ruminantium* of bovine brain endothelial cells. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 195

Interleukins are soluble protein mediators which can trigger cell activation. First involved in T-cell activation and B-cell immunoglobulin production, interleukin 6 (IL6) was shown to induce acute phase proteins by hepatocytes. Thus, IL6 is involved in inflammatory processes which account for most of the pathogeny encountered in cowdriosis. Produced primarily by activated macrophages and endothelial cells, IL6 acts as a "booster" of immune responses. However, when produced constantly and in high quantities, IL6 provokes uncontrolled inflammatory reactions. To test whether IL6 is implied in cowdriosis, a primary culture of bovine brain endothelial cells (BBEC) was infected in vitro by *C. ruminantium*. Infected cells were harvested every day until day 6 in which all cells were lysed. The same experiment was on BBEC which were simultaneously infected and treated with γINF. RNA was purified from these cells and after agarose gel electrophoresis, blotted onto a nylon filter. The filter was probed with a radiolabeled cDNA coding for the bovine IL6. Signals at 1 kb were detected only in RNA of cells collected 4 days after infection treated or not by γINF. All other samples, including RNA of cells which were not infected but treated with γINF, revealed to be negative for IL6 expression. Thus, upon infection by *C. ruminantium* IL6 expression is induced shortly before the cytopathogenic effect occurs. Currently, culture media supernantants of these cells are being tested for their capacity to induce T-cell proliferative responses.

BENSAID (A.), BOURDOULOUS (S.), LERHUN (D.), CALVEZ (D.), DROOGMAN (L.), MARTINEZ (D.), COURAUD (P.O.). Expresión de la interleukina 6 sobre la infección de *Cowdria ruminantium* en células de endotelio cerebral bovino. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 195

Las interlukinas son mediadores de proteínas solubles, capaces de provocar la activación celular. Inicialmente se les atribuyó la activación de células T y la producción de inmunoglobulinas de células B, sin embargo, se ha demostrado que la interlukina 6 (IL6) induce la fase protéica aguda en los hepatocitos. La IL6 se encuentra involucrada en procesos inflamatorios, causantes de una gran parte de la patogenia encontrada en la cowdriosis. Las IL6, producidas en forma primaria por los macrófagos y las células endoteliales, actúan como apoyo para las reacciones inmunológicas. Sin embargo, cuando la IL6 es producida constantemente y en grandes cantidades, se producen reacciones inflamatorias incontrolables. Con el fin de determinar si la IL6 se encuentra involucrada en la cowdriosis, se infectó in vitro un cultivo primario de células de endotelio cerebral bovino (BBEC) con *C. ruminantium*. Las células infectadas se colectaron todos los días, hasta el sexto, día en el que se provocó la lísis de todas las células. El mismo experimento se realizó en BBEC infectadas y tratadas simultáneamente con γINF. El ARN de estas células se purificó, se sometió a una electroforesis en agar gel y luego se secó en un filtro de nylon. Este filtro se probó con un codificador marcado con ADNc para la IL6 bovina. Solamente se observaron señales a 1 kb en las células recolectadas 4 días post infección, tratadas o no con γINF. Todas las otras muestras, incluyendo el ARN de células no infectadas, pero tratadas con γINF, se revelaron negativas a la expresión de la IL6. La IL6 se expresa en las infecciones por *C. ruminantium* poco tiempo después de la aparición del efecto citopatogénico. Actualmente, se prueba la capacidad de inducir respuestas de células T proliferativas con los sobrenadantes de los medios de cultivo de estas células.

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The development of antibody to *Cowdria ruminantium* in mice and its role in heartwater disease

BYROM (B.), MAHAN (S.M.), BARBET (A.F.). Le développement d'anticorps contre *Cowdria ruminantium* chez la souris et leur rôle dans la cowdriose. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 197-201

Les réponses immunitaires contre *Cowdria ruminantium* ont été étudiées en utilisant des souris DBA/2 et Balb/c comme modèle. Les deux souches de souris ont été inoculées avec 1, 10 ou 100 DL_{50} de *C. ruminantium* (stock Crystal Springs). Des anticorps contre *C. ruminantium* ont commencé à se développer dans la deuxième semaine après l'inoculation et le titre d'anticorps dépendait de la dose de *C. ruminantium* inoculée. Le rôle possible des anticorps sur la maladie a été recherché au moyen des tests de neutralisation *in vitro*, utilisant des sérums de souris et de bovins. Les résultats ont montré que les sérums hyperimmuns des souris DBA/2 et Balb/c étaient capables de neutraliser l'infection *in vitro*, celui des souris DBA/2 montrant l'effet neutralisant le plus fort. Deux sérums de bovins, l'un d'un animal infecté au laboratoire et l'autre provenant d'un mélange de sérums de deux animaux infectés naturellement, ont également montré un effet neutralisant.

Mots clés : Bovin - Souris - Cowdriose - *Cowdria ruminantium* - Sérum - Anticorps - Infection expérimentale - Réaction de neutralisation - Réponse immunitaire.

INTRODUCTION

Heartwater disease has never been recognized to exist in mice as a result of natural infection. However a number of *Cowdria ruminantium* field strains of varying pathogenicity for the mouse have been discovered since 1971 when the Kümm strain (5) was first isolated. Pathogenicity of *C. ruminantium* infection in mice resembles that in ruminants and may vary with :

- the strain of *C. ruminantium* used ;

- the strain of mouse used ;

- the route of inoculation (8).

Although the development of antibody to *C. ruminantium* in various species of animals has been well documented in recent years (3, 6, 10) very little is known about its role in the immunity to heartwater. Experiments with transfer of serum of gamma globulins did not confer protection and *in vivo* neutralization tests have had varying results (4, 11). Many of these experiments were done before methods of determining antibody levels had been developed so it is not known whether any antibody was in fact present in the serum used for some of these experiments. However it was assumed that the protective immunity to heartwater was probably cellular and not humoral. To clarify the role of antibody in *C. ruminantium* infection the mouse model was used to investigate the development of antibody, and immune mouse and bovine sera were used to investigate the role of antibody in an *in vitro* neutralization assay.

MATERIALS AND METHODS

Inoculation of mice and collection of sera

To determine how antibody to *C. ruminantium* develops, groups of both Balb/c and DBA/2 mice were inoculated intravenously with 100, 10 and 1 LD_{50} of previously titrated mouse organ homogenate of Crystal Springs strain (2). Representative mice from each group were bled from the retro-orbital sinus at intervals of 2-7 days from day 7 after infection.

Immune antisera

Mouse sera

Hyperimmune mouse sera were obtained by immunizing mice according to the following schedule. Three inoculations were given at 28 day intervals :

- first inoculation : 100 LD_{50} intravenous (i/v) dose given intraperitoneally (i/p) ;

- second inoculation : 500 i/v LD_{50} doses given i/p ;

- third inoculation : 100 i/v LD_{50} doses given i/v.

DBA/2 mice were inoculated with mouse organ homogenate, whereas Balb/c mice were inoculated with cell culture stabilate. Pooled serum from such mice with an IFAT titre of 1/2560 was used in the *in vitro* neutralization assays.

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Bovine sera

Laboratory infection

Because the Crystal Springs strain of *C. ruminantium* is not highly pathogenic for bovines, animal No. 95 was splenectomized. Five weeks after splenectomy this animal was inoculated with 10 ml of passage 6 cell culture material of the Crystal Springs strain. Six days later it developed a temperature reaction which lasted 10 days and reached a maximum of 41.0 °C. *C. ruminantium* was isolated in cell culture from plasma (2) taken on days 1, 2, 3 and 7 of the temperature reaction and sub-inoculation of blood to sheep on days 1, 3 and 7 resulted in death of the sheep from heartwater (confirmed by brain smears). The serum used in the *in vitro* neutralization assays was taken 4 weeks post-inoculation and had an IFAT titre of 1/1280 and a western blot titre of 1/1000.

Field infection

This serum was a pool of equal amounts of serum taken from two bovines living on the Heartwater Project's field station in the Zimbabwe lowveld (a heartwater endemic area). These animals had developed increasingly high IFAT titres to *C. ruminantium* over a two year period. The serum used in the in vitro neutralization assay had an IFAT titre of 1/2560.

All sera were sterilized by filtration before carrying out the neutralization assay.

Indirect fluorescent antibody test

This was performed by the method of SEMU *et al.* (10). Antigen slides were made from bovine endothelial cells infected with the Crystal Springs strain of *C. ruminantium*. The reaction of the mouse sera was determined by anti-mouse polyvalent immunoglobulins FITC labelled antisera (Sigma) and that of the bovine sera by goat anti-bovine IgG FITC labelled antisera (Kirkegaard and Perry, Gaithersburg, MS, USA). Sera were tested at dilutions of 1/20 to 1/10240.

Propagation of *C. ruminantium*

C. ruminantium (Crystal Springs strain) was propagated in bovine aorta endothelial cells maintained on Leibovitz's L15 medium (Gibco BRL, Grand Island, N.Y. USA) as described previously (1). The L15 medium contained 10 % tryptose phosphate broth (Difco Laboratories, Detroit, MI, USA) ; 5 % New Born Calf Serum (NBCS) (ICN Flow, Bucks, UK) ; 100 units/ml penicillin G (CAPS, Harare, Zimbabwe) ; 2.5 µg/ml amphotericin B (Fungizone, Squibb Laboratories, Transvaal, South Africa) ; 0.292 µg/ml L-glutamine (Gibco/BRL) ; and 4.5 µg/ml D-glucose (Gibco/BRL).

Cell culture stabilate

Four 900 cm^2 roller bottles (Costar, Van Nuys, CA, USA) with bovine aorta endothelial cells were infected with the Crystal Springs strain of *C. ruminantium*. When these bottles showed almost complete destruction of the monolayer (approximately ten days after inoculation), the culture supernatant was harvested into sterile centrifuge tubes and centrifuged at 30,000 g for 30 min at 4 °C. The supernate was discarded and the pellet was passed several times through a syringe with a 26 gauge needdle to break up clumps of cellular material. The pellet was then made up to 50 ml in L15 medium as above, but containing 20 % NBCS, and cryopreserved in the presence of 10 % dimethyl sulphoxide (Sigma Chemical Co, St. Louis, MO, USA).

This infection stabilate was titrated out in 25 cm^2 flasks (Costar) of bovine endothelial cells to determine the LD_{50} dose and was used at 100 LD_{50} in neutralization assays.

In vitro neutralization assay

C. ruminantium cell culture stabilate was diluted in L15 medium to give 200 LD_{50}. Equal amounts (1.5 ml) of stabilate and immune serum were mixed and incubated at 4 °C for one hour. Five 25 cm^2 flasks of bovine endothelial cells were then inoculated with 0.5 ml of this mixture. Control cell cultures were inoculated with a similarly treated mixture of stabilate and normal serum. Flasks were incubated on a rocking platform at 37 °C for two hours to adsorb the inoculum, then the inoculum was poured off and the cell monolayers washed twice with 2 ml phosphate buffered saline (PBS) pH 7.4. Five ml of fresh L15 medium was added to each flask and cultures were then incubated at 37 °C on the rocker platform for up to 35 days. Flasks were examined daily for development of cytopathic effect (CPE). Smears were made at intervals from day 11 onwards, stained with Leukostat (Fischer scientific, Orangeburg, NY, USA) and examined for per cent infection. Graphs were plotted (figures 3, 4, 5) representing the average percentage infection rate of five flasks.

RESULTS

In Zimbabwe the Crystal Springs strain was first isolated in 1988 (2). This strain was found to be pathogenic for both Balb/c and DBA/2 mice, with the i/p route requiring a larger dose of organisms than the i/v route to cause death. When infected i/v with the Crystal Springs strain of heartwater Balb/c mice died between 12-19 days post-inoculation ; DBA/2 mice were more resistant, dying at 14-24 days post-inoculation.

Development of antibody in mice

Figures 1 and 2 show that the development of antibody to *C. ruminantium* in both DBA/2 and Balb/c mice began in the second week post infection and the development of peak titre was inoculation dose dependent.

Figure 1 shows that DBA/2 mice inoculated with 100 LD_{50} dose achieved antibody titres of 1/160 to 1/1280 (average 1/618) by day 14 post-infection. An inoculation dose of 10 LD_{50} gave rise to lower antibody titres of 1/160-1/320 (average 1/187) by day 14, while 1 LD_{50} gave antibody levels of between 0-1/320 (1 mouse only) (average 1/20). It was noticed that some DBA/2 mice survived a 10 and 100 LD_{50} inoculation dose. Subsequently these mice were resistant to challenge with 100 LD_{50}. In contrast the DBA/2 mice inoculated with 1 LD_{50} failed to develop high antibody titres and were susceptible to challenge even with the same dose (1 LD_{50}), except for the one mouse which developed an antibody titre of 1/320 after the first inoculation.

Figure 2 shows that Balb/c mice inoculated with 100 LD_{50} achieved antibody levels of 1/160 to 1/640 (average 1/480) by day 18 post-infection. A dose of 10 LD_{50} gave rise to antibody levels of 1/80 to 1/320 (average (1/187) by the same day. These titres were less than those of the DBA/2 mice on day 14. All of these Balb/c mice died. Those inoculated with 1 LD_{50} failed to develop any antibody and were fully susceptible to challenge.

In vitro neutralization assays

Immune serum having a high titre in the IFAT were used to perform an *in vitro* neutralization test in 25 cm^2 flasks of bovine endothelial cells.

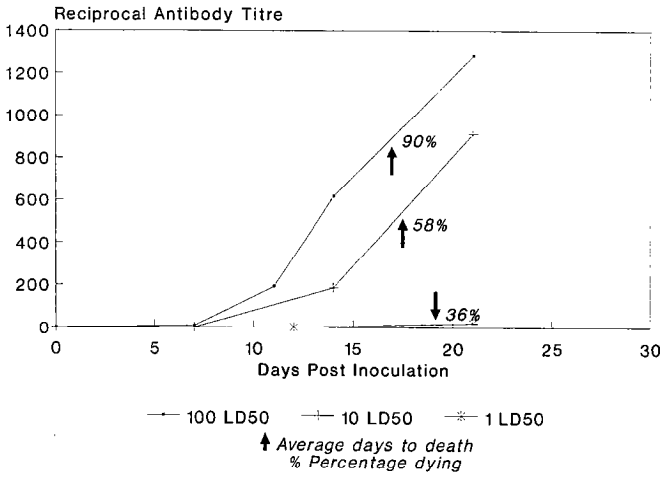

Fig. 1 : Development of antibody to C. ruminantium *in DBA/2 mice.*

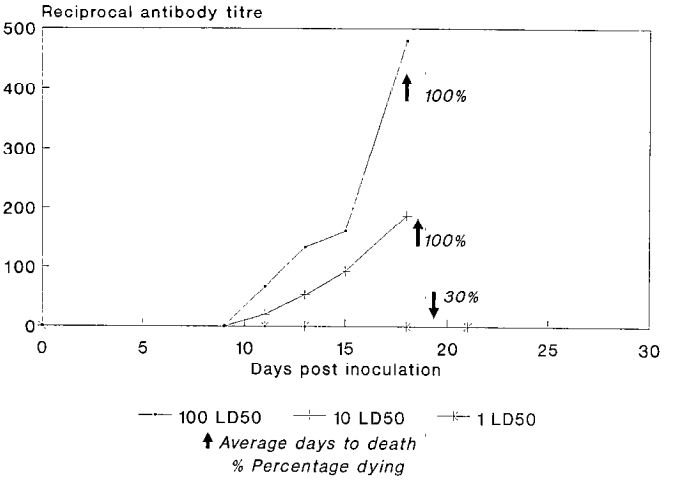

Fig. 2 : Development of antibody to C. ruminantium *in Balb/c mice.*

Figure 3 shows that pooled immune serum from DBA/2 mice had the ability to almost completely neutralize 100 LD_{50} *C. ruminantium* infection of bovine endothelial cells. In fact only one out of 5 flasks inoculated with DBA/2 immune serum/stabilate mixture became infected in this experiment compared with five out of five for the normal serum/stabilate control. This flask only began to show CPE on day 17 post-inoculation, whereas all flasks inoculated wih normal serum/stabilate mixture were showing CPE by day 10 post-inoculation. Infection rates *in vitro* on day 14 post-infection averaged 10.5 % for normal serum/stabilate mixture but only 0.2 % for immune serum/stabilate mixture.

With pooled Balb/c serum (fig. 4) all flasks inoculated with both normal serum/stabilate mixture and immune serum/stabilate mixture became infected. However there

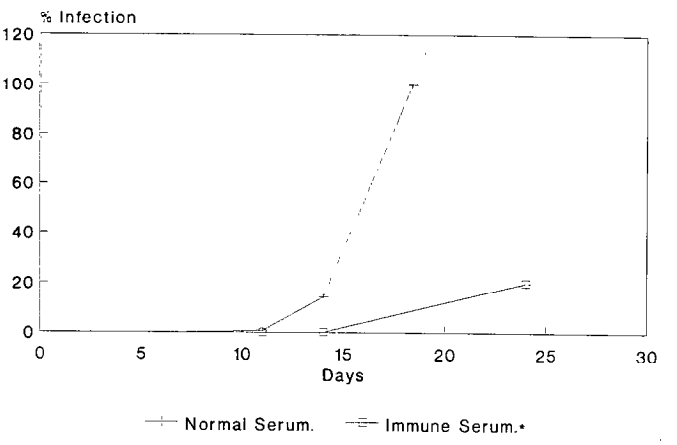

Fig. 3 : In vitro *neutralization. DBA/2 mouse serum. (* IFAT titre 1/2560)*

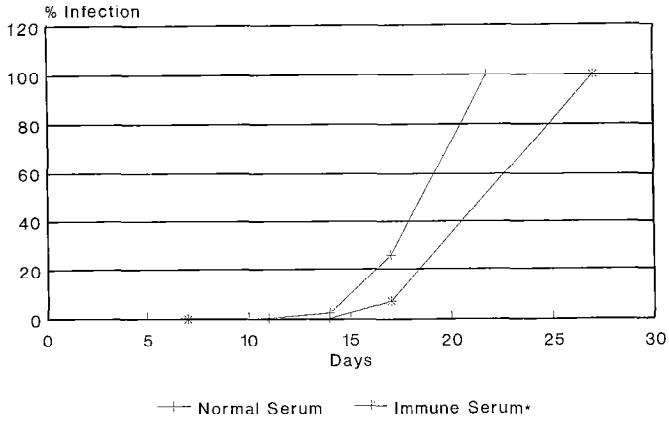

Fig. 4 : In vitro *neutralization. Balb/c mouse serum. (* IFAT titre 1/2560)*

was a difference in the infection rate, with the normal serum/stabilate controls showing a higher average infection rate compared with cultures inoculated with *C. ruminantium* inoculated with immune serum/stabilate.

Bovine sera (fig. 5) also showed a neutralizing effect. Only two out of five flasks inoculated with laboratory infected bovine serum/stabilate became infected and one out fo five flasks inoculated with field bovine serum/stabilate became infected. There was also a delay in the first appearance of CPE in those flasks inoculated with immune serum/stabilate (day 14 for normal serum, day 21 for laboratory infected bovine serum, and day 25 for field infected bovine serum). Infection rates at day 21 post-inoculation averaged 17.6 % for normal serum/stabilate, 0,5 % for laboratory infected bovine serum/stabilate, and nil for field infected bovine serum/stabilate.

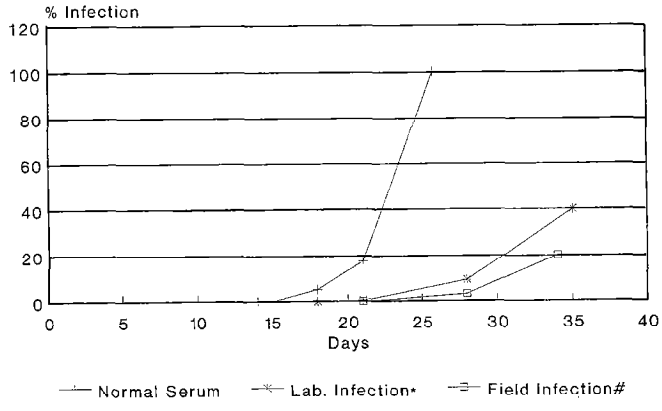

Fig. 5 : In vitro *neutralization. Bovine serum. (*IFAT titre 1/1280 ; # IFAT titre 1/2560)*

DISCUSSION

The experiments presented here have demonstrated that in mice antibody to *C. ruminantium* develops in the second week post-infection and that the antibody level is dependent upon the inoculation dose. In addition, hyperimmune serum from both DBA/2 and Balb/c mice contains antibodies which prevent either the adhesion of *C. ruminantium* to endothelial cells or the entry of *C. ruminantium* into endothelial cells *in vitro*. A similar effect was demonstrated for two bovine sera, one from a laboratory infection, one from field infection.

It is interesting to note that the strain of mouse which had greater resistance to heartwater (DBA/2) was able to produce a higher titre of antibody on initial infection (fig. 1) and also a more effective neutralization (fig. 3). Although immune serum from Balb/c mice had the same IFAT titre as immune serum from DBA/2 mice it was not nearly as efficient in neutralization. This indicates that neutralization ability of the immune sera differs in the two strains of mice and may also differ from the antibody reacting in the IFAT.

It should be also noted that serum from bovines infected in the field (and presumably constantly re-exposed to *C. ruminantium* infection) gave a greater neutralizing effect than serum from a laboratory infected bovine. However in this case there was also a corresponding difference in the IFAT.

The role of antibody in heartwater infections has been debated over a long period of time. Investigators have attempted passive transfer of antibody to susceptible animals, but were unable to confer protection either with serum or large quantities of gamma globulin from immune or hyperimmune animals, whether given simultaneously with the infection, during the incubation period or during the clinical reaction (reviewed by UILENBERG (11)). In this laboratory, experiments with DBA/2 and Balb/c mice have shown that transfer of immune serum or immune serum plus complement failed to protect either DBA/2 or Balb/c mice against an i/v challenge of *C. ruminantium* (data not shown).

Several attempts at *in vitro* neutralization have been made previously, with varying results (reviewed by UILENBERG (11)). In this laboratory we have been unable to demonstrate any neutralizing effect in DBA/2 or Balb/c mice inoculated i/v with a mixture (incubated at 4 °C for one hour prior to inoculation) of immune serum and 100 LD^{50} of either percoll purified EBs (DBA/2 mice) or cell culture stabilate (Balb/c mice) (data not shown). A recent paper by DU PLESSIS (4) reported that incubation of hyperimmune sheep sera with a tick homogenate of the Kümm strain of *C. ruminantium*, inhibited the infectivity of the homogenate for outbred Swiss mice inoculated by the i/p route, but only if the incubation was carried out in the presence of complement. In the absence of complement the immune serum/tick homogenate was fully infective.

The fact that neutralization is not obtained *in vivo* using sera which gives *in vitro* neutralization in the studies of the authors may be related to the possibility that the final antibody titre of the transferred sera is too low to cause effective neutralization or that the coating of *C. ruminantium* by immune antibody actually facilitates an establishment of infection in phagocytic cells e.g. neutrophils and macrophages. It is known that *C. ruminantium* can multiply in neutrophils *in vivo* (7) and infect the peritoneal macrophages of mice (6). *C. ruminantium* EBs incubated with immune serum and then injected into animals by either the i/v or i/p route may be inhibited from entering endothelial cells but would probably be taken up by macrophages or neutrophils by opsonization. If these opsonized *C. ruminantium* EBs were able to multiply in these cells clinical infection would result.

Failure to demonstrate any effect of antibody *in vivo* may also be due to the possibility that *C. ruminantium* infection, once established, could spread directly from cell to cell, as is suspected with some species of *Ehrlichia* (9) and *Rickettsia* (12). If this is the case organisms would escape exposure to antibody.

The results presented here indicate that neutralizing antibody may play an effective role in the protective immunity to heartwater by blocking adhesion to or invasion of endothelial cells. With the imminent development of new vaccines for heartwater, it could be important to determine which epitopes of the heartwater organisms react with neutralizing antibody and to include them in the vaccine.

ACKNOWLEDGEMENTS

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BYROM (B.), MAHAN (S.M.), BARBET (A.F.). The development of antibody to *Cowdria ruminantium* in mice and its role in heartwater disease .*Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 197-201

DBA/2 and Balb/c mice were used as a model to study the immune responses to *Cowdria ruminantium*. Both strains of mice were inoculated with 1, 10 or 100 LD_{50} of the Crystal Springs strain of *C. ruminantium*. Antibody to *C. ruminantium* started to develop in the second week post-inoculation and the titre of the antibody was dependent on the inoculation dose of *C. ruminantium*. The possible role of antibody in heartwater disease was studied by *in vitro* neutralization assays using both mouse sera and bovine sera. Results of these tests show that hyperimmune serum from both DBA/2 and Balb/c mice had the ability to neutralize infection *in vitro*, with the DBA/2 serum showing a greater neutralizing effect. Two bovine sera, one from a laboratory infected animal and one a pool from two animals infected in the field also gave a neutralizing effect.

Key words : Cattle - Mice - Heartwater - *Cowdria ruminantium* - Sera - Antibody - Experimental infection - Neutralization test - Immune response.

BYROM (B.), MAHAN (S.M.), BARBET (A.F.). Aparición de anticuerpos a *Cowdria ruminantium* en roedores y su importancia para la cowdriosis.*Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 197-201

Con el fin de estudiar la respuesta inmune a *Cowdria ruminantium*, se inocularon ratones DBA/2 y Balb/c a 1, 10 o 100 DL_{50}, de la cepa "Crystal Springs" de *C. ruminantium*. Los anticuerpos a *C. ruminantium* aparecieron dos semanas post inoculación, con títulos que variaron de acuerdo a las dosis de inoculación de *C. ruminantium*. El posible papel de los anticuerpos en la cowdriosis, se estudió mediante seroneutralización *in vitro*, tanto con los sueros de ambos tipos de ratón, como con sueros bovinos. Los resultados de estas pruebas muestran que los sueros hiperinmunes de los ratones, tanto DBA/2 como Balb/c, lograron neutralizar la infección *in vitro*, observandose un mayor efecto neutralizante de los DBA/2. El efecto neutralizante se observó también con dos sueros bovinos, uno proveniente de un animal de laboratorio infectado y el otro de un "pool" obtenido a partir de dos animales infectados bajo condiciones de campo.

Palabras claves : Bovino - Ratón - Cowdriosis - *Cowdria ruminantium* - Suero - Anticuerpo - Infección experimental - Reacción de neutralización - Repuesta inmunológica.

STVM-93

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MHC class II molecules induction after infection of bovine brain endothelial cells by *Cowdria ruminantium* *

BOURDOULOUS (S.), BENSAID (A.), MARTINEZ (D.), DURIEU-TRAUTMANN (O.), STROSBERG (A.D.), COURAUD (P.O.). Induction de molécules de classe II du CMH après infection de cellules endothéliales de cerveau de bovin par *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 203

Les molécules de classe II du complexe majeur d'histocompatibilité (CMH) jouent un rôle clé dans l'induction de l'immunité. Chez le bovin, les molécules de classe II du CMH, codées par les loci DR et DQ, sont constituées par deux chaînes protéiques, α et ß, qui sont associées à une chaîne invariante (I) durant leur transport intracellulaire. Des cellules endothéliales bovines (BBEC) de microvaisseaux cérébraux peuvent être cultivées *in vitro* et n'expriment pas de manière constitutive des molécules de classe II du MHC. Néanmoins, après traitement avec de l'interféron γ bovin (INFγ), les BBEC synthétisent des protéines de classe II du CMH. Une infection *in vitro* des BBEC par *C. ruminantium* a été effectuée sur des cellules traitées ou non par l'INFγ. L'expression des molécules de classe II a été suivie, au niveau des ARN, par analyse de "northern blot". Quarante huit heures après l'infection, des transcrits correspondant à la chaîne I ont été détectés sur toutes les cellules infectées. Deux ARNmDQα de 1,3 et 1,5 kb sont présents sur les cellules infectées et traitées à l'INFγ, alors qu'un simple transcrit DQα de 1,5 kb a été observé sur les cellules seulement infectées. D'autre part, les cellules traitées mais non infectées ont montré un simple ARNmDQα de 1,3 kb. La nature et la fonctionnalité de l'espèce moléculaire de 1,5 kb demeurent inconnues, mais elle pourrait représenter un ARNmDQα polyadénylé en un deuxième site en aval du premier site de polyadénylation. En contraste de l'expression précoce des chaînes I et DQα, les ARNmDRα n'ont été détectés que sept jours après l'infection. Il est important de noter qu'une lyse totale des BBEC infectées n'a été obtenue qu'au treizième jour de l'infection, alors que les cellules infectées et traitées par l'INFγ ont montré un effet cytopathogène plus précoce et plus marqué. Les résultats présentés ici montrent que les molécules de classe II du CMH peuvent être induites sur les BBEC après infection par *C. ruminantium*, ce qui nous mène à l'hypothèse que les cellules endothéliales de cerveau peuvent exercer une fonction immune spécifique, comme la présentation d'antigènes. Des expériences supplémentaires doivent être accomplies de manière à évaluer si oui ou non ces cellules infectées peuvent spécifiquement stimuler des cellules T.

BOURDOULOUS (S.), BENSAID (A.), MARTINEZ (D.), DURIEU-TRAUTMANN (O.), STROSBERG (A.D.), COURAUD (P.O.). MHC class II molecules induction after infection of bovine brain endothelial cells by *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 203

Major histocompatibility complex (MCH) class II molecules play a key role in inductions of immunity. In cattle, MCH class II molecules encoded by th DR and DQ loci are constituted by one α and one ß protein chain which are associated to an invariant (I) chain during their intracellular transport. Bovine brain endothelial cells (BBEC) from microvessels can be cultured *in vitro* and do not express constitutively MCH class II molecules. However, after treatment with bovine γ interferon (γ INF), BBEC synthesize MHC class II proteins. *In vitro* infection with *C. ruminantium* of BBEC treated or not by γINF was performed. Expression of class II molecules was monitored at the RNA level by northern blot analysis. Forty eight hours after infection, transcripts corresponding to the I chain were detected on all infected cells. Two DQαmRNA of 1.3 and 1.5 kb were present on INF treated infected cells whereas a single 1.5 kb DQα transcript was observed on infected cells. In the other hand, treated but non-infected cells displayed a single 1.3 kb DQαmRNA. The nature and functionality of the 1.5 kb molecular specie remains unknown but it might represent a DQαmRNA polyadenylated at a second site downstream the first site of polyadendylation. In contrast, to the early expression of I and DQα chains, the DRαmRNA were detected 7 days after infection. It is important to note that total lysis of infected BBEC was obtained 13 days after infection while γINF treated and infected cells displayed an earlier and more market cytopathogenic effect. The results presented here show that MCH class II molecules can be induced in BBEC after *C. ruminantium* infection ; leading to the hypothesis that brain endothelial cells can exert specific immune functions such as antigen presentation. Further work must be accomplished to assess whether or not these infected cells can specifically stimulate T-cells.

BOURDOULOUS (S.), BENSAID (A.), MARTINEZ (D.), DURIEU-TRAUTMANN (O.), STROSBERG (A.D.), COURAUD (P.O.). Inducción de moléculas de clase II del complejo mayor de histocompatibilidad (MHC) después de la infección de células de endotelio cerebral bovino con *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 203

Las moléculas de clase II del complejo mayor de histocompatibilidad (MHC), juegan un papel clave en la inducción de la inmunidad. En el ganado, las moléculas clase II del MHC codificadas en el loci DR y DQ, se encuentran constituídas por una cadena de proteínas α y una de ß, las cuales se encuentran asociadas en una sola (I) cadena durante el transporte intracelular. Las células de endotelio cerebral bovino (BBEC) provenientes de microcapilares, pueden cultivarse *in vitro* y sin que se observe la expresion de moléculas de clase II del MHC. Sin embargo, después de un tratamiento con interferón bovino γ (γ INF), las BBEC sintetizaron proteínas clase II del MHC. Se llevó a cabo una infección *in vitro* de las BBEC con *C. ruminantium*, con o sin tratamiento de γ YINF. A nivel del ARN, la expresión de proteínas de clase II del MHC, se siguió gracias al análisis del "northern blot". Cuarenta y ocho horas después de la infección, las transcripciones correspondientes a la cadena I se detectaron en todas las células infectadas. Se encontraron dos DQαARNm de 1,3 y 1,5 kb en las células infectadas tratadas con INF, mientras que una sola transcripción de DQα, de 1,5, se observó en las células infectadas. Por otro lado, las células tratadas, pero no infectadas, mostraron una DQαARNm de 1,3 kb. Aún no se conocen ni la naturaleza y ni la funcionalidad de la especie molecular de 1,5 kb, pero podría representar una DQαARNm poli-adenílica en un segundo sitio, bajo el primer sitio de poliadenilización. En contraste con la expresión encontrada en las cadenas I y DQα, la DRαARNm se detectó 7 días post infección. Es importante notar que la lísis total de las BBEC infectadas, se obtuvo 13 días después de la infección con *C. ruminantium*, mientras que las células infectadas y tratadas con γ INF mostraron un patrón citopatológico más temprano y marcado. Los resultados muestran que las moléculas clase II del MHC pueden ser inducidas en BBEC después de una infección con *C. ruminantium*. Esto conduce a la hipótesis de que las células endoteliales podrían ejercer funciones inmunes específicas, como la presentación de antígenos. Se recomienda llevar a cabo otros estudios, con el fin de determinar si estas células infectadas pueden o no estimular en forma específica las células T.

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Occurrence of caprine leucocyte antigens (CLA) in Creole goats susceptible/resistant to heartwater

RUFF (G.), MAILLARD (J.C.), CAMUS (E.), DEPRES (E.), MATHERON (G.). Antigènes leucocytaires caprins (ALC) chez les chèvres Créole sensibles ou résistantes à la cowdriose. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 205-207

Certaines lignées de chèvres ont montré une prédisposition génétique à manifester des symptômes cliniques de la cowdriose. Afin d'élucider une implication possible du complexe majeur d'histocompatibilité (CMH) dans la pathogénie de la cowdriose, les antigènes ALC de classe I, codés par le CMH caprin, de plus de 100 chèvres Créole, ont été typés sérologiquement. Le CMH est un système génétique qui influence des processus immunologiques différents, c'est-à-dire sur la réponse immunitaire individuelle. A l'aide de nos allosérums ont été détectés 13 allèles de ALC différents qui se retrouvent également dans les races suisses, deux antigènes non-CMH et un nouveau groupe défini par la combinaison de deux antigènes. Les fréquences alléliques des antigènes ALC détectés étaient différentes entre les groupes résistant et sensible. Il reste à éclaircir si les différences représentent des effets régionaux de population, ou si elles indiquent une pression de sélection par l'agent pathogène. Des tests additionnels sont en cours sur des chèvres résistantes ou sensibles originaires d'un même environnement, ainsi que sur des animaux issus de croisements entre caprins résistants et sensibles.

Mots clés : Caprin - Chèvre Créole - Cowdriose - Antigène - Complexe majeur d'histocompatibilité - Allèle - Sérologie - Immunité.

INTRODUCTION

A genetic predisposition to the manifestation of heartwater disease (cowdriosis) in Creole goats could be demonstrated (6). The caprine major histocompatibility complex (MHC), representing a genetic marker system of individual immunocompetence, was studied regarding its involvement in the pathogenesis of heartwater disease.

The MHC codes for its gene products, the so-called caprine leucocyte (CLA) antigens. They can be differentiated into various classes according to their structure and function. In the goat, class I antigens are expressed on all nucleated cells, class II antigens are primarily expressed on B-cells and on activated T-cells.

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The caprine MHC has been characterized by several techniques. The earliest reports described serological techniques (9, 10, 14), later biochemical methods were applied (3, 4) and in the most recent studies molecular genetic analysis was carried out (12, 13). Up to the present, a total of 27 class I antigens belonging to one locus and one class I antigen belonging to another locus could be detected by serological means. Eighteen of these gene products could also be confirmed using biochemical detection, whereas one antigen could be split. A total of seven class II antigens could be characterized by the serological and biochemical techniques. The application of DNA analysis resulted in 22 different nucleic sequences of the class II (DRB) type. Whether they all code for different gene products remains to be clarified. Three non-MHC antigens have also been described (9).

The authors applied serological characterization of MHC class I products in the present study. Using this technique a high degree of polymorphism can be rapidly detected causing relatively little expenses. Furthermore, class I and class II antigens are closely linked and are thus inherited on the same chromosome. In this way it is possible to conduct linkage studies within family material using the class I antigens as markers in various projects.

MATERIAL AND METHODS

A total of 112 Creole goats belonging to CIRAD-EMVT and INRA have been serologically typed for their CLA class I antigens. Thirty-nine goats were classified as resistant type after challenge and 73 others, including animals native from the Les Saintes archipelago, were presumed to be of the susceptible type before being challenged to heartwater (6).

CLA alloantiserum production, cell isolation and CLA-typing procedures have been carried out as described in detail elsewhere (1, 11).

For the statistical evaluation typing results have been computed. Gene frequencies and relative risks have been calculated using Chi-square analysis with Yate's correction where necessary (5, 7).

RESULTS AND CONCLUSIONS

Of the already known caprine class I antigens, 13 could be detected in the Creole goats. In addition, a new combination of two reagents could be found, characterizing a local breed-specific antigen (Gu1). Furthermore, two of the three non-MHC antigens were present in the Creole goats.

The gene frequency of all the determined CLA class I antigens was estimated for the presumed susceptible and the resistant group. For the antigens with a statistically different gene frequency for the two groups, the relative risk and the probability value was calculated (table I). Four antigens showing significantly different P-values ($P < 0.05$) could be detected. The non-MHC antigen CLY 1.1 (9) and the CLA antigen Be13 occurred in an increased frequency in the resistant group. The CLA antigens Be4 and Be9 were more frequent in the susceptible group.

These differencies might represent pathogen-induced selection pressure, but they might also be due to frequency differencies found at the population level. In order to clarify this hypothesis, further investigations are necessary. Genetic comparison between these two populations of different geographical origin, using other genetic marker systems is presently under investigation (PEPIN, thesis in preparation). Furthermore a crossbreeding programme has been initiated in order to study the inheritance of the different CLA class I marker antigens in the offspring of resistant/susceptible parents. A final number of over 200 offspring is expected and will be challenged with heartwater before May 1994. CLA-typing and challenge testing of these animals will enable a better understanding of the underlying disease mechanisms and the genes linked to susceptibility/resistance in the pathogenesis of heartwater disease.

If the serologically detected MHC antigens will not provide sufficient information about disease association, more refined techniques for analysis of MHC or other genetic marker systems have to be considered. Today, an increasing number of molecular genetic techniques such as RFLP, PCR, sequencing, oligotyping (12) and various DNA probes including microsatellites (8) have already been developed for small ruminants. These sophisticated methods represent powerful tools in association studies with various traits in sheep and goats.

ACKNOWLEDGMENT

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***TABLE I** Gene frequencies, relative risk and P-values of CLA antigens in Creole goats, resistant (R) or susceptible (S) to heartwater (cowdriosis).*

	Specificity	Sera	Gene frequencies			Probabilities		
			R N = 39	S N = 73	χ^2	P (11.d.)	R.R.	Sign.
R	CLY panleuc.	94 N	0.168	0.028	13.28	< 0.001	0.13	+++
	Be 13	154 N	0.108	0.028	4.54	< 0.035	0.22	++
	Be 1	332 K	0.108	0.042	3.51	< 0.065	0.35	+
S	Be 4	Boby	0.08	0.250	9.18	< 0.01	4.29	+++
	Be 9	Kaffea	0	0.078	4.93	< 0.03	–	++
	Be 20-D3	Venus	0.013	0.086	3.51	< 0.065	7.47	+
	Be 22	1 N	0.013	0.086	3.51	< 0.065	–	+

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RUFF (G.), MAILLARD (J.C.), CAMUS (E.), DEPRES (E.), MATHERON (G.). Occurrence of caprine leucocyte antigens (CLA) in Creole goats susceptible/resistant to heartwater. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 205-207

A genetic predisposition to the manifestation of disease symptoms has been demonstrated for heartwater in certain goat lines. In order to clarify a possible involvement of the major histocompatibility complex (MHC) in the pathogenesis of heartwater, over 100 Creole goats have been typed serologically for their CLA class I antigens coded by the caprine MHC. The MHC is a genetic system that influences different immunological processes, i.e. the individual immune response. With our alloantisera we were able to detect 13 different CLA alleles also present in the Swiss breeds, two non-MHC antigens, a new cluster defined by the splitting of two antigens and several specific reaction patterns of single reagents. The allele frequencies of the detected CLA antigens differed for the resistant and susceptible groups examined. Whether the differences represent regional sire effects or indicate pathogen-induced selection pressure remains to be clarified. Additional testing of resistant/susceptible goats originating from common environment as well as of specially crossbred (resistant x susceptible) animals are underway.

Key words : Goat - Creole goat - Heartwater - Antigen - Major histocompatibility complex - Allele - Serology - Protection.

RUFF (G.), MAILLARD (J.C.), CAMUS (E.), DEPRES (E.), MATHERON (G.). Aparición de antígenos leucocitarios caprinos (CLA) en cabras Criolla susceptibles o resistentes a la cowdriosis. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 205-207

En ciertas líneas caprinas se ha demostrado la predisposición genética a la manifestación de los síntomas de la cowdriosis. Con el fin de aclarar la posible acción del complejo mayor de histocompatibilidad (MHC) en la patogénesis de la cowdriosis, se tipificaron serológicamente alrededor de 100 cabras Criolla para el antígeno de CLA clase I, codificado con el MHC caprino. El MHC es un sistema genético que influye sobre procesos inmunológicos diferentes, como por ejemplo la respuesta inmune individual. Gracias a nuestros anti-sueros de misma especie, se pudieron identificar 13 alelos diferentes de CLA, también presentes en razas suizas, dos antígenos no MHC, un nuevo grupo definido mediante la separacíon de dos antígenos y varios patrones específicos de reacción de agentes individuales. Las frecuencias alélicas de la detección de antígenos de CLA difieren para los grupos resistentes y susceptibles examinados. No es claro si las diferencias representan efectos reguladores regionales o indican una presión selectiva patógeno-inducida. Se llevaron a cabo otros exámenes de caprinos resistentes o susceptibles provenientes de un medio ambiente común, así como de una raza producto de un cruce específico (resistente x susceptible).

Palabras claves : Caprino - Cabra Criolla - Cowdriosis - Antigeno - Complejo mayor de histocompatibilidad - Alelo - Serología - Inmunidad.

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Use of microsatellites as genomic markers to study resistance to cowdriosis *

PEPIN (L.), CAMUS (E.), MATHERON (G.), BENSAID (A.). Utilisation de microsatellites comme marqueurs génomiques pour l'étude de la résistance à la cowdriose. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 209

Les chèvres Créole de Guadeloupe sont considérées comme appartenant à une même race. Néanmoins, selon leur origine géographique, elles peuvent être résistantes ou sensibles à la cowdriose. Une étude antérieure a montré que la résistance est probablement sous contrôle génétique. Le but de l'étude actuelle est de fournir les outils de base pour trouver des marqueurs génomiques de chèvre ayant une corrélation avec la résistance à la cowdriose. Afin d'accomplir cette tâche il faut détecter des régions très polymorphiques distribuées de façon égale sur le génome pour servir de repères. Ainsi, des portions du génome impliquées dans la détermination d'un caractère donné peuvent être suivies dans des populations. De tels repères existent, ce sont les microsatellites; ils ont déjà été utilisés avec succès pour cartographier certains traits de la souris et de l'espèce humaine. Des séquences microsatellites sont composées de répétitions de di- ou trinucléotides, le polymorphisme étant basé sur le fait que le nombre de répétitions peut varier entre individus. Si elles sont flanquées par des séquences non-répétitives, elles peuvent être détectées facilement par la technique de réaction en chaîne de polymérase (RCP). Des amorces délimitant cinq microsatellites, caractérisés auparavant dans le génome bovin, ont été appliquées avec succès au génome caprin. De l'ADN de 70 chèvres, dont 30 chèvres Créole, 10 Sahélienne, 10 Guinéenne, 10 de race Saanen et 10 de race Alpine, a été préparé et soumis à la RCP. Du polymorphisme a été détecté dans les cinq satellites et 3, 4, 8, 14 et 15 allèles ont été démontrés respectivement pour chaque microsatellite. D'autres microsatellites ont été trouvés utiles et seront soumis à des tests plus approfondis. En même temps, des familles de chèvres Créole sont constituées par croisements d'animaux résistants et sensibles. La technologie décrite ici sera appliquée à l'ADN de chèvres de la génération F1 pour déterminer si la ségrégation d'une région polymorphe donnée est liée au caractère de la résistance à la cowdriose.

PEPIN (L.), CAMUS (E.), MATHERON (G.), BENSAID (A.). Use of microsatellites as genomic markers to study resistance to cowdriosis. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 209

Creole goats from Guadeloupe are considered to belong to a same breed. However, following their geographical origin, they can be resistant or susceptible to cowdriosis. A previous study showed that resistance to cowdriosis is most likely under genetic control. The aim of the present study is to provide the basic tools in order to find goat genomic markers correlating with resistance to cowdriosis. What is needed to accomplish such a task is to detect highly polymorphic regions evenly distributed through the genome which will serve as landmarks. Thus, portions of the genome involved in determining a given character can be followed in populations. Such useful landmarks exist and are called microsatellites; they have already been used with success to map particular traits in the mouse and human species. Microsatellite sequences are composed of di- or trinucleotide repeats, the polymorphism is based on the fact that the number of repeats can vary between individuals. If flanked by non-repetitive sequences, they can be easily detected by the polymerase chain reaction (PCR) techniques. Primers surrounding five microsatellites, which were previously characterized in the bovine genome, were successfully applied on the goat genome. DNA from 70 goats of the Creole (30), Sahelian (10), Guinean (10), Saanen (10) and Alpina (10) breeds was prepared and subjected to PCR. Polymorphism was detected in all five satellites and 3, 4, 8, 14 and 15 alleles were revealed for each microsatellite, respectively. More microsatellites have been found to be useful and will be tested further. Concurrently, Creole goat families involving the crossing of resistant and susceptible animals are being constituted. The technology described here will be applied on DNA of goats of the F1 generation to assess whether a given polymorphic genetic region segregates with the character of resistance to cowdriosis.

PEPIN (L.), CAMUS (E.), MATHERON (G.), BENSAID (A.). Uso de microsatélites como marcadores genotípicos para el estudio de la resistencia a la cowdriosis. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 209

Actualmente se considera que todas las cabras "Creole" de Guadalupe pertenecen a la misma raza. Sin embargo, si se sigue el origen geográfico, éstas pueden ser resistentes o susceptibles a la cowdriosis. Un estudio anterior mostró que la resistencia a la cowdriosis se debe principalmente a factores genéticos. La finalidad del presente estudio es la de proveer las herramientas básicas para identificar los marcadores genómicos relacinados con la cowdriosis. Para alcanzar dicha meta, deben detectarse las regiones altamente polimórficas que se encuentren distribuídas homogéneamente en el genoma, las cuáles servirán de guía. Se sabe que partes del genoma, involucradas en la determinación de un determinado caracter, pueden ser seguidas en las poblaciones. Las guías mencionadas existen y se conocen como microsatélites. Estos microsatélites se han utilizado con éxito en el seguimiento de caracteres particulares en ratones y humanos. Las secuencias de microsatélites se componen de repeticiones de di o trinucleótidos. El polimorfismo se basa en el hecho de que cantidad de repeticiones puede variar entre los individuos. Si éstas se acompañan de secuencias no repetitivas, pueden ser detectadas facilmente mediante técnicas de reacciones en cadena de polimerasas (PCR). Los "primers" que rodean a cinco microsatélites, caracterizados previamente en el genoma bovino, han sido aplicados exitosamente en el genoma caprino. Se preparó y se sometió a PCR el ADN de 70 cabras, de razas Creole (30), Sahelina (10), Guineana (10), Saanen (10) y Alpina (10). Se detectaron polimorfismos en los cinco satélites y los alelos 3,4,8,14 y 15 se revelaron en cada microsatélite, respectivamente. Se han encontrado más microsatélites útiles, los cuáles serán examinados posteriormente. Paralelamente, se han constituído familias de cabras Creole en relación con los cruces de animales resistentes y susceptibles. La tecnología descrita se aplica al ADN caprino de la F1, con el fin de asegurar que una determinada region genética polimórfica segregue con el caracter de resistencia a la cowdriosis.

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* *Seuls les résumés de cette communication sont publiés dans ce volume.*

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Use of serological response to evaluate heartwater immunization of cattle

LAWRENCE (J.A.), WHITELAND (A.P.), MALIKA (J.), KAFUWA (P.), JONGEJAN (F.). Utilisation de la réponse sérologique pour évaluer l'immunisation de bovins contre la cowdriose. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 211-215

Du vaccin congelé à base de sang contenant la souche Ball 3 de *Cowdria ruminantium* est produit par un projet FAO/DANIDA au Malawi pour l'immunisation de bovins laitiers améliorés contre la cowdriose. L'immunogénicité des lots de vaccin a été quantifiée et des régimes différents d'immunisation ont été évalués par l'utilisation d'immunofluorescence indirecte pour déterminer les taux d'anticorps générés. Des cellules endothéliales infectées, en culture, ont été utilisées comme antigène. La proportion d'animaux montrant une réponse sérologique variait entre différents lots de vaccin testés en même temps dans des groupes homogènes de bovins, ce qui reflète probablement des différences en immunogénicité entre les lots. La proportion d'animaux dans une population homogène donnant une réponse sérologique au même lot de vaccin administré sous des régimes différents variait également. Le test d'immuno-fluorescence indirecte s'est montré être une méthode utile pour évaluer la réponse immunitaire de bovins à l'immunisation et a été adopté comme procédure de routine pour contrôler la qualité du vaccin contre la cowdriose produit au Malawi.

Mots clés : Bovin - Cowdriose - *Cowdria ruminantium* - Sérologie - Vaccin - Immunofluorescence indirecte - Réponse immunitaire - Anticorps - Antigène - Malawi.

INTRODUCTION

Heartwater (*Cowdria ruminantium* infection) was first recognized as an important disease of domestic ruminants in South Africa during the 19th century (18). Its prevention depended entirely on control of the tick vectors, *Amblyomma* spp. until the 1940s, when NEITZ and ALEXANDER (16, 17) developed a method of immunization involving the inoculation of infected sheep blood and treatment of the ensuing disease process, where necessary, with sulphonamides or, more recently, tetracyclines. The methods of production and quality control of a frozen blood vaccine are described by BEZUIDENHOUT (1).

The stock of *C. ruminantium* currently used for immunization in Southern Africa is Ball 3.

Heartwater vaccine stimulates immunity by establishing an active infection. Confirmation that active infection has occurred is conventionally provided by the development of a febrile reaction, and it has been suggested that the strength of the immunity is directly proportional to the severity of the reaction (16). HAIG (10) states that a small percentage of animals (2-5 %) may fail to react after vaccination but proves to be susceptible on subsequent inoculation. However, our experience in Malawi is that the proportion of non-reactors is much higher. Of 673 cross-bred cattle aged six months to three years old, reared with intensive tick control on six farms and vaccinated in 12 groups against heartwater with a single batch of vaccine, only 266 (39.5 %) showed febrile reactions, with rectal temperatures in excess of 39.5°C, when the temperatures were monitored daily for varying periods between Day 7 and Day 26 post-vaccination. The incidence of reactions varied between groups from eight to 67 %. Possible causes for low reaction rates are specific immunity resulting from previous infection, non-specific resistance (7) and genetic resistance (5). Of more concern, however, are the additional possibilities of loss of viability of the vaccine during preparation, storage, transport or thawing, or failure to administer it correctly by the intravenous route (2).

DU PLESSIS and MALAN (6) have described the use of an immunofluorescent antibody (IFA) test, utilizing peritoneal macrophages of mice infected with the Kümm strain of *C. ruminantium* as antigen, in the evaluation of immunization in cattle. Significant differences were recorded between groups of animals in both clinical and serological reactions. This paper describes the use of infected endothelial cell culture (3) as antigen for the same purpose, as it has been found to be easier to prepare and use and to give more specific results (15).

MATERIALS AND METHODS

Experimental design

The study was conducted in four parts. In the preliminary part, experimental high-grade Friesian or Holstein calves, 17 aged 3-5 weeks and 19 aged 6-9 months, were inocu-

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lated intravenously with 5 ml frozen blood vaccine, based on the Ball 3 strain of *C. ruminantium*, purchased from the Veterinary Research Institute, Onderstepoort, Republic of South Africa. Serum was collected on Day 0 and Day 43 for antibody estimation at dilutions from 1:30 to 1:810, and animals were monitored daily from Day 7 to Day 25 for febrile reactions and were treated with tetracyclines where necessary.

In the second part, Friesian, Simmenthal and Brahman crossbred animals aged 1-3 years on two farms, Kabumbu and Kasikidzi, were inoculated intravenously in groups of approximately 20 with 2 ml of one of four frozen blood vaccines. These were the reference vaccine used in the preliminary study and three vaccines produced in Merino sheep in Malawi with the Ball 3 strain from the reference vaccine, using the technique described by BEZUIDENHOUT (1). The viability of each vaccine had been confirmed previously by inoculation into Merino sheep and demonstration of *Cowdria* colonies in brain biopsy material taken at the height of the febrile reaction (20). Serum was collected from the test cattle on Day 0 and Day 42 for antibody estimation at a dilution of 1:30. Animals were monitored daily for febrile reactions, and treated where necessary, from Day 11 to Day 21.

In the third part, two other batches of vaccine, HW004 and HW005, produced in Malawi were titrated in cattle to determine the ID_{50}. Dimethylsulphoxide (DMSO) was omitted from the blood diluent in the preparation of batch HW005. Friesian crossbred cattle aged between six months and three years were inoculated intravenously in groups of 6-10 with 5 ml of vaccine, undiluted or diluted in successive three-fold steps to 1/27 with citrate-lactose-peptone buffer (1). Animals were monitored daily from Day 10 to Day 22 for febrile reactions and treated with long-acting tetracycline if the temperature rose above 39.5 °C. Serum was collected on Day 0 and Day 42 or Day 51 for antibody estimation at 1:30.

Finally, vaccine batch HW004 was titrated in a similar manner in cattle under different regimens of administration. Two titrations were carried out in which a doxycycline implant ("Doximplant B" - George Schwulst Labs. Ltd, Republic of South Africa) was administered subcutaneously at the base of the ear at a dose of 5-8 mg/kg at the same time as the vaccine. Weight was estimated by a weigh band. A third titration was carried out with the vaccine administered by deep intramuscular injection in the neck. In each case a positive control group was vaccinated with undiluted vaccine intravenously without other treatment. The ID_{50} for each vaccine titrated was calculated by the method of REED and MUENCH (20).

Serological testing

Antibody responses were determined by the IFA test using bovine endothelial cell cultures infected with the Senegal stock of *C. ruminantium* (13) as antigen. Bovine endothelial cell cultures were established in The Netherlands from umbilical cord arteries as described previously (12). They were harvested when heavily infected with intracellular colonies of reticulate bodies and large numbers of extracellular elementary bodies of *Cowdria*. In the preliminary study, the material was collected, centrifuged at 10,000 g, resuspended in phosphate buffered saline (PBS), spotted onto microscope slides, fixed in acetone and sent to Malawi. For subsequent studies, endothelial cell cultures heavily infected with *Cowdria* (Senegal stock) were harvested, centrifuged at 10,000 g, resuspended in phosphate-sucrose-glutamate buffer (SPG) (4), frozen at -80 °C and sent to Malawi. The antigen was thawed at 40 °C and 7 µl was applied to a glass slide in a pattern of spots 3-4 mm in diameter using a micropipette. The surplus fluid from each circle was taken up so that only a thin deposit of cells remained (9). Slides were air dried and then fixed in acetone for 15 min. After drying, they were wrapped in tissue paper and cling film and stored at -70 °C.

To carry out the test, antigen slides were thawed at 55 °C before unwrapping. The slides were immersed in glycine buffer pH 2.8 (14) for 10 min. The buffer was prepared from 500 ml 0.2 M glycine in 1.6 % sodium chloride which was titrated with 0.2 M hydrochloric acid in distilled water to pH 2.8 and the volume made up to 1,000 ml (11). Slides were washed twice for 10 min in PBS at pH 7.4 to restore the pH value.

Threefold dilutions of serum from 1:30 to 1:810 were prepared in PBS in microtitre plates and placed on the antigen spots on the slides using 4 mm diameter Whatman No. 3 filter discs dipped in the wells and pressed lightly directly onto the spots. Positive and negative control sera were tested on each slide. Serum obtained from Friesian calf No. 456, experimentally infected at Utrecht with the Senegal strain of *Cowdria*, was used as a positive control serum at 1:200. Negative control serum was obtained from an experimental calf (No. 28) born and raised in The Netherlands and never exposed to *Cowdria*. Slides were incubated at 37 °C in a humidity box for 30 min, after which time the discs were washed off with running PBS and the slides immersed in PBS for 10 min. Following a brief rinse in distilled water and shaking dry, a filter disc containing about 20 µl of a 1:100 or 1:160 dilution of FITC conjugated rabbit anti-bovine immunoglobulin (Miles Scientific USA) was placed on each spot. The conjugate contained a final concentration of 0.2 % Evans blue to reduce background fluorescence (14). After re-incubation for 30 min, the filter discs were washed off as before, and the slides were mounted with 50 % glycerine in PBS under a 22 x 50 mm coverslip. Examinations were carried out using a Leitz incident light fluorescence microscope under a 40 x objective.

RESULTS

Immunofluorescence

With positive sera, *Cowdria* organisms were seen mostly scattered singly or in small colonies throughout the field, although some organisms could be recognized in the cytoplasm of disrupted endothelial cells. They appeared as either solid or ring shaped, fluorescing bodies. With negative sera, the organisms were only faintly visible and did not fluoresce.

Preliminary study

Following inoculation, 11/17 3-5 week old calves developed rectal temperatures in excess of 39.5 °C. In three animals, the reaction persisted for three days and tetracycline treatment was initiated. All animals recovered. Amongst the 6-9 month old group, 12/19 reacted and three died, one on the second day of reaction and the other two at a later stage, despite initiation of tetracycline treatment on the second day of fever. Heartwater was confirmed at post-mortem examination in each case. Three other reacting animals were treated from the second day of fever and recovered.

Paired sera from 21 animals were tested for antibodies by IFA (table I). Two calves less than five weeks old showed titres of 270 on Day 0, possibly representing maternal antibodies. Both underwent febrile reactions following inoculation. The remaining 19 animals were seronegative on Day 0 and had all developed detectable antibodies by Day 42, despite the fact that 10 had shown no febrile reaction.

TABLE I Antibody responses to **Cowdria ruminantium** *after immunization of experimental calves.*

Titre	Sera positive on Day 0	Sera positive on Day 42
<30	19	0
30	0	1
90	0	9
270	2	10
810	0	1
	21	21

Comparison of vaccines

In the second part of the study, monitoring and treatment of febrile reactions was complicated by a number of factors. On Kabumbu Farm, the process of taking temperatures on the first day, Day 11, was very prolonged, and the high ambient temperatures resulted in 50/84 animals having temperatures above 39.5 °C; of these, 46 were treated. On Kasikidzi Farm, temperature monitoring proceeded as planned, but, as a result of a misunderstanding, animals were treated whenever the rectal temperature reached 39.0 °C. Comparison of the serological responses of treated and untreated animals revealed no evidence that treatment had affected antibody production on either farm.

Twenty-six animals were found to have antibodies to *C. ruminantium* on Day 0 and/or to have a history of previous immunization and were discarded from the trial. Paired sera from the remainder were tested by IFA at a dilution of 1:30 (table II). There were significant differences ($P < 0.01$, chi-squared test) on both farms between vaccine batches in the proportion of animals which seroconverted. Batches HW002 (Malawi) and RSA (reference vaccine) were not significantly different, with antibodies developing by Day 42 in 72 to 90 %.

TABLE II Comparative serological responses in calves on two farms to four **Cowdria ruminantium** *vaccines.*

Vaccine	Seroconversion*	
	Kabumbu	Kasikidzi
HW001	4/12 (33 %)[a]	7/20 (35 %)[a]
HW002	11/14 (79 %)[b]	18/20 (90 %)[b]
HW003	3/16 (19 %)[a]	5/21 (24 %)[a]
RSA**	13/18 (72 %)[b]	16/19 (84 %)[b]

** Number with Day 42 titre of 30/number with Day 0 titre of < 30.*
*** Reference vaccine.*
[a] [b] No significant difference between groups with same superscript (chi-squared, $P > 0.05$).

Vaccine titrations

The ID_{50} of vaccine batch HW004 was calculated as 5 ml x $3^{-2.44}$ (table III). Only eleven of the 29 animals which seroconverted experienced febrile reactions. In this titration 1/9 controls seroconverted, possibly as a result of misidentification of animals or serum samples or of naturally acquired infection with *C. ruminantium* or another immunologically related organism. The working dose of the vaccine was set at 2.5 ml, giving a mean concentration of 7.3 ID_{50} per dose, with a 99 % probability from the Poisson distribution that each dose contained at least two immunizing units. Batch HW005, which did not contain DMSO, was very poorly immunogenic.

The immunogenicity of batch HW004 vaccine was markedly reduced when it was administered with the doxycycline implant, and almost abolished when it was administered intramuscularly (table IV).

TABLE III Antibody response of calves to serial dilutions of **Cowdria ruminantium** *vaccines.*

Vaccine dilution	Seroconversion	
	HW004*	HW005
1/1	10/10	1/8
1/3	7/9	0/7
1/9	8/10	0/7
1/27	3/10	0/6
Control	1/9	0/4

* ID_{50} 5 ml x $3^{-2.44}$.

TABLE IV Comparative serological responses of calves to serial dilutions of **Cowdria ruminantium** *vaccine (HW004) administered under different regimes.*

Vaccine dilution	Seroconversion		
	Intravenous + doxycycline Expt. 027	Expt. 031	Intramuscular Expt. 040
Positive control*	9/10[a]	5/8[a]	5/5[a]
1/1	3/8	5/10	1/7
1/3	2/9	6/10	0/6
1/9	6/10	3/9	0/5
1/27	5/9	0/8	0/5
Negative control	0/9	0/8	0/6
ID_{50} = 5 ml x	$3^{-1.18(?)}$	$3^{-1.00}$	> 1

[a] *No significant difference between groups with same superscript (chi-squared, P > 0.05).*
* *Vaccine 1/1, intravenous, without doxycycline.*

DISCUSSION

The IFA test proved to be a more consistent and sensitive indicator of establishment of an immune response to *C. ruminantium* following administration of frozen blood vaccine than the monitoring of febrile reactions. In the preliminary study, 10/19 animals which seroconverted had no febrile reaction, while in the titration of vaccine batch HW004, 18/29 failed to react.

A high level of cross-reactivity was found between *Cowdria* antigens of the Senegal stock and antibodies raised against the Ball 3 isolate, confirming other observations that differences in serotype do not appear to have an important effect in heartwater serology using immunofluorescence with endothelial cell culture antigen (15; JONGEJAN, unpublished results). Antigen slides prepared from a cell culture suspension stored at -80 °C in SPG buffer proved to be more satisfactory than those made directly from fresh cultures; there was much less background fluorescence, and they required less culture material to prepare.

The IFA test demonstrated marked differences in immunogenicity between different batches of vaccine and between different regimens of administration of the same batch of vaccine. It is clearly a useful technique for the evaluation of immunization of cattle. The development of antibodies does not necessarily confirm the development of protective immunity, but it does demonstrate the stimulation of an immune response and thus provides some measure of the immunogenicity of the vaccine, which otherwise could only be assessed by expensive and time-consuming vaccine challenge procedures. The technique has been adopted as a routine quality control procedure for vaccine production in Malawi.

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LAWRENCE (J.A.), WHITELAND (A.P.), MALIKA (J.), KAFUWA (P.), JONGEJAN (F.). Use of serological response to evaluate heartwater immunization of cattle. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2): 211-215

Frozen blood vaccine containing the Ball 3 strain of *Cowdria ruminantium* is prepared by an FAO/DANIDA Project in Malawi for the immunization of improved dairy cattle against heartwater. The immunogenicity of vaccine batches for cattle has been quantified and different regimens for immunization have been evaluated using indirect immunofluorescence to assess antibody responses. Infected endothelial cells grown in culture are used as antigen. The proportion of animals responding serologically has varied between different batches of vaccine tested in homogeneous cattle populations at the same time, presumably reflecting differences in immunogenicity of batches. The proportion of animals in a homogeneous population responding serologically to the same vaccine batch administered under different regimens has also varied. Indirect immunofluorescence testing has proved to be a useful method for assessing the immune response of cattle to immunization and has been adopted as a routine quality control procedure for heartwater vaccine production in Malawi.

Key words : Cattle - Heartwater - *Cowdria ruminantium* - Serology - Vaccine - Indirect immunofluorescence - Immune response - Antibody - Antigen - Malawi.

LAWRENCE (J.A.), WHITELAND (A.P.), MALIKA (J.), KAFUWA (P.), JONGEJAN (F.). Uso de la respuesta serológica para la evaluación de la inmunización contra la cowdriosis en bovinos. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2): 211-215

Con el fin de mejorar la inmunización del ganado contra la cowdriosis, el proyecto FAO/DANIDA en Malawi produce actualmente una vacuna con sangre congelada, a partir de la cepa Ball 3 de *Cowdria ruminantium*. Para verificar las respuestas de los anticuerpos, se cuantificó la inmunogenocidad de los grupos de vacunas en el ganado, al tiempo que se evaluaron los diferentes regímenes de inmunización, mediante la inmunofluorescencia indirecta. La proporción de animales que respondieron serológicamente varió entre los grupos de vacunas examinadas conjuntamente en poblaciones homogéneas de ganado, lo cual refleja posibles diferencias inmunogénicas entre estos grupos. También se observó una variación en la proporción de animales en una población homogénea que presentaron una respuesta serológica al mismo grupo vaccinal, administrado bajo diferentes regímenes. La inmunofluorescencia indirecta demostró ser un método útil para la verificación de la respuesta inmune en el ganado y se ha adoptado como un proceso rutinario de control de calidad para la producción de vacunas contra la cowdriosis en Malawi.

Palabras claves : Bovinos - Cowdriosis - *Cowdria ruminantium* - Serología - Vacuna - Inmunofluorescencia indirecta - Respuesta inmunológica - Anticuerpo - Antígeno - Malawi.

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Cowdriosis in Senegal : some epidemiological aspects

GUEYE (A.), MBENGUE (Mb.), DIEYE (Th.), DIOUF (A.), SEYE (M.), SEYE (M.H.). La cowdriose au Sénégal : quelques aspects épidémiologiques. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 217-221

Les résultats d'une étude sur l'abondance d'*Amblyomma variegatum* dans des zones écologiques différentes et des taux d'infection par *Cowdria ruminantium* dans les nymphes et les adultes de la tique de la zone nord-guinéenne sont présentés. En même temps la séroprévalence a été déterminée. Selon cette étude, le vecteur est le plus fréquent dans la zone nord-guinéenne, suivie par la zone sud-soudanienne et la zone côtière des Niayes. Ailleurs, les populations de la tique sont absentes ou peu importantes. Le taux d'infection dans la zone nord-guinéenne est élevé : 1,1 p. 100 au moins chez les tiques adultes et 7,8 p. 100 chez les nymphes. La séroprévalence dans les zones nord-soudanienne et soudano-sahélienne est très basse à l'intérieur, tandis qu'elle est plus élevée proche de la côte.

Mots-clés : Cowdriose - *Cowdria ruminantium* - Tique - *Amblyomma variegatum* - Épidémiologie - Sérum - Prévalence - Antigène - Immunofluorescence indirecte - Sénégal.

INTRODUCTION

Since the 1970s, the introduction of exotic dairy cattle of high productivity in the Niayes ecological zone has emphasized the importance of cowdriosis as the major pathological constraint to the development of animal production (3). Further findings on the mortality of native goats occurring periodically confirm also the role of this disease (4).

The lack of reliable information on the epidemiology together with the control of this infection as a major objective, justify the ongoing research programme aiming to investigate the most important parameters of the disease : the vector, namely *Amblyomma variegatum* (Fabricius, 1794), the agent and the immune status of the livestock.

The vector distribution has been established by previous studies consisting in collecting ticks on cattle of different ecological zones and on wildlife (7). Studies have been performed in the Niayes area to assess the importance of the infection by *Cowdria ruminantium* (12). As far as the immune status is concerned, data are available for only few ecological zones (13).

This article records the abundance of the vector in the main geographical areas and the tick infection rate in the North Guinean zones; the seroprevalence in the Sudano-Sahelian, North Sudan and the hinterland of the North Guinean zones is also reported.

MATERIAL AND METHODS

Abundance of the vector

The distribution and the abundance of *Amblyomma variegatum* have been established essentially by studies on the population dynamics of this species in different ecological zones of Senegal, *i.e.* :

- Sahelian zone : annual rainfall of 300 to 500 mm, grassland type of vegetation ;

- Coastal region of the Niayes: annual rainfall of 400 to 600 mm , grassland and palm trees (*Elaeis guineensis*) in clay soil depressions ;

- Sudano-Sahelian zone : annual rainfall of 500 to 800 mm ; shrubby grassland ;

- North Sudan zone : annual rainfall of 800 to 1 000 mm ; grassland and woodland ;

- South Sudan zone : annual rainfall of 1 000 to 1 200 mm ; woodland ;

- North Guinean zone : annual rainfall of 1200 to 1850 mm ; woodland and forest.

In each of these zones, apart from the coastal Niayes, 40 cattle, 40 sheep and 40 goats were subjected to a monthly removal of all their body ticks. In the particular case of the Niayes region, the study was limited to cattle and goats since sheep do not graze on natural pastures. To compare the abundance of *A. variegatum* in the different ecological zones, the level of cattle infestation by adult ticks is used as the criterion. In fact, the larvae and nymphs of this species may engorge on several types of hosts, particularly birds.

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Infection rate of *Amblyomma variegatum* in the North Guinean zone

During the rainy season of 1992, unfed adult ticks were collected from cattle grazing in day time on natural pastures. Unfed nymphs were collected according to the same procedure.

The adult ticks and nymphs were fed on rabbits for 4 to 5 days. They were divided into groups of 3 ticks and ground, then suspended in PBS (pH 7.2). The suspension was centrifuged for 5 min at 500 rpm. The supernatant was injected into a sheep by the intravenous route. A total of 30 sheep was used for each tick stage (nymphs and adults). The temperature of the sheep was recorded daily.

All sheep originated from cowdriosis-free areas in Northern Senegal. If we assume that one infected tick can transmit the infection to an inoculated sheep, the following formula can be applied :

$$IRo^* = \frac{\text{Number of tiks infected}^{**}}{\text{Total number of ground ticks infected}} \times 100$$

An infected sheep (showing hyperthermia) may die or survive. In the latter case, it is challenged with blood infected with *Cowdria ruminantium* originating from the same area. If no reaction is observed, it is considered that the previous hyperthermia was caused by tick infection.

Seroepidemiology

In March 1992, blood was collected from animals living in the following ecological zones :

- 451 cattle in the North Sudan zone ;

- 271 cattle in the hinterland of the North Guinean zone (Kedougou) ;

- 149 cattle in the hinterland of the Sudano-Sahelian zone ;

- 354 cattle in the coastal area of the latter zone.

The sera collected were centrifuged at 3 000 rpm for 15 mn. The supernatant was dispensed in 2 ml tubes and stored at -20°C.

Antigen production

A Senegalese strain of *Cowdria ruminantium* originating from the Niayes area has been maintained in endothelial cells (1, 15). A suspension of elementary bodies and morulae is stored at -20°C in 200 µl aliquots.

Indirect immunofluorescence

The test was carried out as described by MARTINEZ *et al.* (15).

The antigen suspension is thawed and diluted 1/100 in PBS (pH 7.4), and a drop of 10 µl is deposited on each spot of special slides for immunofluorescence. The slide is dried and then fixed in methanol. 10 µl of serum (1/80 dilution) is deposited per spot. A positive and negative control serum at the dilution of 1/80 is applied to one spot each of each slide. The slide is incubated for 30 min in a moist chamber at room temperature. The slide is washed for 10 min in PBS and an antibovine IgG conjugate diluted 1/100 in PBS with 0.01 % Evans blue is applied. Slides are again kept in a humid atmosphere for 30 min and washed in PBS, as previously.

They are mounted with glycerol and examined under a fluorescent microscope.

RESULTS

Abundance

The data on tick collection are reported in table I. The parasite burden caused by adult ticks during one year (map 1) allows to define three levels of abundance determined as follows :

Range of data

9,618 - 53 = 9,565.

If we retain 3 classes corresponding to the 3 categories : not abundant, abundant and very abundant, the length of the class is :

9,565 : 3 = 3,188.

Therefore the superior limit of the first class is : 3,188 + 53 = 3,241

not abundant : < 3,241

The second class interval is : [3,241-6,429] : abundant.

The third class interval is : very abundant : > 6,429.

For the different ecological zones we get the following classification :

- not abundant : North Sudan and Sudano-Sahelian zones ;

- abundant : Niayes and South Sudan zones ;

- very abundant : North Guinean zone.

---

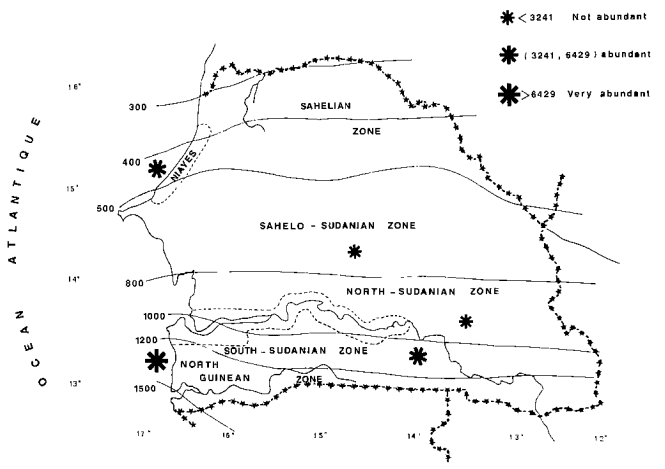
* : IRo = Infection rate observed

** : Number of sheep infected

TABLE I Ticks collected on cattle, sheep and goats.

	Ecological zones					
	Niayes*	Sahelian	Sudano-Sahelian	North Sudan	South Sudan	North Guinean
Cattle (n = 40)	L = 365 N = 1 457 A = 6 834	0	A = 53	A = 89	L = 1 020 N = 3 179 A = 3 294	L = 8 060 N = 3 739 A = 9 618
Sheep (n = 40)		0	A = 2	L = 2	L = 1 131 N = 421 A = 53	L = 837 N = 735 A = 163
Goats (n = 40)	L = 43 834 N = 5 675 A = 525	0	0	L = 1 N = 4	L = 307 N = 141	

L = Larvae
N = Nymph
A = Adult tick
** Study done during 18 months*



Map 1 : Abundance of Amblyomma variegatum.

Infection rates

Nymphs

Seven out of 30 inoculated sheeps contracted the infection, 2 died, 5 recovered and did not react to a new inoculation of infected blood. The observed infection rate is the following :

IRo = 7/90 x 100 = 7.8 %

Adult ticks

One out of 30 inoculated sheep died; no other sheep showed hyperthermia. The infection rate is :

IRo = 1/90 x 100 = 1.1 %

Seroprevalence

The results are given in table II. The seropositivity is low in the Sudano-Sahelian and North Sudan zones. However, the prevalence is higher near the coastal area of the Sudano-Sahelian zone.

TABLE II Ifa test results of cattle of different ecological zones.

Ecological zones	Number of sera	Number of positives	% positives
Sudano-Sahelian			
– Hinterland	149	8	5.3
– Coastal	354	107	30.2
North Sudan	451	31	6.9
North Guinean			
– Hinterland	271	92	33.9

DISCUSSION

The distribution and the abundance of *A. variegatum* in the different ecological zones correspond to the observations made by MOREL (16) on the normal habitat of the species in West Africa. However this tick is not the most important numerically, in comparison with the other species connected with livestock in the South Sudan and coastal Niayes zones (5, 9). In the latter regions, *Boophilus geigyi* AESCHLIMANN and MOREL, 1965 and *B. decoloratus* (KOCH, 1844) are numerically more important. On the contrary, the species is definitely dominant in the North Guinean zone (10).

The observed infection rates are based on a low number of ticks and sheep and their statistical significance remains to be assessed.

The high infection rate of ticks in the North Guinean zone recalls the data recorded in the coastal Niayes. The infection rates of adult ticks are equivalent in these two areas. On the other hand, nymphs are more infected in the Niayes. However the high infection rate of ticks in the coastal North Guinean zone and the abundance of the vector may explain the very high prevalence of infection found previously among cattle in this area. Concerning the seroprevalence, the low positivity rates in the hinterland of the Sudano-Sahelian zone and the North Sudan zone correspond with the scarcity of the vector. The coastal microclimate allows a larger population of this tick. This fact explains the high prevalence in this site.

The seropositivity of the Niayes and coastal North Guinean zones which reaches about 90 % is far higher (13) .

The serological cross reaction between *Cowdria ruminantium* and *Ehrlichia bovis* (2, 14) does not allow an easy interpretation of the results obtained.

Nothwithstanding the low prevalence of the infection in the Sudano-Sahelian and North Sudan belt, no mortality is recorded so far in cattle, even if the disease is recognized in goats of this area.

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GUEYE (A.), MBENGUE (Mb.), DIEYE (Th.), DIOUF (A.), SEYE (M.), SEYE (M.H.). Cowdriosis in Senegal : some epidemiological aspects. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 217-221

The results of a study on the abundance of *Amblyomma variegatum* in different ecological zones and of *Cowdria ruminantium* infection rates in nymphal and adult ticks of the North Guinean zones are given. Joint research is also conducted on the evaluation of seroprevalence. In this study, it appears that the vector is most important in the North Guinean zone, followed by the South Sudan and the coastal Niayes zones. Elsewhere, the tick populations are not significant or absent. The infection rate in the North Guinean zone is high : 1.1 % at least for adult ticks and 7.8 % for nymphs. The seroprevalence in the North Sudan and the Sudano-Sahelian zones is very low in the hinterland whereas the values are higher near the coast.

Key words : Heartwater - *Cowdira ruminantium* - Tick - *Amblyomma variegatum* - Epidemiology - Sera - Prevalence - Antigen - Indirect immunofluorescence - Senegal.

GUEYE (A.), MBENGUE (Mb.), DIEYE (Th.), DIOUF (A.), SEYE (M.), SEYE (M.H.). Aspectos epidemiológicos de la cowdriosis en Senegal. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 217-221

Se describen los resultados de un estudio sobre la abundancia de *Amblyomma variegatum* en diferentes zonas ecológicas, así como las tasas de infección de *Cowdria ruminantium*, tanto en garrapatas adultas y como en estadio ninfal, en diferentes zonas al norte de Guinea. Conjuntamente se llevó a cabo una evaluación de la seroprevalencia. Según nuestros resultados, el vector es más importante en la zona norte de Guinea, el sur de Sudán y las zonas costeras de Niayes. En el resto del territorio las poblaciones de garrapatas son mínimas o nulas. La tasa de infección, en la zona norte de Guinea es elevada : al menos 1.1 p. 100 para las garrapatas adultas y 7.8 p. 100 para las ninfas. La seroprevalencia es baja en las regiones internas del norte de Sudán y en las zonas sudano-sahelinas, mientras que en las zonas costeras es más elevada.

Palabras claves : Cowdriosis - *Cowdria ruminantium* - Garrapata - *Amblyomma variegatum* - Epidemiología - Suero - Prevalencia - Antigeno - Inmunofluorescencia indirecta - Senegal.

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Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms

JONGEJAN (F.), VOGEL (S.W.), GUEYE (A.), UILENBERG (G.). Vaccination contre la cowdriose avec des *Cowdria ruminantium* atténuées *in vitro*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 223-227

Des passages successifs de *Cowdria ruminantium* (stock Sénégal) dans des cultures de cellules endothéliales ombilicales bovines ont produit une perte de virulence sans perte d'immunogénicité, comme il a été démontré antérieurement. Dans une nouvelle expérience, 39 moutons néerlandais ont été immunisés avec des rickettsies atténuées du 21e passage et ont été éprouvés avec le stock homologue et des stocks hétérologues de *C. ruminantium*. Suite à l'immunisation, plusieurs des moutons ont montré une hyperthermie pendant 2 jours au plus, sans présenter une autre réaction clinique à la vaccination. Tous les moutons ont développé des titres élevés d'anticorps contre *Cowdria*. L'épreuve homologue virulente de 10 moutons n'a provoqué aucune réaction clinique, démontrant ainsi une immunité solide. Les réactions aux épreuves hétérologues ont varié entre la presque absence de réaction et la cowdriose mortelle, selon le stock utilisé. Les résultats sont commentés par rapport aux méthodes d'immunisation contre la cowdriose qui existent actuellement. Au Sénégal, 30 moutons sahéliens sensibles ont été immunisés avec des rickettsies atténuées du passage 21. Treize d'entre eux ont présenté une hyperthermie, le seul autre symptôme clinique fut une diarrhée passagère. Les animaux immunisés sont à présent exposés à l'infection naturelle dans les Niayes, région d'où le stock Sénégal a été isolé à l'origine.

Mots clés : Ovin - Cowdriose - *Cowdria ruminantium* - Culture de cellule - Cellule endothéliale bovine - Technique immunologique - Vaccin atténué - Anticorps - Vaccination - Virulence - Hyperthermie.

INTRODUCTION

Protective immunity against cowdriosis can be induced in susceptible ruminants by infection with virulent blood and subsequent treatment of the reaction with antibiotics (1). This type of immunization is practiced in South Africa using virulent sheep blood infected with the Ball 3 stock, followed by treatment with tetracyclines (13). Although useful to control the disease, the vaccine is far from ideal. In addition, the existence of distinct antigenic differences between *Cowdria* isolates (4, 5, 9, 10) may explain the occurrence of clinical cowdriosis in animals that were vaccinated with this vaccine based on the Ball 3 isolate.

Sequential passage of *Cowdria* in bovine endothelial cell (BUE) cultures and the resulting attenuation of a Senegalese isolate of *Cowdria* has been reported recently (12). We have carried out further immunizations of sheep with the attenuated *Cowdria* vaccine. Here we report on the response of European and African sheep to vaccination and of European sheep to homologous and heterologous *Cowdria* challenge under laboratory conditions.

MATERIAL AND METHODS

Cowdria stocks

Seven stocks of *Cowdria ruminantium* were used : a Senegalese isolate, designated "Senegal" (9), two South African stocks, "Welgevonden" (3) and "Ball 3" (7), an isolate from Guadeloupe, "Gardel" (15) and three other stocks from Africa, "Um Banein" from the Sudan (8), "Umpala" isolated by M. ASSELBERGS in Mozambique (unpublished) and the "Lutale" stock from Zambia (9).

All stocks were stored as infective blood stabilates in liquid nitrogen with DMSO as cryoprotectant. The infectivity of the isolates had been tested before in susceptible goats (Saanen breed) by intravenous inoculation of 2 ml aliquots of thawed blood stabilate (9, 10, 14). It had been previously shown that mortality in similar untreated goats was 100 % for the Senegal isolate (12 out of 12), Welgevonden (5/5), Gardel (5/5), Um Banein (4/4) and Lutale (4/4). The Umpala stock was also highly virulent but was tested in two animals only (both died). The Ball 3 isolate appeared somewhat less pathogenic with a mortality rate in untreated goats of 10 out of 13 (10).

Cultivation

The method of cultivation of *Cowdria* has been described before (12). Briefly, bovine umbilical endothelial (BUE) cells were grown in RPMI 1640 medium. Monolayers were inoculated with *Cowdria* (Senegal stock) and incubated on a slowly rocking platform. *Cowdria* growth medium consisted of Glasgow Minimal Essential Medium (GMEM) supplemented with 2.9 g/l tryptose phosphate broth, penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (1.25 µg/ml), HEPES buffer, L-glutamine

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(2 mM) and 10 % newborn calf serum. The growth cycle of *Cowdria* consisted of reticulate bodies (RB) within BUE cells resulting in elementary bodies (EB) which were released into the culture medium (11). *Cowdria* infection of BUE cells was scored as follows : RBs (1+), scanty intracellular colonies, less than 1% of BUE cells infected ; (2+), approximately 10% of the cells infected ; (3+), virtually all cells infected. Score for EBs : (1+), scanty extracellular particles ; (2+), present in large numbers, coinciding with moderate cytopathic effect ; (3+), heavily infected culture supernatant, coinciding with destruction of most BUE cells.

Cultures with 3+EBs were used to passage *Cowdria* onto other BUE cells with an average interval of 10.3 days (range 8-34 days) between passages. BUE culture supernatant heavily infected with elementary bodies (score of 3+) of passage n° 21 (324 days in culture) was centrifuged at 10,000 g for 10 min, washed and resuspended in sucrose-phosphate-glutamate buffer (SPG) (2) and stored at -80°C.

Vaccination

At Utrecht, a total of 39 adult female Tesselaar sheep were inoculated intravenously with attenuated *Cowdria* of passage 21, at a dose of 0.5 ml of culture supernatant, deepfrozen at -80°C in SPG buffer. The animals were monitored by daily temperature records, clinical inspection, as well as collection of blood samples for serology at weekly intervals. All vaccinated animals were challenged by the intravenous route on day 30 post infection with 2 ml of virulent blood stabilates. These had earlier been shown to cause fatal heartwater in control animals. The first group, which consisted of 10 sheep, received an homologous challenge by inoculation of virulent blood stabilate (Cr111) infected with the Senegal stock. The remaining 29 animals were divided into 5 groups of 5 animals and one group consisting of 4 sheep. Each group was challenged with a different *Cowdria* isolate, either Umpala, Lutale, Gardel, Ball 3, Um Banein or Welgevonden, 30 days after the animals had been vaccinated.

In Senegal, 60 local sheep from the northern Sahel zone, where *Amblyomma* ticks and heartwater are rare and sheep have been shown before to be susceptible to the disease (6), were transported to the laboratory in Dakar and maintained free from ticks. Serum was prepared from the animals and tested in the indirect fluorescent antibody test (IFA test). 30 of the animals were then immunized with attenuated EBs of the 21st passage of the Senegal stock, as described above. The animals are presently exposed, since 57 days after immunization, together with the 30 non-immunized controls, to natural infestation by ticks in the coastal Niayes region of Senegal, north of Dakar, where *A.variegatum* and heartwater are common (6).

Immunofluorescence test

BUE cultures infected with *Cowdria* (EB score 3+) were centrifuged at 4 °C for 15 minutes at 15,000 g. Pellets were resuspended in PBS, spotted onto microscope slides, dried and fixed in acetone. The slides were incubated with twofold titrations of sera in PBS starting from 1:80 up to 1:20,480. Positive and negative control sera were also tested. Fluorescein isothiocyanate-labeled rabbit anti-sheep immunoglobulins were used as second antibodies. Fluorescence was observed with an Olympus BH2-RFL microscope.

Monitoring

Serum was prepared for the IFA test prior to immunization and at weekly intervals thereafter. Rectal temperatures were recorded daily and the animals were inspected daily for clinical symptoms. Brain smears of animals that died were examined for clusters of *C. ruminantium* in capillary endothelial cells, after methanol fixation and Giemsa staining.

RESULTS

All sheep vaccinated at Utrecht developed antibodies to *Cowdria* with titres ranging from 640 to at least 5120 as determined by immunofluorescence. Six out of the 39 animals had elevated temperatures for a maximum of 2 days, but no further clinical response to the vaccine was observed.

Challenge of 10 of the sheep (n° 373 to 382) with the virulent homologous Senegal stock, previously shown to be lethal for all non-vaccinated control animals, did not provoke any clinical reaction, demonstrating that these animals were solidly immune (table I).

The other 29 sheep vaccinated at Utrecht with the attenuated material were challenged with heterologous isolates. Reactions varied widely from no clinical reaction at all to fatal cowdriosis, depending on the stock of *Cowdria* used for the challenge (table I). Four out of 5 sheep were fully protected against Umpala, whereas one animal reacted with a transient fever only. Three out of 5 sheep challenged with the Lutale isolate were immune, one was partially immune and the fifth animal required tetracycline treatment to prevent a possibly fatal outcome of the disease. Reactions to Ball 3 and Gardel stocks were similar : 2 out of 5 sheep were immune, whereas the remaining animals were partially immune or required tetracycline[1] treatment. The remaining 4 sheep challenged with the

1. Oxytetracycline (Engemycin®) at 20 mg/kg IM.

TABLE I Clinical reactions of sheep vaccinated with attenuated **Cowdria ruminantium** *(Senegal stock) to challenge with homologous and heterologous* **Cowdria** *isolates.*

Sheep number	Challenge stock	Incubation period (days)	Maximum temp. (°C)	Duration of fever (days)	Time to death (days)	Outcome	IFA titre*
373	Senegal	–	–	–	–	no reaction = immune	≥ 5120
374	Senegal	–	–	–	–	no reaction = immune	≥ 5120
375	Senegal	–	–	–	–	no reaction = immune	≥ 5120
376	Senegal	–	–	–	–	no reaction = immune	2560
377	Senegal	–	–	–	–	no reaction = immune	2560
378	Senegal	–	–	–	–	no reaction = immune	≥ 5120
379	Senegal	–	–	–	–	no reaction = immune	640
380	Senegal	–	–	–	–	no reaction = immune	640
381	Senegal	–	–	–	–	no reaction = immune	640
382	Senegal	–	–	–	–	no reaction = immune	≥ 5120
394	Umpala	17	40.6	2	–	partially immune	≥ 5120
412	Umpala	–	–	–	–	no reaction = immune	≥ 5120
408	Umpala	–	–	–	–	no reaction = immune	2560
396	Umpala	–	–	–	–	no reaction = immune	2560
393	Umpala	–	–	–	–	no reaction = immune	≥ 5120
400	Lutale	2	41.2	5	–	Engemycin treatment	640
386	Lutale	9	40.1	2	–	partially immune	2560
406	Lutale	–	–	–	–	no reaction = immune	≥ 5120
398	Lutale	–	–	–	–	no reaction = immune	≥ 5120
388	Lutale	–	–	–	–	no reaction = immune	≥ 5120
403	Gardel	15	40.7	3	–	Engemycin treatment	2560
401	Gardel	15	41.1	3	–	Engemycin treatment	2560
402	Gardel	16	40.5	6	–	partially immune	1280
409	Gardel	–	–	–	–	no reaction = immune	2560
405	Gardel	–	–	–	–	no reaction = immune	1280
399	Ball 3	13	41.7	4	–	Engemycin treatment	≥ 5120
390	Ball 3	14	40.7	3	–	Engemycin treatment	≥ 5120
397	Ball 3	15	41.0	3	–	partially immune	≥ 5120
391	Ball 3	–	–	–	–	no reaction = immune	≥ 5120
383	Ball 3	–	–	–	–	no reaction = immune	≥ 5120
395	Um Banein	9	40.9	5	14	fatal heartwater**	≥ 5120
411	Um Banein	10	41.6	5	–	Engemycin treatment	≥ 5120
404	Um Banein	11	41.9	4	–	Engemycin treatment	≥ 5120
389	Um Banein	12	41.1	3	–	Engemycin treatment	640
387	Um Banein	12	41.5	2	–	Engemycin treatment	≥ 5120
384	Welgevonden	9	41.7	8	19	fatal heartwater**	≥ 5120
407	Welgevonden	12	41.7	6	18	fatal heartwater**	≥ 5120
410	Welgevonden	12	40.4	1	14	fatal heartwater**	≥ 5120
392	Welgevonden	12	41.6	3	15	fatal heartwater**	640

* *All sheep were negative for* Cowdria *antibodies prior to vaccination ; the IFA titre was determined four weeks after vaccination but prior to challenge inoculation.*
** *Heartwater confirmed by the demonstration of rickettsial inclusion bodies in brain crush smears.*

Um Banein isolate required treatment, after one of them had died. Finally, the attenuated vaccine did not protect at all against challenge with Welgevonden, resulting in death due to cowdriosis of all 4 vaccinated sheep.

Antibody levels after vaccination did not correlate with the level of protection induced.

In Senegal, 13 of the 30 vaccinated sheep had a febrile response after vaccination, and a temporary diarrhoea

was observed. No other clinical signs were noticed and none of the animals was treated. Results of exposure to field challenge will be reported later.

DISCUSSION

In the first report on vaccination with live attenuated *Cowdria ruminantium* the vaccinated animals were challenged with the homologous virulent Senegal stock (12). In this study it is confirmed that a solid protective immunity can be induced in sheep (n = 10) against a lethal challenge with the homologous isolate. In view of possible replacement of current vaccination using virulent blood with in vitro attenuated organisms, it was important to determine reponses to heterologous challenge under laboratory conditions. It was found that responses to heterologous *Cowdria* challenge varied depending on the isolate used. For instance, on the one hand 4 out of 5 sheep were protected against challenge with the Umpala isolate, whereas on the other hand 4 out of 4 sheep died due to challenge with the Welgevonden isolate.

It has been demonstrated previously that antigenic differences between stocks of *Cowdria* play an important role in small ruminants (5, 9, 10). For instance, cross-immunity experiments in goats have shown that 3 out of 5 goats immunized with the virulent Senegal stock died of heartwater after challenge with the Welgevonden isolate (10). Therefore, the fatal outcome of heterologous challenge of sheep with the Welgevonden isolate in the present study could be expected, although immunological differences between the two isolates appear to be much more pronounced in goats than in sheep (10).

Responses of vaccinated sheep to challenge with Ball 3, Lutale, Gardel and Um Banein isolates were heterogenous. Two or three animals in each group were fully protected, whereas the remaining animals were partially immune or required treatment, apart from all 5 sheep challenged with the Um Banein isolate, which all reacted severely. This was surprising in view of the fact that complete cross-protection between this stock and Ball 3, Lutale and Gardel isolates has been reported in goats (10, 14, 15). Finally, 4 out of 5 sheep challenged with the Umpala isolate from Mozambique were protected, indicating a high level of cross-immunity between this isolate and the attenuated *Cowdria* from Senegal.

It can be concluded that antigenic differences are an important factor in the development of improved vaccination methods to prevent cowdriosis. It should however also be stressed that lack of cross-protection between stocks may be more pronounced in small ruminants than in cattle. It is therefore important to determine the extent of heterologous field challenge in cattle vaccinated with attenuated *Cowdria*, in addition to experiments with small ruminants. The attenuated vaccine is currently tested in a field trial using sheep in Senegal, the results of which will be reported elsewhere. Further experiments are underway to determine whether attenuation of other isolates can also be achieved. Finally, it remains to be shown if *Amblyomma* ticks feeding on vaccinated animals will transmit avirulent or virulent rickettsiae.

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JONGEJAN (F.), VOGEL (S.W.), GUEYE (A.), UILENBERG (G.). Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 223-227

Sequential passage of *Cowdria ruminantium* (Senegal isolate) in cultures of bovine umbilical endothelial cells has resulted in loss of virulence without loss of immunogenicity, as previously demonstrated. We have carried out further immunization of 39 Dutch sheep using in vitro attenuated rickettsiae of passage 21 and challenged these animals either with the homologous or with heterologous *Cowdria* stocks. After vaccination several sheep developed elevated rectal temperatures for a maximum of 2 days, but no further clinical response to the vaccine was observed. All sheep developed high titres of antibodies to *Cowdria*. Challenge of 10 sheep with the homologous virulent stock did not provoke any clinical reaction, demonstrating that these animals were solidly immune. Reactions to heterologous challenge varied from virtually no reaction to fatal heartwater depending on the stock of *Cowdria* used. These results are discussed in relation to currently available vaccination methods against cowdriosis. In Senegal 30 susceptible sahelian sheep were immunized with attenuated rickettsiae of passage 21. Hyperthermia was seen in 13, the only other clinical symptom was a temporary diarrhoea. The immunized animals are at present exposed, together with 30 controls, to field challenge in the Niayes, the area where the Senegal isolate was originally isolated.

Key words : Sheep - Heartwater - *Cowdria ruminantium* - Cell growth - Bovine endothelial cell - Immunological technique - Attenuated vaccine - Antibody - Vaccination - Virulence - Hyperthermia.

JONGEJAN (F.), VOGEL (S.W.), GUEYE (A.), UILENBERG (G.). Vacunación contra cowdriosis mediante el uso de organismos de *Cowdria ruminantium* atenuados *in vitro*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 223-227

Se ha demostrado que los pasajes seguidos de *Cowdria ruminantium* (aislamiento de Senegal) en cultivos de células de endotelio umbilical bovino, resulta en la pérdida de virulencia, sin pérdida de inmunogenicidad. Se llevó a cabo la inmunización de 39 ovejas holandesas, mediante el uso de rickettsias atenuadas in vitro, al pasaje 21. Estos animales se probaron con series homólogas o hetérologas de *Cowdria*. Después de la vacunación, varias ovejas presentaron temperaturas rectales elevadas, durante un máximo de dos días, pero no se observaron otros signos clínicos secundarios a la vacuna. Todas las ovejas desarrollaron títulos altos de anticuerpos contra *Cowdria*. Diez (10) ovejas fueron sometidas a una serie homóloga virulenta, sín presencia de reacciones clínicas, lo que demuestra la sólida inmunidad de estos animales. Las reacciones a las series heterólogas variaron de la ausencia de reacción hasta cowdriosis fatal, según el tipo de serie. Estos resultados se discuten en relación a los métodos existentes de vacunación contra la cowdriosis. En Senegal, se inmunizaron 30 ovejas sahelinas susceptibles, con rickettsias atenuadas al pasaje 21. En trece de ellas se observó hipertermia. El otro síntoma clínico presente fue una diarrea pasajera. Los animales inmunizados se encuentran actualmente expuestos, junto con 30 controles, a pruebas de campo en Niayes, zona de origen del aislamiento senegalés.

Palabras claves : Ovino - Cowdriosis - *Cowdria ruminantium* - Cultivo de célula - Célula endotelial bovina - Técnica inmunologica - Vacuna atenuada - Anticuerpo - Vacunación - Virulencia - Hipertermia.

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Protection of goats against heartwater acquired by immunization with inactivated elementary bodies of *Cowdria ruminantium* *

MARTINEZ (D.), MAILLARD (J.C.), COISNE (S.), SHEIKBOUDOU (C.), BENSAID (A.). Protection des chèvres contre la cowdriose acquise par immunisation à l'aide de corps élémentaires inactivés de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 229

Lors de 2 expériences, 4 et 5 chèvres ont été vaccinées par 2 injections en sous-cutané d'une préparation de corps élémentaires de *Cowdria ruminantium* (stock Gardel), mélangés à l'adjuvant de Freund. Tous les animaux vaccinés, ainsi que 4 témoins, ont été éprouvés par voie intraveineuse avec 5 ml de surnageant d'une culture de cellules endothéliales bovines en lyse, infectée par le même stock de *Cowdria*. Toutes les chèvres ont réagi par une hyperthermie. Deux des 4 chèvres d'un groupe vacciné et 4 des 5 chèvres de l'autre ont survécu, tandis que tous les témoins sont morts dans les 7 à 12 jours. Les animaux vaccinés qui sont morts, ont survécu plus longtemps que les témoins. Les animaux vaccinés protégés et non protégés n'ont pas montré de différences dans les titres d'anticorps. De plus, des sérums d'animaux ayant survécu, inactivés par la chaleur ou non, n'ont pas neutralisé l'infection par *Cowdria* de cellules endothéliales bovines *in vitro*. Les mécanismes responsables de la protection des chèvres immunisées sont encore inconnus mais l'hypothèse que des populations de lymphocytes T-helper ont été stimulées semble probable. Cette méthode d'immunisation avec des organismes tués aidera la recherche d'antigènes protecteurs contre la cowdriose.

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In two experiments, 4 and 5 goats were vaccinated by giving 2 subcutaneous injections of a preparation of inactivated elementary bodies of *Cowdria ruminantium* (Gardel stock) mixed with Freund adjuvant. All vaccinated animals together with 4 naive controls were challenged intravenously with 5 ml of supernatant of a lysing culture of bovine endothelial cells infected with the same stock of *Cowdria*. All goats developed a high temperature. Two out of 4, and 4 out ot 5 vaccinated goats survived the challenge whereas all naive control animals died within 7 to 12 days. Vaccinated goats which died, survived longer thant the controls. No difference in antibody titres was observed between protected and non protected vaccinated goats. Moreover, immune sera from surviving goats, whether heat inactivated or not, were unable to neutralize the infection of bovine endothelial cells by *Cowdria in vitro*. Mechanisms conferring protection to the immunized goats are unknown at the moment but the hypothesis that T-helper lymphocytes populations have been elicited seems to be likely. This method of immunization with dead organisms will help in the search of protective antigens against cowdriosis.

MARTINEZ (D.), MAILLARD (J.C.), COISNE (S.), SHEIKBOUDOU (C.), BENSAID (A.). Protección en cabras contra la cowdriosis adquirida mediante inmunización con cuerpos elementales inactivados de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 229

Se llevaron a cabo dos experimentos, en los cuales se vacunaron 4 y 5 cabras mediante la inyección subcutánea de una preparación de cuerpos elementales inactivados de *Cowdria ruminantium* (stock Gardel), mezclados con adyuvante de Freund. Todos los animales vacunados, así como 4 controles sanos, fueron tratados con 5 ml IV del sobrenadante de una cultura de lísis de células de endotelio bovino, infectadas con el mismo stock de *Cowdria*. Todas las cabras presentaron un aumento de la temperatura. Dos de las cuatro y cuatro de las cinco cabras vacunadas sobrevivieron al tratamiento, mientras que todos los controles sanos murieron en siete a doce días. Las cabras vacunadas que murieron, sobrevivieron más tiempo que los controles. No se encontraron diferencias en los títulos de anticuerpos entre animales vacunados protegidos y no protegidos. Además, los sueros inmunes de las cabras sobrevivientes, inactivados o no mediante calor, no fueron capaces de neutralizar la infección *in vitro* de *Cowdria*, en células de endotelio bovino. Por el momento se desconocen los mecanismos que confieren la protección a las cabras inmunizadas, sin embargo se plantea la posible acción de los linfocitos T de ayuda. Este método de inmunización con organismos muertos, podría utilizarse en la búsqueda de antígenos protectores contra la cowdriosis.

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Progress towards a vaccine against *Theileria parva* : relevance for heartwater research

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McKEEVER (D.J.). Le progrès vers un vaccin contre *Theileria parva* : Pertinence pour la recherche sur la cowdriose. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 231-235

Beaucoup de progrès ont été enregistrés durant les 10 dernières années en ce qui concerne la caractérisation de l'immunité bovine contre *Theileria parva*. Il n'est plus à démontrer que les bovins devenus immunisés après infection peuvent se débarrasser d'infections ultérieures par le déploiement de lymphocytes T cytotoxiques (LTC) spécifiques pour le parasite. De plus, des anticorps neutralisants sont produits à des titres élevés contre la surface du sporozoïte après des infections multiples par le parasite, et peuvent neutraliser l'infection *in vitro*. Bien que cela ne soit vraisemblablement pas significatif dans les circonstances naturelles, on a tiré profit de cette dernière observation pour créer un candidat prometteur pour un vaccin neutralisant basé sur une forme recombinante de l'antigène de surface majeur des sporozoïtes de *T. parva*. On essaie actuellement d'identifier le(s) antigène(s) cible(s) des LTC spécifiques pour *T. parva*, et après réussite un vaccin amélioré visant aussi bien les stades infectieux que pathogènes sera à portée. L'élucidation de la base de l'immunité des ruminants contre *Cowdria ruminantium*, agent causal de la cowdriose, est encore à un stade relativement peu avancé. Néanmoins, on applique déjà à la cowdriose plusieurs des principes et des techniques ayant mené à la compréhension de l'immunologie de *T. parva*, et cela devrait permettre un progrès rapide dans le développement d'un vaccin contre *C. ruminantium*.

Mots clés : Bovin - Cowdriose - *Cowdria ruminantium* - Vaccin - Tique - *Theileria parva* - Lymphocyte - Anticorps - Antigène - Infection expérimentale - Immunité.

INTRODUCTION

Despite the current trend in many Western nations towards the consumption of plant rather than animal proteins, livestock remain a major source of nutritional protein in many Third World countries. The tick-borne diseases of cattle and small ruminants, which include theileriosis, cowdriosis, anaplasmosis and babesiosis, are probably the greatest disease constraint to the improvement of livestock productivity in these countries. The most significant among these diseases on the African continent, in terms of economic losses and restriction of livestock development, are East Coast fever (ECF) and heartwater. Both of these conditions are most severe in animals introduced to endemic locations from areas that are free of the disease.

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ECF is a lymphoproliferative disease of cattle caused by the protozoan parasite *Theileria parva,* which is characterized by high mortality in naive animals. The disease is distributed over large areas of eastern, central and southern Africa, and its principal vector is the brown ear tick *Rhipicephalus appendiculatus.* Heartwater is endemic in a much larger area, occurring throughout most of sub-Saharan Africa, and is also present on some islands to which the major vector *Amblyomma variegatum* has spread (29). Its economic significance is intensified by the fact that sheep and goats are affected as well as cattle. Because young cattle possess an innate resistance to the causal agent *Cowdria ruminantium* (26), heartwater is rarely observed in indigenous livestock in endemic areas, and presents as a problem chiefly in susceptible animals that have been moved to areas where the agent is present.

Control of both diseases is largely dependent on the use of acaricide-based tick control strategies and immunisation by infection and treatment, the administration of live organisms along with chemotherapeutic agents (3, 31). However, the expense of acaricides coupled with the numerous disadvantages associated with infection and treatment have prompted a search for improved vaccines against these diseases.

Immune responses of cattle to *C. ruminantium* are not well understood (34,36). The agent survives intracellularly within vascular endothelial cells, and the pathogenesis of the disease that it causes is believed to be the result of increased capillary permeability (8, 10, 27, 30). After inoculation with *C. ruminantium,* antibody responses are detected in cattle at the height of the febrile response (32), and these are probably generated in response to organisms released following rupture of infected endothelial cells. However, experiments involving the transfer of immune serum or purified g-lobulins have yielded no evidence that antibodies influence the course of infection (1, 9), and there is no apparent correlation between antibody titre as measured by indirect immunofluorescence antibody tests and the immune status of the animal (11). These observations, together with the intracellular location of the agent, have led to the belief that cell-mediated immune mechanisms are required for protection of immune animals from rechallenge.

The life cycle of *T. parva is* more complex than that of *C. ruminantium,* with the parasite progressing through schizont, merozoite and piroplasm stages in the mammalian host. After inoculation by the tick, sporozoites rapidly

invade lymphocytes and differentiate to schizonts (17, 33). This is the pathogenic stage of the parasite, and is associated with uncontrolled proliferation of infected lymphocytes. This feature of the disease is responsible for the majority of its clinical signs (18). Bovine immune responses to *T. parva* are well understood, and much of the available information is derived from studies in animals immunised by infection and treatment. This paper will attempt to summarize the work that has led to the current understanding of bovine immunity to *T. parva,* and to highlight those aspects of it that may be of relevance to heartwater.

IMMUNITY TO *THEILERIA PARVA*

Although serum antibodies against all stages of *T. parva* can be observed in immune cattle, a number of observations indicate that they are not important for the elimination of challenge infections. As observed for heartwater, the transfer of immune serum does not influence the course of infection in naive cattle (22, 35). Several aspects of the immunity seen in cattle after infection and treatment immunisation with *T. parva* suggest that protective mechanisms are directed at the schizont-infected cell. These include an apparent requirement for the development of this stage of the parasite for the generation of effective immunity, the common occurrence of a low schizont parasitosis in immune animals before the clearance of infection, and the observation that cattle immunized by infection and treatment resist challenge with up to $5x10^8$ schizont-infected cells (16, 37). There are no indications that these mechanisms are antibody-dependent. EMERY (13) demonstrated that in spite of generating schizont-specific antibody titres equivalent in magnitude to those produced by conventionally immunized animals, cattle immunised with heat-killed schizont-infected cells or partially purified schizont antigens were not protected against challenge. These observations gave rise to the belief that immunity against *T. parva* was cell-mediated. This was supported by the observation (13) that immunity could be transferred in the cellular fraction of immune thoracic duct lymph, although this fraction did contain B lymphocytes.

Characterization of cellular immune responses against *T. parva* were greatly facilitated by successful infection of bovine lymphocytes with the parasite *in vitro* (5). This allowed PEARSON *et al.* (28) to establish that bovine lymphocytes proliferated in the presence of autologous infected cell lines, and in addition, that lymphocytes derived from immune but not naive cattle developed cytolytic activity in these cultures. A subsequent analysis of the nature of this cytolytic activity confirmed that it was directed at parasite antigens rather than those induced by culture conditions (15). These developments clearly implicated parasite-specific cytotoxic cells in immunity to ECF, and prompted a number of studies of *in vivo* cytolytic responses to infection or immunization. EMERY *et al.* (14) demonstrated that naive cattle generated cytolytic activity only during the terminal stages of lethal infection and that it was not restricted to autologous parasitised lymphocytes, killing in addition allogeneic infected cells and mouse tumour cells. Conversely, in immune cattle undergoing challenge, killing activity was observed around the time of remission of infection, and this was restricted to autologous infected cells. This restriction, coupled with the subsequent localization of the activity to the T cell population of blood lymphocytes, provided a strong indication that parasite-specific killing activity in *T. parva* immune cattle was mediated by class I MHC-restricted cytotoxic T lymphocytes (CTL).

Confirmation of this hypothesis was made possible by the availability of serological reagents for the typing of bovine class I MHC antigens. Three international workshops have grouped these reagents into over thirty specificities, the majority of which are believed to be encoded by one locus, known as BoLA-A (6). The use of these reagents in conjunction with a knowledge of parentage allows the identification of cattle that are MHC haplo-identical or that share individual class I MHC antigens.

In a study of parasite-specific CTL activity in 10 immune cattle that were heterozygous at the BoLA-A locus, it became clear that killing was only observed when target cells shared at least one class I MHC antigen with the donor animal (21). The abrogation of killing by the addition of class I MHC-specific mAbs confirmed that these molecules were indeed the restricting elements. In a later study, it was established that *T. parva*-specific cytolytic activity resides in the $CD8^+$ T cell fraction of bovine lymphocytes. Kinetic analysis of CTL activity in the blood of immune cattle under challenge has shown that peak activity is associated with the disappearance of schizonts from peripheral blood lymphocytes (21), providing further evidence that CTL play a role in protection.

LYMPHATIC CANNULATION

A major advantage held by ruminant immunologists is the capacity to cannulate lymphatics and collect lymph fluid over long periods. It is accepted that immune responses are initiated in the lymph node that drains the site of antigen entry, and the activity within these nodes is reflected in phenotypic and functional changes in efferent lymph cell populations. These systems have been exploited in the study of bovine immune responses to *T. parva*. EMERY (12) examined the kinetics of infection in lymph of naive calves after lethal challenge. He observed a dramatic 7-8 fold increase in cell output that peaked 3-4 days after infection. This was accompanied by an increase in the proportion of blasting cells. Parasitised cells were first detected 8 days after challenge and 60-65 % of lymph cells were parasitised by day 14. Functional parameters

were not examined in these experiments, although it was known that parasite-specific cytolytic activity is not a feature of primary infections of cattle with *T. parva* (14). More recently, we have studied CTL activity in the lymph of immune cattle under challenge with the parasite (McKEEVER, TARACHA, INNES, MacHUGH, AWINO, GODDERIS and MORRISON, submitted). In kinetic studies, we have observed that CTL activity is more marked and peaks one day earlier in lymph than in blood. Furthermore, by limiting dilution analysis of the frequency of CTL precursors, we have established that at the peak of the nodal response to challenge, as many as 1:32 of efferent lymph lymphocytes are parasite-specific CTL. This is up to 25 times the frequency observed in PBM at the same time.

The large numbers of *T. parva*-specific CTL present in responding lymph highlighted the possibility of evaluating their capacity to clear challenge infections *in vivo.* By complement lysis of CD8- lineages in responding lymph, it was possible to prepare up to 7.5 x 10^9 $CD8^+$ T cells from an overnight collection of the fluid. In this way, large numbers of CTL were transferred between immune and naive identical twin calves, such that peak CTL activity in the donor coincided with the emergence of a lethal schizont parasitosis in the recipient. In two such experiments the recipient cleared the challenge after transfer, while challenge control animals developed lethal infections. These observations provide conclusive evidence that *T. parva*-specific CTL, the major cellular effector population in immune cattle, can clear the parasite after challenge.

In the search for an improved vaccine against *T. parva,* considerable effort is now focused on the identification of the parasite components that provoke specific CTL responses. These studies are based on the use of *T. parva*-specific CTL clones and appropriate target cells sensitised either by incubation with peptide fractions of parasitised cells or by the expression of parasite genes from a variety of vector systems.

ALTERNATIVE APPROACHES

In spite of the evidence outlined above for a major role for CTL in recovery and protection of immune cattle from *T. parva,* it should be remembered that immune responses that are protective in the field need not necessarily dictate the nature of an effective vaccine. As mentioned earlier, there is considerable evidence that serological responses against *T. parva* do not play a significant role in protection. However, high titres of antibody against the sporozoite surface are present in sera from cattle in endemic areas or those repeatedly exposed to infected ticks under laboratory conditions (24). These sera neutralise the infectivity of sporozoites *in vitro* and *in vivo.* Their dominant target specificity is a 67 kDa antigen (p67) on the sporozoite surface, and monoclonal antibodies raised against this antigen also effectively neutralise infectivity (25). These observations suggested that immunization with this antigen might give rise to a protective immune response directed at the sporozoite stage of the parasite. The gene that encodes the antigen was cloned and expressed in *Escherichia coli* as a fusion protein with the NS 1 antigen of influenza virus (23), which is a powerful inducer of helper T cell responses (2). The recombinant product has now been used in immunization trials in cattle, and results have been extremely promising; in initial experiments 13 of 21 immunized animals were protected against a measured challenge (23). Further development of this product as a first generation improved vaccine against T. *parva is* underway. These results emphasise the value of exploiting recombinant technology and antigen delivery systems to generate protective responses against antigens that may not be protective under natural circumstances.

RELEVANCE FOR FUTURE STUDIES IN HEARTWATER

Many of the systems and techniques that have led to the current understanding of bovine immunity to *T. parva* are applicable to the development of improved vaccines against heartwater. Major advances have been made in recent years in the molecular and antigenic characterization of *C. ruminantium,* and in the development of *in vitro* techniques for its culture. An immunodominant surface antigen has been described that is serologically conserved among isolates of the agent (19, 20), and this represents a promising candidate for a neutralising vaccine. Protective immunity in the field is likely to be based on cellular mechanisms, and the capacity to infect autologous endothelial cells with the agent (4, 7) has set the stage for a rapid evaluation of the role of cell-mediated responses in immunity of ruminant species to heartwater. If these responses are implicated in protection, the important task will be the identification of the antigens involved in their induction. Because *C. ruminantium is* a less complex organism it is possible that this task will prove less arduous than that of identifying relevent antigens of *T.parva,* and the prospect of an improved vaccine for heartwater may be somewhat less that remote.

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Such progress has been made in the last decade in the characterization of bovine immunity to *Theileria parva*. The evidence is overwhelming that cattle that become immune through infection can clear subsequent challenge infections by deploying parasite-specific cytotoxic T lymphocytes (CTL). Furthermore, high titres of neutralising antibodies are generated against the sporozoite surface after multiple exposure to the parasite, and these can neutralise infection *in vitro*. Although unlikely to be of relevance under natural circumstances, the latter observation has been exploited to generate a promising candidate neutralising vaccine based on a recombinant form of the major surface antigen of the *T. parva* sporozoites. Efforts are under way to identify the target antigen(s) of *T. parva*-specific CTL, and when this has been achieved, an improved vaccine targeted on both infective and pathogenic stages of the parasite will be within reach. The elucidation of the basis of immunity of ruminants to *Cowdria ruminantium*, the causal agent of heartwater, is at a comparatively early stage. However, many of the principles and techniques that have led to the current understanding of the immunology of *T. parva* are already being applied to heartwater, and these should enable rapid progress to be made in the development of a vaccine against *C. ruminantium*.

Key words : Cattle - Heartwater - *Cowdria ruminantium* - Vaccine - Tick - *Theileria parva* - Lymphocyte - Antibody - Antigen - Experimental infection - Protection.

McKEEVER (D.J.). Avance de la vacunación contra *Theileria parva* : importancia para la investigación de la cowdriosis. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 231-235

En la última década han habido importantes progresos en la caracterización de la inmunidad bovina contra *Theileria parva*. Parece evidente que los bovinos inmunizados mediante infección, pueden resistir a infecciones futuras, gracias a linfocitos T citotóxicos específicos para el parásito (CTL). Aún más, después de una exposición múltiple al parásito, se generan títulos altos de anticuerpos neutralizantes contra la superficie del esporozoito, lo que permite la neutralización *in vitro* de la infección. Aunque la importancia de lo anterior bajo condiciones naturales es dudosa, parece prometedora para la fabricación de una vacuna neutralizante, basada en una forma recombinante del antígeno mayor de superficie de los esporozoitos de *T. parva*. Actualmente se realizan esfuerzos para determinar el o los antígenos clave CTL-específicos para *T. parva*, lo que una vez adquirido, permitirá la realización de una vacuna específica tanto para el estadio infectivo, como patogénico del parásito. La comprension de las bases de la inmunidad contra *Cowdria ruminantium* (agente causal de la cowdriosis o enfermedad de "heartwater") en rumiantes, se encuentra en un estadio análogo. Sin embargo, muchos de los principios y de las técnicas que han conducido a la comprensión de la inmunología de *T. parva*, se han aplicado a la cowdriosis y deberían permitir un progreso rápido en el desarrollo de una vacuna contra *C. ruminantium*.

Palabras claves : Bovino - Cowdriosis - *Cowdria ruminantium* - Vacuna - Garrapata - *Theileria parva* - Linfocito - Anticuerpo - Antígeno - Infección experimental - Inmunidad.

A. F. Gomes[1]

The tick vectors of cowdriosis in Angola

GOMES (A.F.). Les vecteurs de la cowdriose en Angola. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 237-243

Parmi les espèces d'*Amblyomma* qui sont des vecteurs prouvés de *Cowdria ruminantium*, six ont été trouvées en Angola. *A. pomposum* est l'espèce la plus importante et la plus répandue. *A. variegatum*, prédominante dans les provinces de Cabinda et de Moxico, et *A. astrion*, qui se trouve dans la zone littorale et la zone de transition et sur le plateau de Camabatela, ont une importance moindre. *A. sparsum* et *A. tholloni*, parasites spécifiques d'hôtes sauvages, sont considérés comme des vecteurs accessoires. *A. hebraeum* a été introduit récemment dans le territoire mais sa distribution actuelle est inconnue. Les hôtes, la répartition, l'écologie et l'abondance saisonnière de ces espèces sont passés en revue.

Mots clés : Cowdriose - *Cowdria ruminantium* - Vecteur - Tique - *Amblyomma* - Hôte - Distribution naturelle - Variation saisonnière.

INTRODUCTION

Cowdriosis (heartwater) is a major tickborne disease of ruminants, caused by the rickettsial organism, *Cowdria ruminantium*, and is transmitted by 3-host ticks belonging to the genus *Amblyomma*. Transmission is transstadial although one case of transovarial transmission has been reported by BEZUIDENHOUT and JACOBSZ (4). ANDREW and NORVAL (2) investigated the role of males in the transmission of this rickettsia and demonstrated the transstadial (nymph to adult) and intrastadial transmission.

In Angola cowdriosis is a major disease problem for cattle and small ruminants originating from disease - free areas and sometimes a serious problem for local populations of small ruminants as well. Amongst the proven experimental vectors there exists *Amblyomma astrion, A. pomposum, A. sparsum, A. tholloni* and *A. variegatum*. *A. hebraeum* has been introduced recently in Camabatela plateau. *A. variegatum* has been proved to be an efficient vector of cowdriosis by DAUBNEY in 1930 (6) ; *A. tholloni* by MACKENZIE and NORVAL (16) ; *A. sparsum* by NORVAL and MACKENZIE (21) ; *A. pomposum* by NEITZ in 1947 (18) and afterwards by SERRANO (27) in Angola; *A. astrion* by UILENBERG and NIEWOLD (33) and *A. hebraeum* by LOUNSBURY (15). Besides these species, *A. splendidum, A. nuttalli* and *A. compressum* have also been recorded in Angola. *Amblyomma* ticks existing in this country have been reviewed previously by SERRANO (26).

METHODS

The present review is based on the material collected and identified by SOUSA DIAS (1949-1957) (28), SERRANO (1961-1974) (26, 27) and by the author (since 1975). The results of some of the detailed surveys accomplished by the author on the livestock areas are presented in table I. Ticks were identified according to descriptions by ROBINSON (25), SOUSA DIAS (28), HOOGSTRAAL (13), WALKER (34) and WALKER and OLWAGE (36).

Ecological zones were classified according to DINIZ and AGUIAR (12), faunal districts according to TAYLOR (30) and vegetation according to BARBOSA (3). Concerning the climate the Thornthwaite's classification presented by DINIZ (11) was followed.

TABLE I Records of **Amblyomma** *species on bovines in some of the livestock areas surveyed.*

Area	No. of collections recorded	No. of collections containing			
		A. astrion	*A. hebraeum*	*A. pomposum*	*A. variegatum*
Camabatela plateau	30	3	1	11	1
Cela	64	—	—	24	—
Dombe Grande	40	22	—	—	—
Huambo province	80	—	—	44	—
Huila province	202	—	—	146	—

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RESULTS

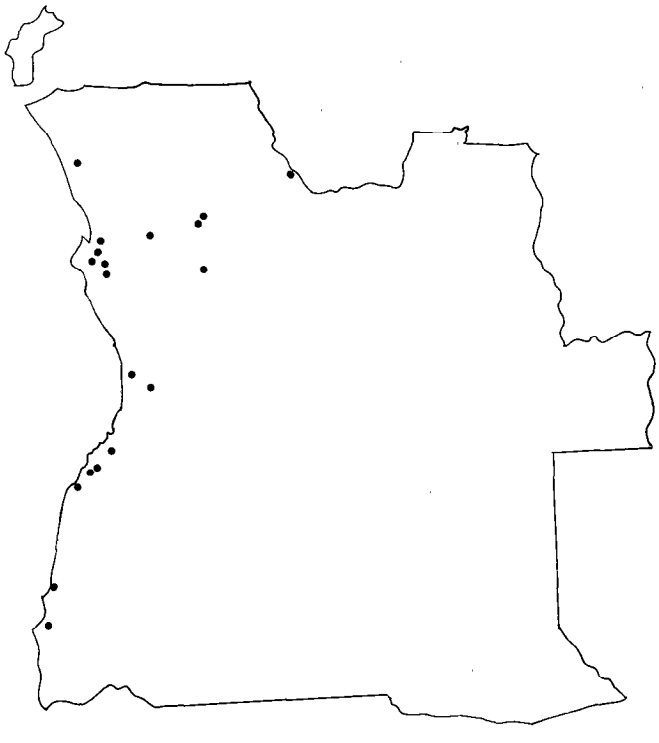
Amblyomma astrion Dönitz, 1909

Hosts

In Angola *A. astrion* is primarily a parasite of the African buffalo (*Syncerus caffer*) but it also parasitizes frequently domestic ruminants especially in the regions where both exist. It was also recorded from the wart hog (*Phacochoerus aethiopicus*). Immatures are found predominantly on buffalo although they have also been seen on domestic ruminants.

Distribution and ecology (map 1)

A. astrion was recorded in Zaire, Bengo, Cuanza Norte, Cuanza Sul, Malanje, Benguela and Namibe provinces (north-west or the country, especially in the littoral and transition strips, both warm and dry). Its Southern limit in Angola and probably in the continent is formed by the Curoca river (Namibe province). The existence of this tick in areas with a rainfall less than 250 mm p.a. (e.g. Dombe Grande, Benguela province, Curoca, Namibe province) would be explained by the microclimate existing there.



Map 1 : Localities at which Amblyomma astrion *has been collected.*

Altitude : 0 - 1,000 m.

Ecological zones : II, III, IV, V, VII, XII.

Faunal districts : this tick was collected mainly in the southwest arid district.

Vegetation : forest - savanna mosaic ; thicket - savanna mosaic, tree and shrub savanna ; tree and shrub steppes.

Climate : *A. astrion* predominates in the semi-arid zone, but it exists from arid to humid climates. Rainfall : 100-1,400 mm p.a..

Seasonal occurrence

In Dombe Grande adults were collected all along the year but the largest collections were recorded from October to March.

Comments

PETNEY et al. (23) considered *A. astrion* as a vector of secondary importance on cowdriosis epidemiology. Nevertheless, its role would be more important because this tick can survive in areas where its main host, the buffalo, is disappearing and being replaced by domestic ruminants (e.g. Dombe Grande, Benguela province). In São Tomé and Principe islands, *A. astrion* is a major parasite of domestic ruminants (32). Also, African buffaloes are also known to remain carriers of *C. ruminantium* for long periods (1).

Amblyomma pomposum Dönitz, 1909

DIAS (7, 8, 10) asserted that the tick classified as *A. pomposum* in Angola is in fact *A. superbum* and considers *A. pomposum* as a species apparently restricted to East Africa. Until this nomenclatural problem would be solved, we continue to denominate this tick as *A. pomposum*, as have done SOUSA DIAS (28) and SERRANO (26).

Hosts

In Angola cattle are the dominant domestic host, but *A. pomposum* has also been found on sheep, goats, horses, donkeys, pigs and dogs. Nymphae are commonly seen on bovines and small ruminants. Adults infests also a wide range of wild hosts but are found predominantly on ungulates. They have been recorded on the following hosts : buffalo (*Syncerus caffer*), eland (*Taurotragus oryx*), kudu (*Tragelaphus strepsiceros*), roan antelope (*Hippotragus equinus*), zebra (*Equus burchelli*), red lechwe (*Kobus lechwe*) banded mongoose (*Mungos mungo*), wart hog (*Phacochoerus aethiopicus*) and pangolin (*Manis tricuspis*). SERRANO (26) indicates that immatures prefer small rodents. BORGHT-ELBL (5) reveals that these forms parasitize birds and reptiles as well as

carnivores and ungulates. DIAS (9) identifies nymphs from a lion (*Panthera leo*).

Distribution and ecology (map 2)

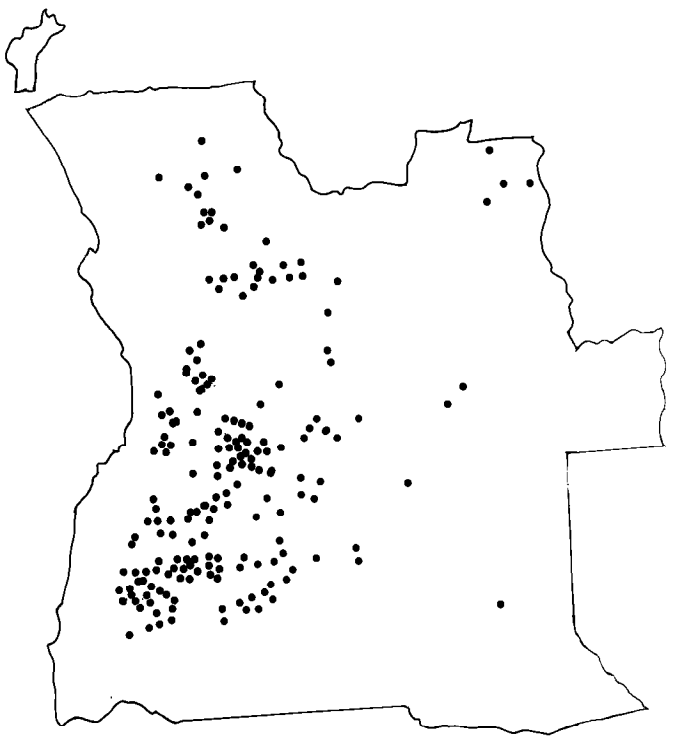
A. pomposum constitutes the most widely distributed *Amblyomma* species in Angola. It predominates in the highlands, where the climate and vegetation are more favourable. It has been collected in the following provinces : Uige, Malanje, Lunda Norte, Cuanza Norte, Cuanza Sul, Bie, Huambo, Benguela, Moxico, Cuando Cubango, Huila, Namibie and Cunene.

Altitude : it occurs above 500 m, but the regions of maximum abundance are located beyond 1,000 m.

Ecological zones : *A. pomposum* prevails in the zones VIII and IX, but it exists also in the zones V, X, XII, XIII. In the zones V and XVI it is scarce.

Faunal districts : it predominates in the Rhodesian highland district. Vegetation : it is especially a tick of woodlands.

Climate : *A. pomposum* prefers a humid climate. It is absent from arid areas and from almost all semi-arid areas. It prevails in areas where annual rainfall is higher than 1,000 mm but it was also recorded from areas receiving 800 mm p.a.



Map 2 : Localities at which Amblyomma pomposum *has been collected.*

Seasonal occurrence

Adults occur throughout the year, but heavier loads are present on cattle during the rainy season, from October-April. Substantial variation in the maximum abundance and in the timing of peak occurrence exists between regions with different climatic characteristics.

Comments

Economically *A. pomposum* is the most important *Amblyomma* species in Angola. It is the major vector of cowdriosis and its distribution has influenced the distribution of this disease which is more prevalent in the areas where *A. pomposum* exists. The tick itself causes abscess formation, lameness, anaemia, weight loss and is also associated with dermatophilosis.

A. pomposum becomes scarce in eastern Angola where it is substituted by *A. variegatum*. This rarefaction is probably due to the association between ticks, climate and particular vegetation types.

Amblyomma sparsum Neumann, 1899

Hosts

In Angola *A. sparsum* has never been recorded on domestic mammals, although YEOMAN and WALKER (37) reported the adult stage on cattle in Tanzania and NORVAL (19) stated that small numbers of nymphae may occur on cattle, sheep and goats in Zimbabwe. In Angola adults were collected from rhinoceros (*Diceros bicornis*), buffalo (*Syncerus caffer*), tortoises, large snakes and man, and nymphae from python (*Python sebae*) and tortoises. MOREL (17) noted that adults are parasites of both reptiles and mammals and asserted that *A. sparsum* has its place between *A. nuttalli* and ruminant *Amblyomma* (*A. variegatum* and *A. hebraeum* groups) in the series of the gradual adaptation of reptile ticks to mammals.

Distribution and ecology (map 3)

A. sparsum was recorded in south-west and south-east Angola (Benguela, Namibe, Huila and Cuando Cubango provinces).

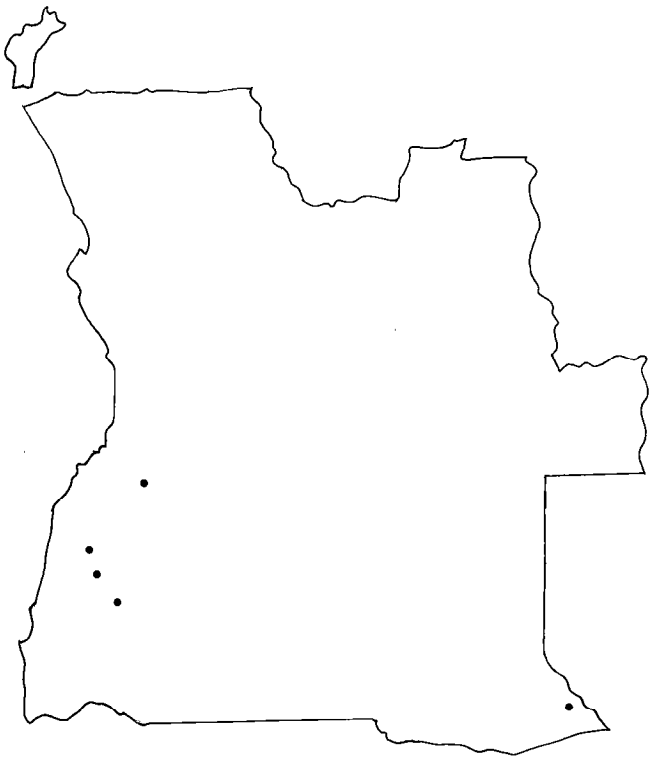
Altitude : 200-1,500 m.

Ecological zones : IV, V, XVI.

Faunal districts : although this tick has been collected predominantly in the south-west arid district, it exists also in the Rhodesian highland district.

Vegetation : woodlands; tree and shrub savanna.

Climate : *A. sparsum* exists in semi-arid and sub-humid areas with an average annual rainfall ranging from 200 to



Map 3 : Localities at which Amblyomma sparsum *has been collected.*

1,000 mm. BORGHT-ELBL (5) affirms that in areas where rainfall is less than 500 mm p.a. this tick probably survives in very humid microhabitats such as marshland and inundated areas along streams and rivers and lake shores.

Seasonal ocurrence

Insufficient data are available.

Comments

A. sparsum cannot be regarded as an important vector of cowdriosis because it is essentially restricted to wildlife. Nevertheless, in addition to than buffaloes, one of its reptile hosts, a tortoise, is known to be an experimental carrier of *C. ruminantium*. According to PETNEY *et al.* (23) it is an accidental vector.

Amblyomma tholloni Neumann, 1899

Hosts

In Angola adults of *A. tholloni* were collected only from elephants. We do not have any collection containing immature stages, but in Zimbabwe larvae and nymphae have been recorded from cattle, sheep and goats (16, 19). It is extremely unlikely that adults will attach on ungulates under field conditions (20).

Distribution and ecology (map 4)

A. tholloni was collected in the north-west (Zaire and Bengo provinces). It occurs in association with the African elephant and undoubtedly its distribution is more extensive than that mapped in map 4.

Altitude : 100-1,000 m.

Ecological zones : II, V.

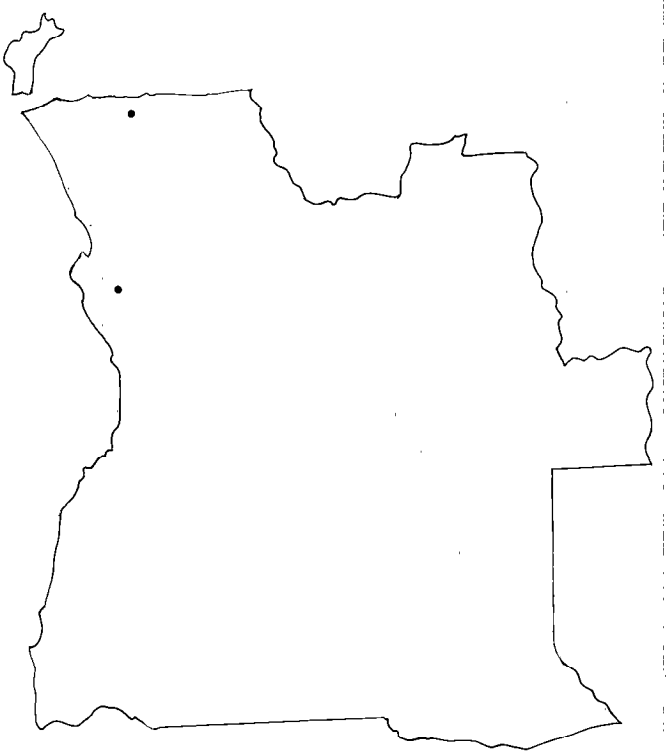
Faunal districts : southern Congo savanna district and southwest arid district.

Vegetation : forest-savanna mosaic; tree and shrub savanna.

Climate : *A. tholloni* was found in semi-arid and sub-humid zones. The rainfall ranges from 500 to 1,400 mm.

Seasonal occurrence

Few data are available to estimate its seasonal abundance.



Map 4 : Localities at which Amblyomma tholloni *has been collected.*

Comments

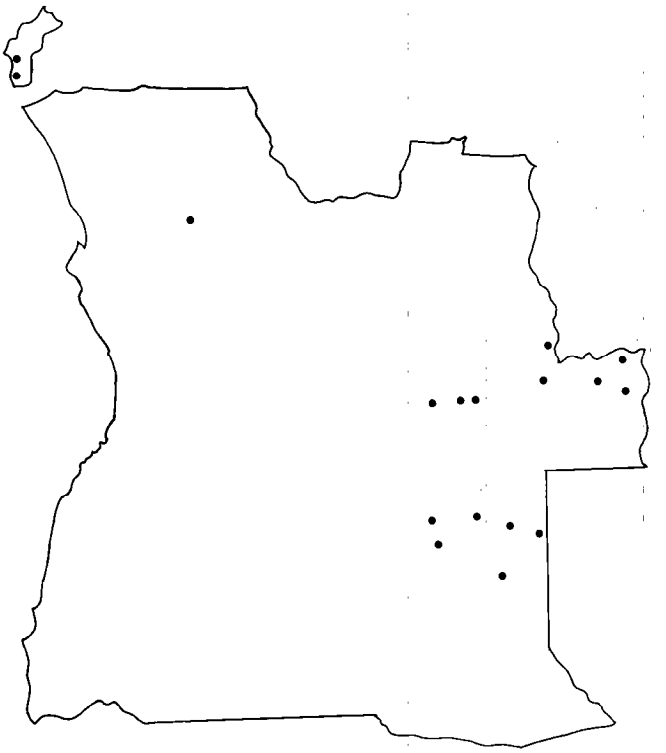
Adults of *A. tholloni* are found mainly on the African elephant (13, 19, 31, 35, 37). The immature stages are less host-specific and parasitize domestic ruminants and probably also wild ungulates making it possible that cowdriosis could be transmitted from wild to domestic hosts (16, 20). The knowledge that *A. tholloni* transmits *C. ruminantium* may be of value in understanding the origin of the disease in areas adjoining game reserves (16, 20). PETNEY *et al.* (23) classified this tick as an accidental vector of cowdriosis.

Amblyomma variegatum (Fabricius, 1794)

Hosts

In Angola *A. variegatum* occurs mainly on domestic ruminants, especially cattle, though sheep and goats are important hosts also.

Adults were also collected from horses, pigs, donkeys, buffalo (*Syncerus caffer*), kudu (*Tragelaphus strepsiceros*) and wart hog (*Phacochoerus aethiopicus*). Immatures predominate on domestic ruminants and dogs, but are also very common on birds, particularly ground-feeding ones (Galliformes, Gruiformes).



Map 5 : Localities at which Amblyomma variegatum *has been collected.*

Distribution and ecology (map 5)

A. variegatum is the most common *Amblyomma* of intertropical Africa on domestic and wild mammals. In Angola it predominates in the East (Moxico province), but it is also present in Cabinda and Camabatela plateau. On this plateau it was recorded only in one collection (table I).

Altitude : 0-1,500 m.

Ecological zones : I, VII, XIII, XIV, XV.

Faunal districts : southern Congo savanna district and Rhodesian highland district.

Vegetation : forest-savanna mosaic ; woodlands; tree and shrub savanna. On Camabatela plateau it inhabits thicket-savanna mosaic with a high grass cover. MOREL (17) indicates that egg-laying and resting places are at ground level, at the foot of graminaceous tufts or in thickets sometimes slightly in the ground.

Climate : sub-humid and humid. Rainfall : 800-1,600 mm p.a.

Seasonal occurrence

Adults occur mainly during the rainy season. PEGRAM *et al.* (22) indicate that in southern Africa nymphae occur from May to September and larvae from March to May. The tick undergoes only one generation per year.

Comments

A. variegatum is considered by PETNEY *et al.* (23) a major vector of cowdriosis. However, in Angola this species does not assume a great importance because it exists basically in regions with low densities of livestock. As predicted with the CLIMEX computer-based system (29) almost all the country constitutes a potential region for the distribution of *A. variegatum*. Nevertheless, this tick is confined to the east and in Cabinda Province. In the center and south-west, where almost all the Angolan livestock is concentrated, this species is replaced by *A. pomposum*. The exclusion of *A. variegatum* from these areas may be due to the interspecific competition between the 2 species. RECHAV *et al.* (24) demonstrated that this phenomenon occurs between *A. hebraeum* and *A. variegatum*, so it is possible that the same could happen between *A. variegatum* and *A. pomposum*, for example on the Camabatela plateau, where *A. pomposum* dominates and *A. variegatum* is very scarce.

Amblyomma hebraeum Koch, 1844

Amblyomma hebraeum is the major vector of cowdriosis in the southern part of the African continent, i.e. in South Africa, eastern Botswana, southern Zimbabwe and southern Mozambique (36). One isolated collection was made from cattle during the survey accomplished in 1982 on the Camabatela plateau, north-west Angola.

The most effective way of introducing a new species is the transportation of ticks on their hosts. *A. hebraeum* was probably introduced with cattle imported from Botswana in the course of the year 1981. This tick has long survival times and longevity of *A. hebraeum* male ticks while attached to the host is shown to be within the range 149-244 days (mean 177 days) (14). In 1984 the Camabatela plateau had around 18,000 bovines but afterwards these animals have been decimated during the civil war. We do not know if this tick is established itself in the region and if so, what its distribution is at present.

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GOMES (A.F.). The tick vectors of cowdriosis in Angola. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 237-243

Amongst the *Amblyomma* species presently known to be capable of transmitting *Cowdria ruminantium* six have been recorded in Angola. *A. pomposum* is the most important and widely distributed. Of secondary importance are *A. variegatum*, which prevails in Cabinda and Moxico provinces, and *A. astrion* which occurs in the littoral and transition strips and on Camabatela plateau. *A. sparsum* and *A. tholloni* which are specific parasites of wild hosts are considered accidental vectors. *A. hebraeum* was introduced recently in the territory but its distribution is unknown at present. For each of these species the hosts, distribution, ecology and seasonal abundance are listed.

Key words : Heartwater - *Cowdria ruminantium* - Vector - Tick - *Amblyomma* - Host - Natural distribution - Seasonal variation - Angola.

GOMES (A.F.). Las garrapatas que actúan como vectores de la cowdriosis en Angola. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 237-243

De todas las especies de *Amblyomma*, reconocidas como vectores de *Cowdria ruminantium*, seis han sido identificadas en Angola. La de mayor distribución es *A. pomposum*. En segundo lugar se encuentra *A. variegatum*, presente en las provincias de Cabinda y Moxico y *A. astrion*, la cual se encuentra en las zonas litorales y adyacentes, así como en la meseta de Camabatela. *A. sparsum* y *A. tholloni*, parásitos específicos de los animales silvestres, se consideran como vectores accidentales. Recientemente se introdujo en el territorio *A. hebraeum*, aunque se desconoce la amplitud de su distribución. Se da una lista de los huéspedes, de la distribución, la ecología y la abundancia estacionaria de cada especie.

Palabras claves : Cowdriosis - *Cowdria ruminantium* - Vector - Garrapata - *Amblyomma* - Huesped - Distribución natural - Variación estacional - Angola.

Poster

Ultrastructure of brain microvasculature in goats with experimental *Cowdria ruminantium* infection*

G.L.M. Mwamengele [1]

S. Larsen [2]

MWAMENGELE (G.L.M.), LARSEN (S.). L'ultrastructure de la microvasculature cérébrale de chèvres infectées expérimentalement avec *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 245

Afin d'étudier les lésions de la microvasculature cérébrale dans la cowdriose, 14 chèvres tanzaniennes ont été infectées par inoculation intraveineuse avec le stock Ball-3 de *Cowdria ruminantium*. Elles ont été suivies sur le plan clinique pendant la période d'incubation et la réaction fébrile, et sacrifiées lorsque les températures ont commencé à baisser. Cinq chèvres saines ont été utilisées pour déterminer la meilleure procédure pour la fixation du cerveau par perfusion et pour servir de témoins. La perfusion a été effectuée par l'artère carotide sous anesthésie générale au pentobarbitone, utilisant du glutaraldehyde de pH 7,4 à 3 p.100, à 500 mOsm. Des prélèvements de tissu cérébral ont été pris pour microscopie classique et électronique. Des signes variables de désordres du système nerveux central et un hydropéricarde peu important se sont développés chez toutes les chèvres infectées. Deux changements neuropathologiques différents ont été observés : des colonies de *Cowdria* dans des cellules endothéliales vasculaires, sans autres changements, et des petites infiltrations périvasculaires de cellules mononucléaires. Aucun signe de vasculite ou d'une perméabilité vasculaire anormale n'a été observé. Plusieurs phagocytes périvasculaires renfermaient des inclusions cytoplasmiques inhabituelles, se présentant comme des agrégations de particules irrégulièrement arrondies, associées à une membrane, de 0,25 à 0,4 µm de diamètre, ayant dans quelques cas une structure interne évocatrice de mitochondries partiellement dégradées. Néanmoins, ces agrégations ne semblaient pas enfermées de façon convaincante à l'intérieur de membranes, comme il est à à prévoir en cas d'autophagocytose. Une autre interprétation hypothétique est qu'elles représentent des stades abortifs de *C. ruminantium* qui tentent de se développer en dehors des vaisseaux et qu'une réponse immunitaire cellulaire, développée pendant et après la période d'incubation, limite ce deuxième cycle dans l'hôte et provoque des infiltrations périvasculaires mononucléaires.

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**Ce texte, dont seuls les résumés sont publiés dans ce volume, a fait l'objet d'un poster.*

MWAMENGELE (G.L.M.), LARSEN (S.). Ultrastructure of brain microvasculature in goats with experimental *Cowdria ruminantium* infection. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 245

To study microvascular brain lesions in heartwater, 14 Tanzanian blended goats were infected by IV inoculation with the Ball-3 strain of *Cowdria ruminantium*, monitored clinically during the incubation and febrile periods and killed when temperatures started to drop. Five, clinically healthy goats were used to establish the optimal procedure for perfusion fixation of brains and serve as control. Perfusion was done through the carotid artery under general pentobarbitone anesthesis using 3 % glutaraldehyde at pH 7.4 and 500 mOsm. Samples of brain tissue were collected for light and electron microscopy, complete necropsis were performed and various other tissues taken for light microscopy. Variable signs of CNS disorder and mild hydropercardium developed in all infected goats. Two, essentially different, neuropathologic changes were observed : colonies of *Cowdria* organisms in endothelial cells of otherwise unaltered vessels and small, perivascular mononuclear cell infiltrations. No signs of vasculitis or abnormal vascular permeability were found. Several perivascular phagocytes contained unusual cytoplasmic inclusions presenting as aggregations of irregularly rounded, membrane-bound particles, 0.25-0.4 µm in diameter, in some cases with an internal structure reminiscent of party degraded mitochondris. However, the aggregations were not convincingly enclosed within membranes as expected in case of autophagocytosis. Another, hypothetical, interpretation is that they represent abortive stages of *C. ruminantium* attempting to develop extravascularly and that possibly a cell-mediated immune response, developed during and after the incubation period, limits this second cycle within the host and results in the perivascular mononuclear cell infiltrations observed.

MWAMENGELE (G.L.M.), LARSEN (S.). Ultraestructura microvascular cerebral en cabras infectadas experimentalmente con *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 245

Con el fin de estudiar las lesiones microvasculares a nivel cerebral en la cowdriosis, 14 cabras de Tanzania fueron infectadas con IV inoculaciones con la cepa Ball-3 de *Cowdria ruminantium*. Los animales se siguieron clinicamente durante la incubación y los períodos febriles y se sacrificaron cuando se inició la disminución de la temperatura. Cinco cabras clinicamente sanas se utilizaron como control y también para establecer la técnica óptima para la fijación de la perfusion cerebral. La perfusion se administró en la arteria carótida, bajo anestesia general con pentobarbitona, con glutaraldeído al 3 p. 100, a pH 7,4 y 500 mOsm. Se colectaron muestras de tejido cerebral para microscopía de luz y electrónica. Se llevaron a cabo necropsias completas, con examen microscópico de varios tejidos. Todas las cabras presentaron signos variables de desórdenes del SNC y de hidropericardio leve. Se observaron principalmente dos cambios neuropatológicos : colonias de organismos de *Cowdria* en células endoteliales de vasos sin ninguna otra alteración y pequeñas infiltraciones perivasculares en células mononucleares. No se observaron signos de vasculitis o de anormalidad de la permeabilidad vascular. Algunos fagocitos perivasculares contenían inclusiones citoplásmicas poco usuales, organisadas en agregados de partículas irregularmente redondeados, con membrana limítrofe, de diámetro de 0.25 a 0,4 µm y en algunos casos se observó una estructura interna reminiscente de degradación parcial de mitocondrias. Sin embargo, los agregados no se encontraron suficientemente incluídos dentro de las membranas, como podría esperarse en los casos de autofagocitosis. Otra hipótesis de interpretación, es que estos agregados representen estados abortivos de *C. ruminantium* para desarrollarse extra celularmente, con una posible respuesta inmune de mediación celular, desarrollada durante y después del período de incubación. Se observaron los límites de este segundo ciclo dentro del huésped así como los resultados de las infiltraciones perivasculares de mononucleares.

Poster

An *in vitro* study of the life cycle of *Cowdria ruminantium* *

L. Prozesky [1]

A. Hart [1]

M.S. Brett [1]

PROZESKY (L.), HART (A.), BRETT (M.S.). Une étude *in vitro* du cycle de vie de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 247

Le cycle de vie de *Cowdria ruminantium* a été étudié dans des cellules SBE 189 par microscopie classique et électronique. Des cultures ont été infectées avec un inoculum synchronisé et fixées et préparées entre 15 min et 111 h post-inoculation (PI). Après 12 h, des grands corps réticulaires seuls ou en petits groupes ont été identifiés dans des vacuoles intracytoplasmatiques entourées de membranes. Ils se développaient graduellement dans des corps réticulaires plus petits avec une structure interne plus granuleuse. De 66 à 75 h PI, il y avait une augmentation importante de la taille des colonies. La plupart des colonies contenaient des corps réticulaires, bien que quelques corps intermédiaires et opaques aux électrons étaient visibles. Des corps réticulaires solitaires extracellulaires avec une couche ressemblant au peptidoglycan ont été observés 84 h PI. Après 90 h, des corps intermédiaires et opaques aux électrons étaient présents en abondance et cela coïncidait avec la lyse des cellules de culture. Le cycle de développement de *Cowdria ruminantium* durait donc environ 4 jours, dans cette étude.

PROZESKY (L.), HART (A.), BRETT (M.S.). An *in vitro* study of the life cycle of *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 247

The life cycle of *Cowdria ruminantium* was studied in SBE 189 cells by light and electron microcospy. Cultures were infected with a synchronized inoculum and fixed and processed form 15 min to 111 h post-inoculation (PI). After 12 h, single or small groups of large reticulate bodies were identified in intracytoplasmic membrane-bound vacuoles. The latter gradually developed into smaller reticulate bodies with a more granular internal structure. From 66 to 75 h PI, there was a significant increase in colony size. Most colonies contained reticulate bodies even though a few intermediate to electron-dense bodies were evident. Single extracellular reticulate bodies with a peptidoglycan-like layer was observed 84 h PI. At 90 h, abundant intermediate and electron-dense bodies were observed which coincided with lysis of tissue culture cells. In this study, *Cowdria ruminantium*, therefore, had a developmental cycle of approximately 4 days.

PROZESKY (L.), HART (A.), BRETT (M.S.). Estudio *in vitro* del ciclo de vida de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 247

Se estudió el ciclo de vida de *Cowdria ruminantium* en células SBE 189, tanto mediante microscopía de luz como electrónica. Los cultivos fueron infectados con inóculos sincronizados, luego fijados y procesados de 15 min a 111 h post inoculación (PI). Doce horas después, se identificaron en las vacuolas de las membranas intracitoplásmicas cuerpos reticulares solos o en pequeños grupos. Estos últimos se transformaron progresivamente en cuerpos reticulares más pequeños, con una estructura interna más granular. Sesenta y seis a setenta y cinco horas PI, se observó un aumento significativo en el tamaño de las colonias. La mayoría de las colonias contenía cuerpos reticulares, aunque se observaron algunos cuerpos intermedios y densos en electrones. Ochenta y cuatro horas PI se observaron cuerpos reticulares extracelulares únicos, con capas de pseudo peptidoglicanos. A las 90 h, se observaron abundantes cuerpos intermedios y densos en electrones, lo cual coincidió con la lísis de las células del cultivo. En este estudio, *Cowdria ruminantium* presentó un ciclo de desarrollo de aproximativamente 4 días.

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** Ce texte, dont seuls les résumés sont publiés dans ce volume, a fait l'objet d'un poster.*

Retour au menu

Session

Dermatophilose

SESSION DERMATOPHILOSE : INTRODUCTION

Dermatophilosis is an exudative skin disease of great economical importance, affecting numerous wild and domestic animal species as well as man. The disease is associated with the presence of the bacterium Dermatophilus congolensis *(Actinomycetes), which was first described in the Congo by VAN SACEGHEM in 1915. Despite much research since that time, dermatophilosis continues to seriously limit ruminant production, especially in the tropics. Although less important, it is also a problem in the temperate regions where it affects mainly sheep and horses.*

Several aspects of the disease such as diagnosis, cultivation of the bacterium and treatment are well known. In contrast, many aspects of the epidemiology and the immunological or immunopathological mechanisms remain obscure.

As far as epidemiology is concerned, it appears from the great number of observations made during the last decades, that the disease is a complex interplay between numerous intrinsic and environmental factors. Recent studies conducted in the Caribbean and elsewhere by several groups have improved the understanding of the epidemiology of dermatophilosis and led to a better classification of risk factors among which the breed and the presence of biting arthropods were confirmed as being of primary importance. In particular, the key role of adult Amblyomma variegatum *in the development of severe dermatophilosis in the region was pointed out. However, although the major risk factors were undoubtedly identified during these epidemiological studies, their respective role in the pathogenesis of the disease is still poorly understood. A better knowledge of the epidemiology of dermatophilosis has nevertheless allowed some progress in reproducing experimentally a chronic or an extensive dermatophilosis in small ruminants, by associating a bacterial infection with the infestation by adult* Amblyomma variegatum *ticks of susceptible ruminants in a humid environment. Despite this progress, a reliable experimental model of severe dermatophilosis is not yet available and it is likely that other factors considered as being of secondary importance such as direct sun light, are in fact important in the development of lesions in association with major risk factors. The improvement of this animal model is essential for the study of immunological mechanisms and the evaluation of vaccines.*

Up till now, vaccination trials have all failed whatever the method used: inoculation by several routes of whole bacterial cultures, inactived or not, and mixed or not with an adjuvant. A complete understanding of immunological mechanisms at the skin level is necessary before efficient vaccines can be developed. Concurrently, the genetic diversity of D. congolensis *must be studied. Antigenic variability has been revealed by cross-protection studies and the evaluation of its impact is essential in a programme of vaccine development.*

At the moment, the control of dermatophilosis is based on the rearing of genetically resistant breeds of ruminants and on tick control.

Rearing resistant breeds is obviously the best method to control a disease, however resistant breeds are not always sufficiently productive. As an example, high population growth associated with rural depopulation because of draughts has resulted in an increase of the demand in milk around towns in many developing countries, particularly in Africa. This production will not be satisfied by local breeds without an improvement of their genetic background by crossing with exotic breeds. However, there are many examples during the last decades of attempts to cross local breeds with highly productive exotic breeds having catastrophic results due to dermatophilosis. In the case of dermatophilosis of ruminants associated with A. variegatum *in Africa and the Caribbean region, this problem can be overcome by strict control of this particular tick using acaricides. Nevertheless, such control is time consuming, expensive and polluting. Furthermore, in other farming systems such as breeding merino sheep for wool production in Australia and South America, the methods of control are not well identified. There is therefore a need for new efficient and safe methods for controlling dermatophilosis.*

Recent progress in ruminant immunology and immunogenetics have given a new breath to the research on dermatophilosis. Research is in progress on the understanding of immunological mechanisms involved in the development and the resolution of dermatophilosis at the skin level in order to develop a vaccine, with particular attention being paid to the influence of A. variegatum, *UV light and malnutrition. Concurrently, polymorphic systems are being analysed at the DNA level in order to identify markers correlating with resistance or susceptibility to the disease. Such markers, once identified, could be used in selection programmes.*

Finally, in addition to the availability of powerful technological tools which make research more and more efficient, a better identification of the groups working on dermatophilosis with their research priority, and a better collaboration between some of these groups, are major improvements that will undoubtedly serve the advancement of research on this disease.

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Heterogeneity among *Dermatophilus congolensis* isolates demonstrated by restriction fragment length polymorphisms *

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FAIBRA (D.T.). Le polymorphisme des longueurs des fragments de restriction démontre l'hétérogénéité parmi des souches de *Dermatophilus congolensis* . *Revue Élev. Méd. vét. Pays trop.*,1993, **46** (1-2) : 253-256

L'existence de différences antigéniques et de virulence entre souches de *Dermatophilus congolensis* est connue. Afin de comprendre l'épidémiologie de la dermatophilose, il est important de pouvoir différencier entre les souches du germe. On a étudié vingt souches isolées sur le terrain à partir de bovins au Tchad et au Cameroun, ainsi qu'une souche américaine de référence, sur le polymorphisme des longueurs des fragments de restriction. Après digestion de l'ADN par l'enzyme de restriction *BamHI* et blotting selon Southern, une sonde d'ADN ribosomal consistant du plasmide pMC5, porteur d'une insertion d'ADN de *Mycoplasma capricolum* de 4,8 kb codant pour les RNA ribosomaux 5S, 23S et une partie des 16S, a permis de distinguer 6 ribotypes chez *D. congolensis*, selon les profils obtenus par hybridation des ADN ribosomaux. Certains ribotypes peuvent avoir une large répartition géographique. Par ailleurs, des souches appartenant à au moins 5 ribotypes peuvent être trouvées dans un même troupeau, ce qui peut partiellement expliquer le peu de succès obtenu lors d'essais de vaccination contre la dermatophilose sur le terrain.

Mots clés : Dermatophilose - *Dermatophilus congolensis* - Souche - ADN - Enzyme de restriction - Polymorphisme enzymatique - Southern blotting - Sonde à ADN - ADN/Ribosome - Hybridation d'ADN - *Mycoplasma capricolum* - Cameroun - Tchad.

INTRODUCTION

Dermatophilus congolensis, an ubiquitous actinomycete, is the causative agent of dermatophilosis, a worldwide cutaneous disease affecting both domestic and wild animals and occasionally man. This disease causes important economic losses in humid and subhumid tropical countries, more particularly West and Central Africa, Madagascar and the Caribbean islands. Dermatophilosis is also a main problem for sheep in Australia and New Zealand.

The genus *Dermatophilus* contains only one species : *D. congolensis* (5). But strain variation is recognized through some studies. In 1975, LLOYD and OJO (7) had found by means of the agar gel precipitation reaction 5 serologically different types among 14 strains (including 7 obtained from donkeys). These strains were isolated from a dermatophilosis outbreak which occurred in Western State of Nigeria. The donkey isolates included three of these types.

More recently in 1990, in the examination of the dose-response of rabbits to *D. congolensis* infection, HOW and LLOYD (6) found a ten-fold difference in the minimum infective dose of zoospores between a Scottish ovine strain (SS 18 C) and a Caribbean bovine strain (FD 11). They also noted an increased severity of the Caribbean strain lesions at the highest dose. These authors concluded that there is a difference in virulence between those strains of *D. congolensis* (FD 11 is more virulent than SS 18 C). In a recent vaccination trial in sheep in Australia differences in terms of virulence and antibody response were demonstrated between the two ovine *D. congolensis* strains used in the study (3).

In a recent biochemical profile of 92 strains of *D. congolensis* we have revealed 7 biotypes based on 3 discriminatory biochemical tests, Gamma Glutamyl Transferase, haemolysis of red blood cells of sheep and hydrolysis of gelatin (4).

There is a need for epidemiological studies to possess and strengthen alternative methods of discriminating among *D. congolensis* strains involved in enzootic and epizootic dermatophilosis. Several molecular typing methods have in recent years gained acceptance for analysing relationships between strains of a wide variety of pathogens. Some of these techniques offer the possibility of strain differentiation at the genomic level and thus are potentially very powerful epidemiologic tools (8). Genes coding for ribosomal ribonucleic acid (rRNA) are among the most conserved genes in procaryotic cells.

The restriction fragment length polymorphism (RFLP) of DNA fragments containing rRNA genes for *D. congolensis* isolates might serve as a distinguishing criterion to investigate the epidemiology of this microorganism.

In this study we show differences between 20 *D. congolensis* strains based on the hybridization pattern of DNA samples cleaved by endonucleases.

1. Laboratoire de Farcha, Ndjaména, Tchad. (Travail effectué lors d'un séjour au CIRAD-EMVT, Laboratoire "PATHOTROP", 10 rue Pierre-Curie, 94704 Maisons-Alfort Cedex, France).

* Presenté par J.C. Maillard.

MATERIALS AND METHODS

Dermatophilus congolensis isolates

The designation, the geographic origins and the dates of isolation of 20 field strains and the reference strain ATCC 14637 of *D. congolensis* tested are shown in table I. The field strains were collected from outbreaks of dermatophilosis occurring in some herds of northern Cameroon and southern Chad from 1986 to 1991. All of the field strains were isolated from skin lesions of zebu cattle affected by epizootic dermatophilosis.

TABLE I List of **Dermatophilus congolensis** *strains used in the study.*

No	Strain	Source
1	Dis. 1	isolated in 1989 from Dissing herd (Chad)
2	Dis. 2	idem
3	Dis. 5	idem
4	Dis. 7	idem
5	Dis. 16	isolated in 1990 from Dissing herd (Chad)
6	Dis. 11b	idem
7	N2	isolated in 1990 from Ngara herd (Chad)
8	N20	idem
9	NG18	isolated in 1990 from Ngondon herd (Chad)
10	D4	isolated in 1989 from Doué herd (Chad)
11	D8	idem
12	NG16	isolated in 1990 from Ngondon herd (Chad)
13	NG12	isolated in 1989 from Ngondon herd (Chad)
14	D22	isolated in 1990 from Doué herd (Chad)
15	F3004	isolated in 1988 from Chari-baguirmi (Chad)
16	ATCC* 14637	reference strain
17	G2	isolated in 1987 from Garoua (Cameroon)
18	NAS32	isolated in 1988 from Nassarwo (Cameroon)
19	W8588	isolated in 1985 from Wakwa (Cameroon)
20	W8753	isolated in 1987 from Wakwa (Cameroon)
21	W9028	isolated in 1990 from Wakwa (Cameroon)

* ATCC : American Types Cultures Collection.

Cultivation of the strains

After isolation, all strains were cloned. Growth of *D. congolensis* for DNA isolation was in tryptose broth with horse serum incubated at 37 °C with shaking and stirring with glass beads. *D. congolensis* cells were harvested after 72 h growth by centrifugation and the pellets were washed once and used for DNA extraction.

DNA extraction

DNA was extracted by lysis of bacterial pellets with lysozym and SDS. Then a standard phenol chloroform extraction with ethanol precipitation was repeated twice.

DNA yield was determinated by spectrophotometric analysis. The DNA concentration of the 21 *D. congolensis* purified DNA samples varied from 0.5 to 3 µg/µl.

Restriction enzyme digestion of DNA and separation of fragments

2 µg of each DNA sample were digested with the following restriction endonucleases : *BamHI*, *EcoRI* and *PstI*.

Digested DNA fragments were separated electrophoretically on agarose gel stained with ethidium bromide and transferred to a nylon filter by the method of SOUTHERN (10).

DNA blots were probed with plasmid pMC5 carrying a 4.8 kilobases insert of *Mycoplasma capricolum* DNA coding for the 5S, 23S, and part of 16S rRNAs (1). Cloned rDNA probes were labelled by using the "random priming" method.

The blots were hybridized, washed, air dried and exposed to X Ray films. Lambda DNA digested by *PvuII* served as molecular weight markers.

RESULTS AND DISCUSSION

In an attempt to find an enzyme which gave a reasonable number of well-separated bands, we examined the restriction patterns generated by *EcoRI*, *PstI* and *BamHI*.

The result with *BamHI* was most encouraging (fig. 1). With most *D. congolensis* strains, this enzyme gave two or three fragments hybridizing with the probe. The strains were grouped in 6 ribotypes according to their hybridized rDNA patterns. The diagrams of each of the 6 ribotypes observed within the isolates were shown in figure 2.

The ribotype 1 is distributed in almost all of the herds from which *D. congolensis* strains were collected (table II). The herds were about 100-200 km distant from each other. Five of the 6 ribotypes (ribotypes 1, 2, 3, 4 and 6) were found in the Dissing herd. Heterogeneity between the *D. congolensis* strains in the same flock is important. These findings agree with the previous observations of LLOYD and OJO in 1975 (7) showing variation of *D.congolensis* strains in the field. In their study, they observed that 3 of the 5 identified *D. congolensis* serogroups occurred in the same outbreak of dermatophilosis in donkeys.

These findings may in part explain the failure of field vaccination trials carried out in the past in Chad by PROVOST et al. (9) and CHENEAU (2) and more recently in Australia by ELLIS *et al.* (3).

In conclusion, this study shows that ribotyping can be a valuable tool for characterization of *D. congolensis*. This

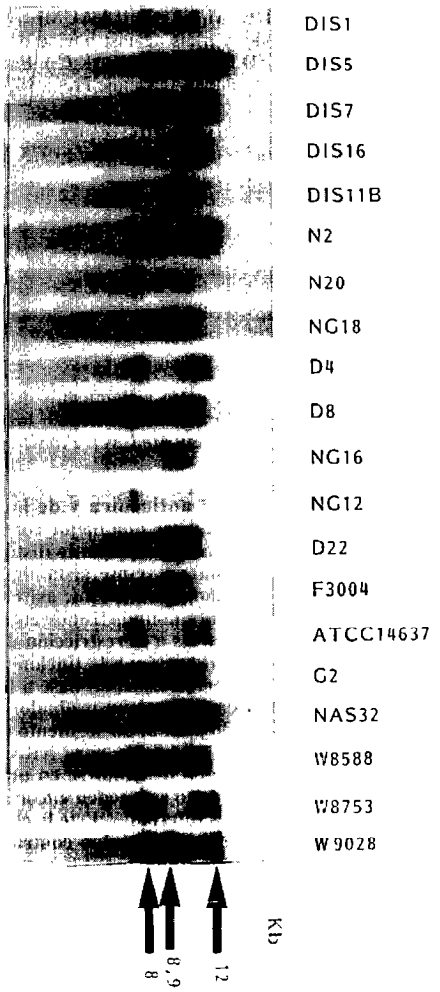

Figure 1 : RFLP patterns of Dermatophilus congolensis *strains studied.*

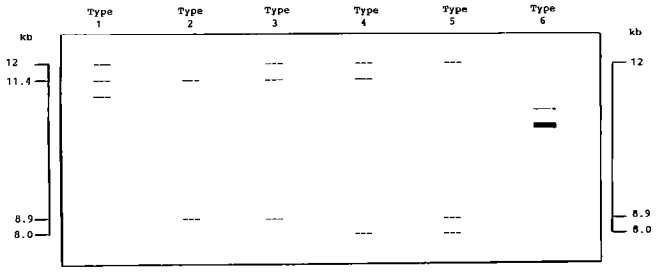

Figure 2 : Pattern of each of the 6 identified ribotypes of Dermatophilus congolensis.

genomic fingerprinting technique emphazises DNA restriction site heterogeneity between isolates, a characteristic presumably more stable than those studied by traditional phenotypic characterization techniques such as serotyping and biotyping.

TABLE II Ribotype grouping of **Dermatophilus congolensis** *strains.*

Ribotype groups	Composition
Ribotype 1 (6 strains)	Dis16, Ng18, Ng16, D22, G2 and Nas32
Ribotype 2 (2 strains)	Dis1, N20
Ribotype 3 (4 strains)	Dis5, D4, Ng12, ATCC14637
Ribotype 4 (4 strains)	Dis7, D8, W8588, W8753
Ribotype 5 (2 strains)	N2, W9028
Ribotype 6 (2 strains)	Dis11b, F3004

In particular ribotyping compares highly conserved rRNA genes and their adjacent sequences, genes not subject to frequent mutation.

ACKNOWLEDGEMENTS

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FAIBRA (D.T.). Heterogeneity among *Dermatophilus congolensis* isolates demonstrated by restriction fragment length polymorphisms. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 253-256

There is evidence of antigenic diversity and of differences in virulence in *Dermatophilus congolensis*. For the understanding of the epidemiology of dermatophilosis it is important to distinguish between strains of the organism. Twenty field isolates from cattle in Chad and Cameroon, and an American reference strain, have been examined on restriction fragment length polymorphisms. After restriction enzyme digestion of DNA by *BamHI* and Southern blotting, a rDNA probe consisting of plasmid pMC5 carrying a 4.8 kb insert of *Mycoplasma capricolum* DNA coding for the 5S, 23S and part of 16S rRNA allowed to distinguish 6 ribotypes of *D. congolensis*, based on their hybridized rDNA patterns. Particular ribotypes may be distributed over a wide geographical area. On the other hand, strains belonging to at least 5 different ribotypes may be found in one herd; this may partly explain the lack of success in immunization against dermatophilosis in the field.

Key words : Dermatophilosis - *Dermatophilus congolensis* - Strain - DNA - Restriction enzyme - Enzyme polymorphism - Southern blotting - DNA probe - Ribosomic DNA - DNA Hybridization - *Mycoplasma capricolum* - Cameroon - Chad.

FAIBRA (D.T.). Heterogeneidad entre aislamientos de *Dermatophilus congolensis*, demostrada mediante polimorfismos en los fragmentos de restricción de la longitud. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 253-256

Se ha demostrado la diversidad antigénica y de la virulencia de *Dermatophilus congolensis*. Para lograr una mejor comprensión de la epidemiología de la dermatofilosis, es importante distinguir entre las cepas del organismo. Se examinaron veinte aislamientos de campo provenientes de ganado de Chad y Camerún, así como una cepa Americana de referencia, mediante polimorfismos en los fragmentos de restricción de la longitud. Gracias a la restricción de la enzima de digestión de ADN por parte del *BamHI* y a la tinción "Southern", se identificaron 6 ribotipos de *D. congolensis*, en base a los patrones de hibridación del ADNr. El test consiste en un probador de ADNr, que transporta mediante un plásmido pMC5, un segmento de ADN codificado de *Mycoplasma capricolum* de 4,8 kb, para ARNr de 5S, 23S y parte del 16S. Los ribotipos pueden distribuirse en una amplia zona geográfica. Por otro lado, en un hato se pueden encontrar cepas provenientes de hasta cinco ribotipos diferentes. Este hecho podría explicar parcialmente el fracaso de la inmunización contra dermatofilosis en condiciones de campo.

Palabras claves : Dermatofilosis - *Dermatophilus congolensis* - Cepa - ADN - Enzyma de restrición - Polimorfismo enzimatico - Southern blotting - Sonda de ADN - ADN Ribosomico - Hibridación de ADN - *Mycoplasma capricolum* - Camerún - Chad.

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Culture filtrate proteins of *Dermatophilus congolensis*

EL JACK (M.A.), AMBROSE (N.). Culture de protéines filtrées de *Dermatophilus congolensis*. *Revue Élev. Méd. vét. Pays trop.*, 1992, **46** (1-2) : 257-261

Lors d'études antérieures sur les antigènes de *Dermatophilus congolensis*, très peu d'attention a été accordée aux hyphes et aux produits d'excrétion/sécrétion (PES) des bactéries en croissance active. Nous avons cultivé quatre isolats de *D.congolensis* dans un milieu liquide synthétique, sans sérum, à base de RPMI 1640. Les PES ont été préparés à partir du liquide de culture infectée, par diafiltration et ultrafiltration. Ces méthodes ont produit des quantités de PES suffisantes pour étudier les profils de polypeptides des quatre isolats par SDA-PAGE et Western immunoblotting. Les quatre isolats ont produit un grand nombre de polypeptides en culture, dont la plupart étaient produits par tous les quatre. Néanmoins, des poplypeptides propres à chaque isolat étaient également produits. Des études par Western immunoblotting sur des sérums mélangés d'animaux du Ghana affectés de façon chronique, ont montré que certains polypeptides dans les PES d'un isolat du Ghana étaient antigéniques. Lorsque les mêmes sérums ont été testés sur les PES d'un isolat écossais, certains polypeptides du même poids moléculaire que ceux dans l'isolat ghanéen, ainsi que d'autres de poids moléculaires différents, ont été reconnus. Ceci indique que des isolats de *D.congolensis* de régions géographiques différentes produisent des PES avec des déterminants antigéniques communs.

Mots clés : *Dermatophilus congolensis* - Protéine - Technique de culture - Polypeptide.

INTRODUCTION

Dermatophilus congolensis is a Gram positive actinomycete bacterium which causes the skin disease dermatophilosis. It has a worldwide distribution, and a wide host range including a wide variety of animal species and man.

D. congolensis has a multiphasic-life cycle which was described by ROBERTS (8) and ABU-SAMRA (1). Infective zoospores or cocci give rise to branching hyphae which undergo longitudinal and transverse divisions to produce filaments constaining cocci. The branching hyphae invade the living non-cornified layer of the epidermis, and may be regarded as the proliferative stage of the bacterium. The role and significance of hyphae and their excreted-secreted products (ESP) in the immunology and pathogenesis of this disease are yet to be fully determined. Apart from the investigation of exo-antigens by KWAPINSKI (5), no detailed, systematic studies on hyphae and ESP of this organism have been carried out. However, several different approaches and media have been adopted in research on various *D. congolensis* antigens.

The main objective of this work was to study the protein composition of ESP produced by actively growing *D. congolensis* in a defined synthetic serum free medium by the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. We have attempted to characterize and compare individual isolates from different geographical regions by examining their protein profiles. We have tested sera from chronically infected cattle for the presence of antibodies against ESP of two isolates to show that unique and cross-reactive antigens occur in these isolates.

MATERIALS AND METHODS

D. congolensis isolates

Four isolates of *D. congolensis* were used in this study (table I). They were recovered from naturally infected cattle and sheep. They had been passaged in blood agar and broth culture twice in our laboratory.

TABLE I Origin of **D. congolensis** *isolates used in this study.*

Isolate	Host	Origin
A5N	Sheep	Scotland, U.K.
Gh89	Cattle	Ghana, Africa
Zambia	Cattle	Zambia, Africa
Paynters, Antigua	Cattle	Antigua, West Indies

Growth conditions and preparation of ESP

Stabilates of the isolates were grown, without shaking, in 75 cm^2 non-inhibitory tissue culture flasks (Nunc) containing 15 ml of wholly synthetic serum free liquid culture medium [RPMI 1640, 0.05 % sodium pyruvate, 0.025 % sodium metabisulfate and 0.001 % ferrous sulfate] at

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37 °C in air containing 5-10 % CO_2 for 48 h. The ESP were collected from infected culture fluid of each *D. congolensis* isolate. Phenyl methyl sulphonylfluoride (PMSF) and ethylene diamine tetra acetic acid (EDTA) were added at 1 mM and 5 mM final concentration, respectively. The culture fluid was centrifuged at 3,000 x g for 15 min at 4 °C to remove bacteria. The supernatants were filtered through a 0.45 µm Millipore low protein binding filter and then desalted and washed three times, by diafiltration, with 3 volumes of phosphate buffered saline (pH 7.4) containing PMSF and EDTA. Finally the supernatants were concentrated by ultrafiltration and stored at -20 °C until they were used. Millipore membranes whith a 10,000 dalton cutt-off were used for diafiltration and ultrafiltration.

Polyacrylamide Gel Electrophoresis (PAGE)

Separation of proteins in the ESP according to their molecular weight was achieved using 1.5 mm thick slab gels. A 4.5 % stacking gel, and a 7-20 % gradient separating gel were used. Samples were boiled for 5 min in sample buffer, containing 0.1 % dithiotreitol, 10 % SDS and a trace of bromophenol blue, and loaded into each gel track. Molecular weight markers (6-200 kDa) were run on the same gel. Electrophoresis was carried out at 100 volts overnight. Gels were fixed in ethanol and acetic acid. Several different ESP preparations of each isolate were prepared at different times and each preparation of ESP was run on at least 3 occasions.

Western immunoblotting

Proteins from SDS-PAGE gels of the A5N and Gh89 isolates were electrotransferred onto nitrocellulose membranes (0.45 µm pore size) using a Bio-Rad semidry transfer cell. The gels were soaked in transfer buffer for a few minutes beforehand. Electrophoresis was carried out at 200 mA for 90 min. After transfer the nitrocellulose membrane was blocked with 5 % dried milk powder in buffer overnight. The membrane was then divided as necessary, and probed overnight with gentle shaking at room temperature in a 1:50 dilution of pooled chronic sera from 9 dermatophilosis infected cattle from Ghana, West Africa. The membranes were washed 3 times for 15 min each with PBS-0.01 % Tween. The strips were screened for antibody binding by incubation with biotin conjugated goat antibovine IgG, the strips were then washed in PBS/Tween 3 times, 15 min/each. The blots were then incubated in streptavidin alkaline phosphatase in 4 % normal goat serum for 1 hour at 37 °C. Blots were washed again in PBS/Tween and incubated in the substrate bromo-chloro-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) to visualize the reaction.

RESULTS

The procedures for desalting, washing and concentrating the culture fluid and the use of protease inhibitors were necessary for detailed electrophoretic analysis of proteins in ESP. Preliminary results using untreated culture fluid were poor and desalting by dialysis followed by freeze-drying produced indistinct protein bands. The results shown here are all of samples that were treated by diafiltration and ultrafiltration. As shown in figures 1 and 2, it is evident that the isolates used in this study produced excreted-secreted products (ESP) during their growth in supplemented RPMI 1640. The ESP obtained by this method were satisfactory for the purposes of characterizing *D. congolensis* isolates by their SDS-PAGE polypeptide profiles and for testing their antigenicity by Western immunoblotting. There was a satisfactory reproducibility between different ESP preparations of each isolate. The different isolates produced a number of peptides of the same molecular weight and several that were unique to each isolate.

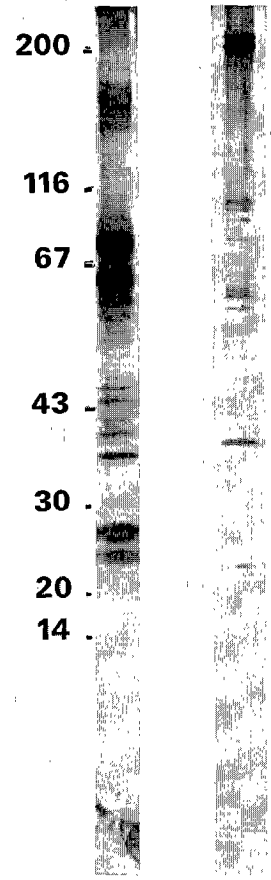

Figure 1 : The ESP of a Scottish isolate (lane 1) and a Ghanaian isolate (lane 2) separated by SDS-PAGE. The Gh89 ESP was diluted to reveal the bands above the 200 kDa marker. Molecular weight standards are shown on the left.

Figure 2 shows the polypeptide profiles of the ESP of the four *D. congolensis* isolates. The points of note are that bands shared by all four isolates were numerous between 15 and 70 kDa, for example the bands at 25, 29, 36, 44, 66 and 70 kDa.

The Ghanaian isolate, Gh89 in lane 2 of figure 1 had double bands with molecular weight of more than 200 kDa, and in lane 3 of figure 2 had bands at 94, 80 and 82 kDa bands that were not found in any of the other 3 isolates. The Scottish isolate, A5N in lane 1 of figure 1 and lane 2 of figure 2 had unique bands at 120, 84, 76, 70 and 58 kDa.

The Antiguan and Zambian isolates in lanes 4 and 5 of figure 2 respectively had very similar profiles as detailed by the silver staining ; the only unique bands were at 67 kDa for the Antiguan isolate and the 69 kDa in the Zambian isolate.

Figure 3 shows the Western immunoblotting results of ESP of Gh89 (lane 1) and A5N (lane 2) tested using pooled chronic cattle sera. The sera contained antibodies against a number of antigenic bands in the ESP of both of the isolates for example bands at 25, 27, 33 and 47 kDa were antigenic and are common to both isolates. The difference between them being that in the Gh89 ESP one

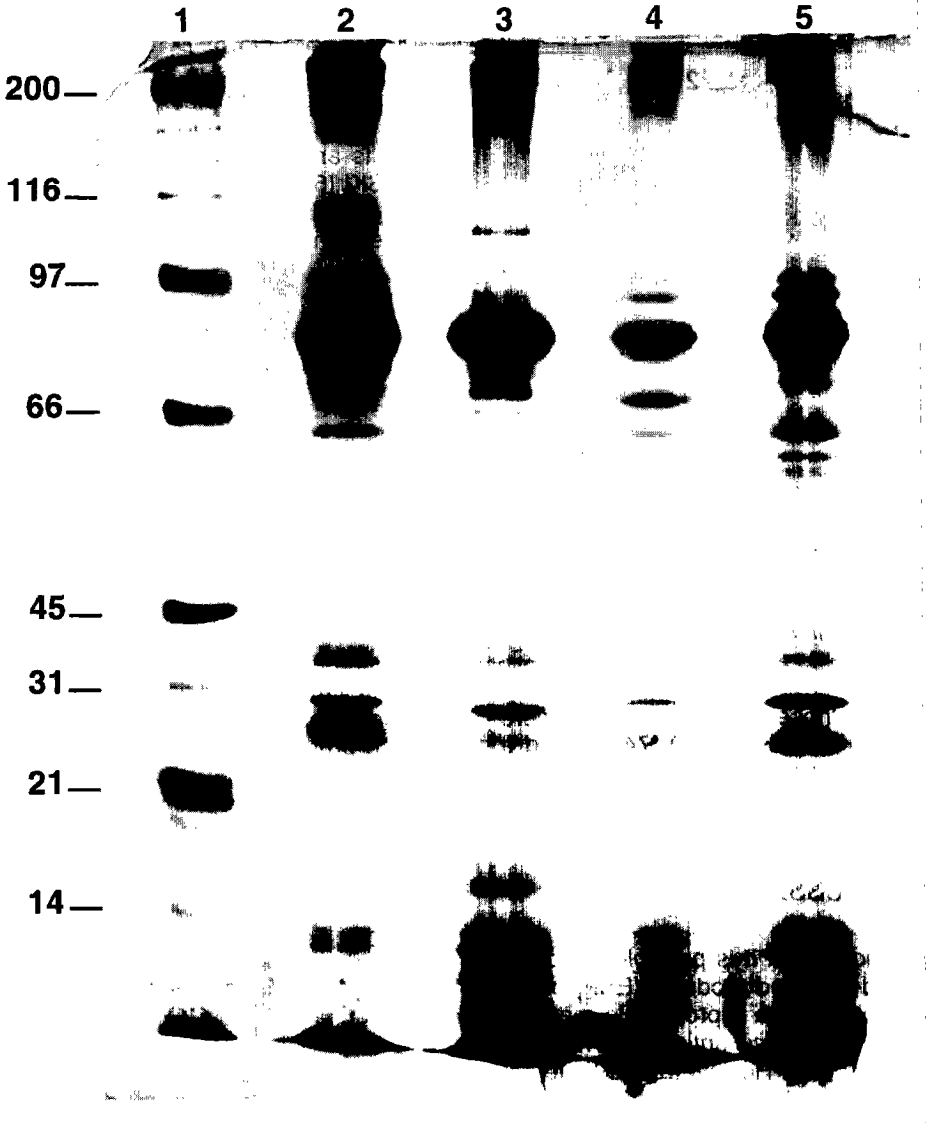

Figure 2 : The ESP of four isolates separated by SDS-PAGE. Lane 2, Scottish ; lane 3, Ghanaian ; lane 3, Antiguan ; lane 4, Zambian. Molecular weight standards are shown on the left.

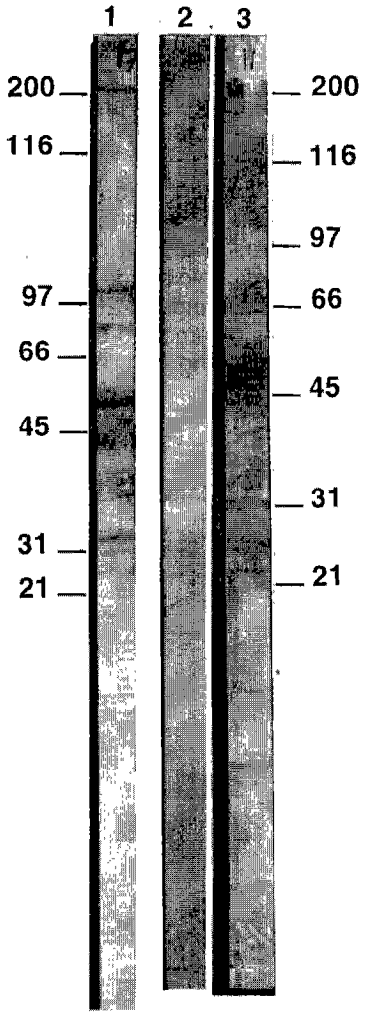

Figure 3 : Western immunoblot of ESP of Ghanaian (lane 1) and Scottish (lane 3) isolates both probed with pooled sera from chronically infected cattle from Ghana. The 2 lanes were taken from different blots, the molecular weight standards are shown to the left and right. Lane 2 is an example of a control using ESP from the Scottish isolate probed with commercial newborn calf serum.

band above 200 kDa, and those at 91 and 59 kDa were antigenic and in A5N ESP bands at 120, 74, 70 and a large band at 52 kDa were antigenic.

DISCUSSION

In this study we have shown that it is possible to collect and concentrate excreted-secreted products (ESP) of different *D. congolensis* isolates in a reproducible manner. Researchers working *D. congolensis* antigens have referred to their complexity (5, 7, 10). However there have not been any attempts to pursue their characterization and there has not been a common approach to antigen preparation. We have attempted to overcome some of these problems by cultivating this organism under controlled conditions in a synthetic serum free liquid culture medium, the main component of which is standardized and commercially available. The medium may be suitable for a wide range of studies on the biology of *D. congolensis*.

Other researchers working on ESP of actinomycetes, such as FIFIS et al. (3), have used prolonged periods of cultivation in which some extracellular products may be degraded. We have overcome this problem by using a short period of culture, by the addition of protease inhibitors to all stages of protein preparation, minimising the time taken to concentrate the ESP and keeping the temperature of the solutions low. Diafiltration and ultrafiltration as a method of ESP preparation, is superior to dialysis and concentration by freeze drying.

All four isolates of *D. congolensis* studied produced a number of proteins in their ESP, the majority of which are common to all isolates. However, we have also observed differences in the polypeptide profiles of the isolates. Our results are therefore in agreement whith those of KWAPINSKI (5) and the recent studies of MAKINDE (6), HOW and LLOYD (4) and SUTHERLAND et al. (9) that there is more than one strain of this organism.

Our Western immunoblotting results illustrate that in sera against one of those isolates there were antibodies to common antigens of the same molecular weights in both isolates. In addition there were bands in both isolates that were unique, indicating that A5N has antigenic determinants in common with Gh89, but they occur on peptides of different molecular weights. It can be concluded that the ESP of isolates probably have both shared and common antigens. If the unique antigens are host protective then this could be one reason why attempts to produce an effective vaccine against dermathophilosis have no been successful.

Proteins secreted by many pathogens play an important role in virulence and tissue invasion as they can be toxins or enzymes. Antibody production is often directed against these proteins. Growing mycobacteria have been shown to release proteins into their surroundings and there is evidence that these antigens evoke protective T-cell responses (2). Therefore it is of considerable importance to determine the role and significance of hyphae and ESP in the immunology of *D. congolensis* infections.

CONCLUSION

Four isolates of *D. congolensis* from different geographical regions were grown in a serum free synthetic culture medium based on RPMI 1640.

Diafiltration and ultrafiltration were used to desalt, wash and concentrate culture fluid containing excreted-secreted products (ESP) of each isolate. Proteins in the ESP were separated by SDS-PAGE and tested for antigenicity by immunoblotting with pooled sera from chronically infected cattle.

The excreted-secreted products of the four isolates contained a large number of polypeptides. Many of these were produced by all four isolates. Other polypeptides appeared to be uniquely produced by only one isolate.

Pooled chronic sera contained antibodies against a number of ESP polypeptides from the isolate the animals were exposed to and from an isolate from a different geographical area. This indicates that ESP of different isolates have shared antigenic determinants.

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EL JACK (M.A.), AMBROSE (N.). Culture filtrate proteins of *Dermatophilus congolensis*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 257-261

In previous studies on the antigens of *Dermatophilus congolensis* very little attention has been given to the hyphae and to excreted-secreted products (ESP) of actively growing bacteria. In this study we have grown four isolates of *D. congolensis* in a serum free synthetic liquid culture medium based on RPMI 1640. Diafiltration and ultrafiltration were used to prepare ESP from infected culture fluid. These methods produced sufficient quantities of ESP that the polypeptide profiles of the four isolates could be examined by SDS-PAGE and Western immunoblotting. The four isolates produced a large number of polypeptides in culture, most of which were produced by all four isolates. However each isolate produced polypeptides that were unique to it. Western immunoblotting studies using pooled sera from chronically affected animals from Ghana showed that a number of polypeptides in ESP of a Ghanaian isolate were antigenic. When the same sera was tested against ESP from a Scottish isolate a number of polypeptides of the same molecular weight as those in the Ghanaian isolate and some at different molecular weights were recognized. This indicates that isolates of *D. congolensis* from different geographical areas produce ESP with shared antigenic determinants.

Key words : *Dermatophilus congolensis* - Protein - Culture technique - Polypeptide.

EL JACK (M.A.), AMBROSE (N.). Cultivos de filtrados proteínicos de *Dermatophilus congolensis*. *Revue Élev. Méd. vét. Pays trop.*, 1992, **46** (1-2) : 257-261

En estudios anteriores sobre los antígenos de *Dermatophilus congolensis*, se ha dado poca importancia a las hifas y a los productos de excresión y secresión (ESP) de las bacterias en crecimiento activo. En el presente trabajo, se cultivaron cuatro muestras de aislamientos de *D. congolensis* en medios de cultivo no séricos, líquidos y sintéticos, basados en RPMI 1640. Para preparar los ESP, a partir de cultivos fluídos, se utilizó tanto la diafiltración como la ultrafiltración. Estos métodos produjeron suficiente cantidad de ESP como para permitir el examen del perfil polipeptídico de los cuatro aislamientos, gracias a la inmuno-tinción de SDS-PAGE y al Western blotting. El cultivo de los cuatro aislamientos produjo una gran cantidad de polipéptidos, la mayoría de los cuales fue producida por cada una de las cuatro muestras. A pesar de esto, también se observó que cada muestra produjó algunos polipéptidos propios. La tinción mediante el Western blotting de un pool de sueros provenientes de animales ganenses portadores de una infección crónica, mostró que varios de los polipéptidos de los ESP de las muestras ganenses presentaban características antigénicas. Cuando estos mismos sueros fueron sometidos a un examen contra ESP provenientes de un aislamiento escocés, se identificaron varios polipéptidos, tanto de mismo peso molecular que aquellos del aislamiento ganense, como de peso molecular diferente. Esto indica que los aislamientos de *D. congolensis* provenientes de diferentes zonas geográficas producen ESP con determinantes antigénicos comunes.

Palabras claves : *Dermatophilus congolensis* - Proteina - Técnica de cultivo - Polipéptido.

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Experimental dermatophilosis in murine models of immunodeficiency

SASIAK (A.B.), SEBESTENY (A.), HRIVNAK (G.), LLOYD (D.H.). Utilisation de modèles souris pour la dermatophilose expérimentale. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 263-269

Des souris gnotobiotiques ayant des déficiences immunitaires congénitales ont été infectées avec *Dermatophilus congolensis*, agent pathogène cutané. Des souris sans thymus (nues), avec une déficience en cellules T, se sont montrées moins sensibles que des souris nues qui portaient également la mutation beige (bg/nu) ayant des défauts de cellules tueuses et de granulocytes, l'équivalent murin du syndrome de Chediak-Higashi. La présence additionnelle chez d'autres souris avec la mutation beige, du gène d'immunodéficience lié au chromosome X, qui cause une réduction de la réponse des cellules B, n'a pas augmenté la sensibilité. Des souris BALB/c possédant la mutation nue et montrant une déficience de macrophages, avaient un niveau modéré de sensibilité, plus élevé que celui de souris nues non consanguines mais moins que celui des souris beiges-nues. Les lésions sur les souris à poils avaient un aspect différent de celles sur les souris nues (nu et bg/nu). Sur les souris à poils, des croûtes minces se développaient et guérissaient rapidement, tandis que les lésions sur les souris nues commençaient comme des nodules et se changeaient ensuite en croûtes. Les souris nues BALB/c développaient des lésions atypiques, ressemblant à des ulcères. Des souris axéniques nues et beiges-nues ont montré les mêmes types et mêmes durées d'infection que les animaux gnotobiotiques, ce qui suggère que l'intervention par des bactéries, d'une flore cutanée limitée, ne jouait pas de rôle majeur dans la défense contre *D. congolensis*. Néanmoins, une analyse bactériologique a montré que *D. congolensis* pouvait survivre dans l'intestin de souris axéniques. Ce travail accentue l'importance de mécanismes immunitaires non-spécifiques dans la résistance à *D. congolensis*, tels que l'hyperprolifération épidermique et le neutrophile.

Mots clés : Souris - Dermatophilose - *Dermatophilus congolensis* - Infection expérimentale - Immunodéficience - Lésion - Maladie de la peau - Gène - Résistance aux maladies.

INTRODUCTION

Successful infection by the epidermal pathogen *Dermatophilus congolensis* is a complex process dependent on various factors including the host immune response and the presence of an inhibitory bacterial skin flora (10). The immune response in dermatophilosis is mediated mainly by neutrophils (14, SASIAK and JENKINSON, in preparation). Specific immune responses have also been shown to play a part and T lymphocytes are known to accumulate under the site of infection (1, 3) but their role in the healing process is not known. In the first experiment described in this study, the relative importance of T cells in resistance to experimental *D. congolensis* infection, *in vivo,* was studied by the use of congenitally athymic mice.

Environmental competition between commensal and pathogenic bacteria is one of the factors which can affect disease severity. In the natural state, infection with *D. congolensis* does not occur in isolation and, in trying to infect, the organism has to compete with the other bacteria and yeasts which have already colonized the skin. Germ-free mice provide a means of examining whether the presence of other organisms can affect the infectivity of *D. congolensis*. In the second experiment, germ-free, immunodeficient mice were infected with *D. congolensis* and the course of the resulting lesions studied.

MATERIALS AND METHODS

Animals

Gnotobiotic (known, limited bacterial flora) and germ-free mice with quantitative or functional deficiencies of various populations of immune cells were used in both experiments (table I). All mice, except for the inbred BALB/c nude group, were bred from a random genetic background. During each experiment, all the animals were housed in a single isolator.

Bacteriology

The presence of existing skin, faecal and environmental organisms was monitored using standard bacteriological techniques. Swab samples were taken from the floor of the isolator and from the skin of several mice ; faecal samples were also examined at the start and finish of each experiment. The germ-free faecal samples were incubated in nutrient broth and in cooked meat medium. Positive samples were cultured on blood agar. Swabs from the gnotobiotic isolator were either processed immediately or left overnight at room temperature in transport medium before being cultured on aerobically and anaerobically on blood agar plates, at 37 °C. Identification of any positive samples was carried out to species level, where possible, using conventional techniques supplemented by the API system (Bio-Mérieux).

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TABLE I Types of mice used.

Experiment 1 : Gnotobiotic mice		
Mice	n	Immune deficiency
nu+	5	Normal control, heterozygote with nude (nu) gene.
nu/nu	5	Homozygote for nude gene ; athymic.
nu/nu-bg/bg	5	Athymic with additional functional neutrophil and NK cell deficiency, and Chediak-Higashi-like syndrome given by beige (bg) gene.
nu/nu-bg/bg-xid	5	As above, with T-independent B cell functional deficiency from x-linked immunodeficiency (xid) gene.
BALB/c-nu/nu	5	Athymic with functional macrophage deficiency.
Experiment 2 : Germ-free mice		
Mice	n	Immune deficiency
nu+	4	Normal control, heterozygote with nude (nu) gene.
nu/nu	4	Homozygote for nude gene ; athymic.
nu/nu-bg/bg	3	Athymic with additional functional neutrophil and NK cell deficiency,and Chediak-Higashi-like syndrome given by beige (bg) gene.

Inoculation procedure

The mice were inoculated with motile zoospores of a Scottish ovine isolate (SS18C) of *D. congolensis*. The zoospore suspensions were prepared as described by HOW and LLOYD (7) and were checked for motility by microscopic examination. The concentrations and purity of the inocula were monitored by a spread plate method. After use in the isolator, the residual inocula were again checked for viability by culture on blood agar.

The hair was clipped only from the backs of the haired mice in experiment 1. The clipping of the mice required several passes of the clipper blades. This did not cause any visible trauma but may have altered the skin surface equilibrium by plucking the hair rather than by cutting it. The skin of the inoculated sites, on all the mice, was swabbed with ether before inoculation. *Dermatophilus* was applied to the skin by dipping a sterile cotton-wool swab into the zoospore suspension and rolling it over the skin for one minute.

Observation and scoring of lesions

The appearance of the skin and of any lesions present was recorded for up to 10 days after inoculation. Lesions that appeared at the inoculated sites were scored for severity on a scale of one to four (0, normal skin; 1, small focal lesions, slight inflammation ; 2, focal lesions affecting less than 50 % of the inoculated area, moderate inflammation ; 3, semi-confluent areas of infection [involving more than 50 % of inoculated area], severe inflammation ; 4, confluent infection, any spread of infection outside the infected area, severe inflammation).

Histopathology

Skin samples were taken aseptically, from euthanased, infected mice, at the end of the observation period. A further set of samples was taken from uninfected mice, both gnotobiotic and germ-free, as controls.

The skin on the back was excised, mounted on card, fixed in modified Bouin's fixative and processed for paraffin wax embedding. Sections were cut and stained with haematoxylin and eosin (H & E), Giemsa, Methyl-green-Pyronine (for plasma cells) and Gram's method.

Statistics

The differences between the mean lesion scores from the different groups, in both experiments, were examined by means of Student's t test.

RESULTS

Bacteriology

Experiment 1 : Gnotobiotic mice

The bacterial flora on the skins of the gnotobiotic mice consisted mainly of *Streptococcus* sp. *(Streptococcus faecalis* and another unidentified *Streptococcus)* together with *Lactobacillus plantarum, Bacillus macerans* and a catalase positive, DNAase and coagulase negative, *Staphylococcus* sp. Isolator surface sampling showed only colonies of a *Streptococcus* sp. No organisms were isolated from skin swab samples from the haired mice either before or after inoculation. At the end of the gnotobiotic experiment, swab samples taken from the lesion sites on nu/nu-bg/bg and nu/nu-bg/bg-xid mice revealed the presence of *D. congolensis* and a *Streptococcus* sp.

Experiment 2 : Germ-free mice

No organisms were present in faecal samples from the germ-free mice at the beginning of the experiment but *D. congolensis* was isolated from faecal samples at the end. No organisms were isolated from skin swab samples at the end of the germ-free experiment.

Monitoring of the inocula after removal from the isolators showed that viability had been maintained. Semi-quantita-

tive assessment of inoculated blood plates and colony counting of the inocula in the 2nd experiment indicated that no significant loss of viability had occurred. In the first experiment, one of the aliquots of inocula became contaminated with *Streptococcus* sp. from the mouse skin during the inoculation procedure.

The concentration of zoospores in the inoculum in the first experiment was 7 x 10^{12} cpu/ml and in the second, 4 x 10^{4} cpu/ml.

Clinical observations and lesion scores

Experiment 1 : Gnotobiotic mice

The time course and appearance of the lesions on the skin of the haired and hairless mice were different (table II). The haired mice developed thin crusts, with no visible inflammation, which detached from the skin by 4 days after inoculation. Hair then began to slowly regenerate in the alopecic areas after the scabs had fallen.

TABLE II Patterns of lesion development on mice in Experiment 1.

Day	Nos. Affected	Clinical observations
Day 1		
nu+	0/5	Normal skin
nu/nu	1/5	Slight nodules on one animal only.
nu/nu-bg/bg	1/5	Slight nodules on one animal only.
nu/nu-bg/bg-xid	1/5	Slight nodules on one animal only.
BALB/c-nu/nu	0/5	Normal skin
Day 3		
nu+	5/5	Extensive, thin crusts on all mice.
nu/nu	3/5	Small nodules visible.
nu/nu-bg/bg	5/5	Larger areas of nodules and skin thickening.
nu/nu-bg/bg-xid	5/5	Large areas of nodules turning into crusts.
BALB/c-nu/nu	5/5	Small nodules present.
Day 7		
nu+	2/5	Lesion healing, scabs falling or reduced.
nu/nu	2/5	Almost negative, 1 mouse has hairless patches.
nu/nu-bg/bg	5/5	Crusted lesions beginning to heal.
nu/nu-bg/bg-xid	4/5	Crusted lesions beginning to heal.
BALB/c-nu/nu	3/5	Ulcer-like lesions appear.
Day 10		
nu+	0/4	No. scabs remaining, hair regrowth started.
nu/nu	1/4	Small nodules on one mouse only.
nu/nu-bg/bg	4/4	Healing scabs.
nu/nu-bg/bg-xid	3/4	Healing scabs.
BALB/c-nu/nu	3/4	Healing lesions.

The hairless mice developed nodules at the site of inoculation which were surrounded by areas of inflammation and skin thickening. When the nodules developed into crusts these tended to have raised margins. The above stages in the progression of a severe lesion in one of the mice in the nu/nu-bg/bg-xid group can be seen in photos 1 to 3. At 3 days after inoculation the skin on the back

Photo 1 : Development of a lesion on a mouse of the nu/nu-bg/bg-xid group. At 3 days after inoculation the skin is thickened and erythematous. Nodules have appeared.

Photo 2 : The same lesion at 4 days after inoculation, a thick scab has formed.

Photo 3 : By 7 days after inoculation, the lesion is healing and the scab is getting smaller.

Photo 4 : Unusual ulcer-like lesions seen on one of the BALB/c nude mice 7 days after inoculation.

was thickened and reddened (photo 1) and by 4 days had begun to show crusting (photo 2). After 7 days the lesion was healing and the scab was smaller, surrounded by a raised margin (photo 3).

The BALB/c-nu/nu mice developed atypical lesions which began as barely visible nodules which then went on to become open ulcers with raised margins, by 7 days after infection (photo 4).

There were significant differences in the severity of lesion scores between the groups of mice. By 3 days after infection the group mean score of the haired (nu+) mice was significantly higher than that of those in all the other groups ($p < 0.001$). At this time the nu/nu mice had the lowest lesion scores of all the groups ($p < 0.01$). By 7 days afler infection the nu/nu-bg/bg mice had the highest lesion scores of all the groups ($p < 0.01$) and the nu/nu group mice had the lowest ($p < 0.01$). There was no difference between the scores from the nu/nu and nu+ groups at 7 days after infection (fig. 1).

Towards the end of the experiment, one of the nu/nu group developed small patches at the inoculated site where it appeared that the residual hair had fallen out. These patches went on to become small crusts and may have represented a sub-clinical or delayed infection.

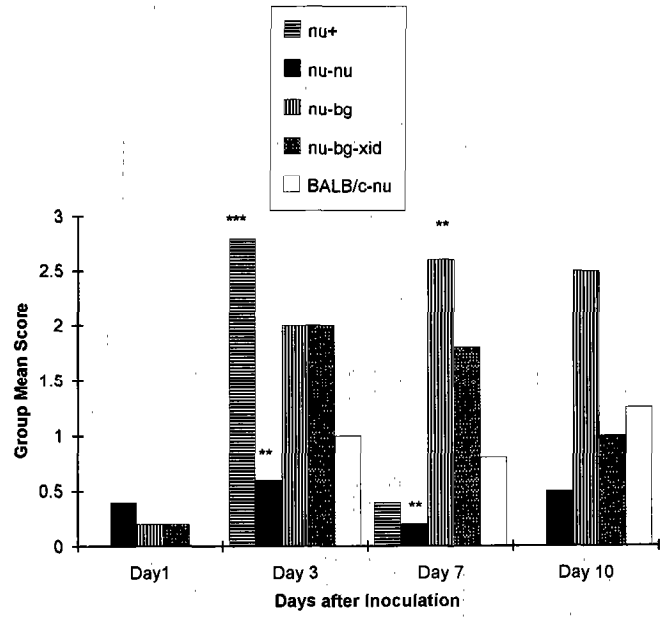

*Figure 1 : The patterns of lesion development and persistence in the groups of normal and immunodeficient gnotobiotic mice. The groups of haired mice (nu+), nude mice (nu/nu), nude-beige mice (nu/nu-bg/bg), nude-beige-B-cell deficient mice (nu/nu-bg/bg-xid) and BALB/c- nude mice (BALB/c-nu/nu) are shown along the x axis. Each group was observed for 10 days after infection and the columns represent the various days post-infection. Blank spaces indicate a zero score on a particular day (e.g. for the nu+ group on days 1 and 10). (***= $p < 0.001$, **= $p < 0.01$.)*

Experiment 2 : Germ-free mice

The haired mice showed no signs of infection throughout the experiment. Traces of infection were seen on 3 of the 4 nu/nu mice, these consisted mainly of tiny, barely visible nodules at the infected sites. A lesion which resembled a bite mark appeared on one of the nu/nu mice at 7 days after infection. The nu/nu-bg/bg mice had more severe infections with crusts visible over most of the infected site in 2 of the 3 mice. In this group also, a lesion which looked like a bite mark appeared on one of the mice at 7 days. These marks may have been the result of self-trauma due to irritation of the infected area. No other bite marks were seen on the mice and therefore the accidental appearance, at the infected sites, of marks due to fighting is unlikely.

At days 3 and 7 after infection the scores for the nu/nu-bg/bg mice were significantly higher ($p < 0.01$ and $p < 0.05$) than those of the nu/nu mice (fig. 2).

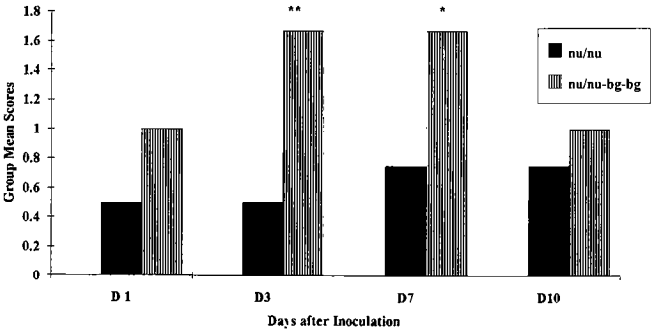

*Figure 2 : The lesion scores for germ-free normal and immunodeficient mice. Axes as figure 1. No lesions were seen on the haired (nu+) group throughout the experimental period. Lesions on the nude-beige mice (nu/nu-bg/bg) were more pronounced than those on the nude (nu/nu) mice. (**= $p < 0.01$ *= $p < 0.05$).*

Histopathology

Large differences were seen in the structure of the hair follicles of the nude and haired mice, reflecting their grossly different appearance. The follicles of the nude mice were convoluted with truncated hair shafts and often contained "keratin pearls". The epidermal layer tended to be slightly thicker in the nude mice. The uninoculated nude mice also tended to have greater numbers of mast cells in the dermis when compared with the uninoculated haired mice.

No differences attributable to the bacteriological status of the mice, i.e. gnotobiotic or germ-free, could be seen.

Very few histology specimens showed clinically apparent lesions, exceptions being the large crusted lesion on one of the nu/nu-bg/bg-xid mice, the ulcer-like lesions on one of the BALB/c nu/nu mice and the small localized "bite marks" on the nu/nu and nu/nu-bg/bg germ-free mice. However, many of the samples without visible lesions showed signs of localized inflammatory changes. Acanthosis and hyperkeratosis were the most common signs of past or continuing inflammation but only in rare instances were these areas associated with an underlying cellular infiltrate. This infiltrate was either of mast cells or, less commonly, of neutrophils. Greater numbers of dermal blood vessels seen in association with the hyperplastic areas were further evidence of inflammatory change.

Mast cells appeared to be present in the dermis of inoculated mice, of all types, in greater numbers when compared with specimens from uninoculated controls. Occasionally, clusters of mast cells could be seen directly underneath the areas of epidermal hyperplasia.

The histological examination of the large, crusted lesion (photo 3) revealed a healing scab underrun by new epidermal growth. Extensive areas of hyperplasia and dermal thickening were seen at the margins of the lesion. Neutrophils could be seen emerging from nearby blood vessels and migrating through the hyperplastic epidermis in regions where the new *stratum corneum* was not yet complete. The bulk of the lesion consisted of a mass of keratin layers interleaved with neutrophil debris. Within this structure the remnants of hair follicles were delineated by the filaments of *D. congolensis* growing within them.

The ulcer-like lesions seen in the BALB/c-nu/nu mice were associated with the presence of Gram-positive cocci, mostly single but also found in pairs or small clusters. The lesion itself consisted of a mass of neutrophil debris with scanty keratin. The epidermis beneath most of the mass of neutrophil debris was all but absent and the lesion extended into the underlying fatty tissue. Neutrophils could be seen migrating from the dermis, through the remains of the epidermis, into the infected area. At the edges of the "ulcer", parakeratosis was evident. The "bite mark" lesions on the germ-free mice were similar to the "ulcer" lesion in the absence of *D. congolensis* in Gram stained sections and the lack of involvement of hair follicles. In both lesions, the epidermis at the margins was hyperplastic but there was no regrowth of fresh epidermis underneath the main part of the lesion. Both types of lesion involved considerable neutrophil infiltration but this was the only point of similarity with the lesion on the nu/nu-bg/bg-xid mouse, which had clearly been caused by *D. congolensis*.

DISCUSSION

In this study, the absence of T cell mediated immunity in the nude mice seemed to have little influence on susceptibility to *Dermatophilus* infection. This is surprising in the

light of other reports which mention the increased susceptibility of nude mice to other infections such as fungi and yeasts (4, 8, 15). One possible explanation may lie in the idea of T cell mediated specific immunity as a back-up for the faster system of neutrophil influx in response to cytokine release from damaged epidermal cells. Infection with *D. congolensis* in the athymic mice appeared to have been successfullly contained by non-specific immune responses and perhaps the T cell response was not needed. However, the observation of a delayed, although very minor, infection on one of the nude mice suggests that a subclinical infection had occurred and had then become apparent before being dealt with, probably by the aforementioned non-specific immune defences.

There is evidence that athymic mice may have some residual gamma-delta T cell activity. Epidermal cells bearing the T cell marker Thy1 are found in nude mice. Unlike the $Thy1^+$ dendritic cells found in the epidermis of normal mice, these cells have limited expression of the genes coding for the $\gamma\delta$ T cell receptor. They also have other functional and structural differences which suggest that they are at a very early stage of differentiation. However, in spite of this, they are able to respond to low doses of interleukin-2 (12) and these cells may be able to play a role, albeit an inefficient and limited one, in the non-specific, protective response of the skin to *D. congolensis*.

In contrast, the beige gene seemed to be the factor which most influenced susceptibility to experimental infection with *D. congolensis*. The beige mutation is found in several species and corresponds to the human immunodeficiency disease Chediak-Higashi syndrome. There are several functional immune abnormalities resulting from the beige mutation in mice and it is uncertain which of them was the cause of the increased lesion severity seen in these mice. The underlying problem in beige mice appears to be a defect affecting the formation and morphology of granular cell organelles, including lysosymes, melanosomes, mast cell granules and platelet storage granules. In some of these cases the presence of characteristic "giant" granules is accompanied by functional deficiencies. The neutrophils of beige mice have reduced chemotactic and bactericidal capacity but their macrophages have been demonstrated to have a normal capacity to secrete lysosymal enzymes (reviewed in (2)). Histological study of the lesion seen on a nu/nu-bg/bg-xid mouse confirmed that neutrophils were still the predominant cells infiltrating the site of infection. The establishment of infection in spite of the influx of neutrophils suggests that they may have been inefficient at clearing the pathogen from the inoculated site.

Another important immune deficiency in these mice is the defect in endogenous NK cell activity which has led to their use in cancer research (6). An ineffective NK cell response could have been another factor in the establishment of severe lesions in the mice carrying the beige gene. Nothing is known of the role of NK cells in the immune response to *D. congolensis* and this area may be a fertile one for investigation. The appearance of the healing lesion also highlighted the importance of the growth of new epidermal tissue forming a protective wall between the infected tissue and the dermis.

The presence of the x-linked immunodeficiency gene did not increase the susceptibility of beige-nude mice to *D. congolensis*. The xid mutation causes a deficiency of T-independent B cell responses, with a B cell population that has the appearance of an immature phenotype (16) and the absence of the unusual B cell subset which carries the T cell marker, CD5 (5). $CD5^+$ B cells are found mainly in the peritoneal cavity and appear to be a separate lineage from the majority of B cells (11). In the context of immunity to *D. congolensis* these cells may not play an important part and thus explain the failure of the xid mutation to influence the outcome of infection.

The presence of commensal skin flora, such as *Bacillus* and *Staphylococcus* sp. has been shown to inhibit the growth of *D. congolensis in vitro* (9, 13). However, the lack of skin flora of the germ-free mice did not appear to affect the course of the lesions induced by experimental *D. congolensis* infection. Thus suggesting that, in this case, immune defences were more important than inhibitory bacteria. Competitive studies will be needed to further elucidate the situation, especially as the *D. congolensis* inoculum titres could not be equalized in this set of experiments. The infective dose was much heavier in the 1st experiment and may have overcome any resistance offered by the gnotobiotic skin flora.

The histopathological evidence of slight inflammation suggests that subclinical reaction to *Dermatophilus* was widespread. The focal nature of the inflammatory response also suggests that it was not due to any reaction against the inoculation process but may have indicated a subclinical infection of the skin. This may have been the case seen with one of the nude mice in experiment 1, where hair was lost from apparently normal skin and small nodules then developed at the hairless sites. The presence of *D. congolensis* in the gut of the germ-free mice also suggests that the bacterium is able to survive in previously unsuspected sites, which may help to explain its recurrence in apparently unaffected animals.

This work has raised interesting questions about the role of specific, T cell-mediated immunity in protection against *D. congolensis*. Increasingly, immunology is being led back to its roots and the importance of its less glamorous components, such as non-specific inflammation is being realized.

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SASIAK (A.B.), SEBESTENY (A.), HRIVNAK (G.), LLOYD (D.H.). Experimental dermatophilosis in murine models of immunodeficiency. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 263-269

Gnotobiotic mice with congenital immune deficiencies were infected with the skin pathogen *Dermatophilus congolensis*. Athymic (nude) mice with T cell deficiency were less susceptible than nude mice which also carried the beige mutation (beige-nude) with NK cell and granulocyte defects, as part of the murine equivalent of Chediak-Higashi syndrome. The additional presence of the x-linked immunodeficiency gene in other beige mutant mice, giving reduced B cell responsiveness, did not increase their susceptibility. BALB/c mice with the nude mutation and evidence of macrophage insufficiency, had a moderate level of susceptibility, greater than that of outbred nude mice but less than that of beige, nude mice. The appearance of the lesions on the haired mice was different from that on those with hairless skin (nude and beige-nude). On the haired mice thin crusts developed and healed rapidly, while on the hairless mice the lesions started as nodules and later progressed to crusts. The nude BALB/c mice developed atypical lesions, which resembled ulcers. Germ-free nude and beige-nude mice showed the same types and time course of infection as the gnotobiotic animals, suggesting that bacterial interference, by a limited skin flora, did not play a major role in defence against *D. congolensis*. However, bacteriological analysis indicated that *D. congolensis* could survive in the gut of germ-free mice. This work emphasizes the importance of non-specific immune mechanisms, such as epidermal hyperproliferation and the neutrophil, in resistance to *D. congolensis*.

Key words : Mice - Dermatophilosis - *Dermatophilus congolensis* - Experimental infection - Immunodeficiency - Lesion - Dermatology - Gene - Disease resistance.

SASIAK (A.B.), SEBESTENY (A.), HRIVNAK (G.), LLOYD (D.H.). Dermatofilosis experimental en modelos murinos de inmunodeficiencia. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 263-269

Se infectaron ratones gnotobióticos inmunodeficientes con *Dermatophilus congolensis*, patógenos de la piel. Los ratones desnudos (sin timo) con deficiencia de células T, fueron menos susceptibles que los ratones desnudos portadores de una mutación "beige" ("beige"-desnudos), con células NK y defectos granulocitarios, como parte de un equivalente murínico del sindrome de Chediak-Higashi. La presencia adicional del gen de inmunodeficiencia x-de unión en otros ratones "beige-mutantes", causante de una disminución en la respuesta de células B, no aumentó la susceptibilidad. Ratones BALB/c desnudos y con insuficiencia de macrófagos, presentaron un nivel moderado de susceptibilidad, mayor que aquel de los ratones desnudos, pero menor que los mutantes "beige"-desnudos. La aparición de las lesiones en los ratones con pelo fue diferente que en aquellos sin pelo (tanto desnudos como "beige"-desnudos). En los primeros, se desarrollaron finas costras que sanaron rapidamente, mientras que en los segundos, las lesiones se iniciaron como nódulos y evolucionaron luego hacia costras. Los ratones BALB/c desnudos desarrollaron lesiones atípicas, semejantes a úlceras. Los ratones desnudos libres de gérmenes y los "beige"-desnudos mostraron el mismo tipo y curso de infección que los animales gnotobióticos, sugiriendo que una interferencia bacteriana, mediante una flora dérmica limitada, no juega un papel importante en la defensa contra *Dermatophilus congolensis*. El analísis bacteriológico indica que *D. congolensis* sobrevive en el intestino de ratones libres de gérmenes. Este trabajo da énfasis a la importancia de los mecanismos de inmunidad no específica, como la hiperproliferación epidérmica y los neutrófilos, en la resistencia a *D. congolensis*.

Palabras claves : Ratón - Dermatofilosis - *Dermatophilus congolensis* - Infección experimental - Inmunodeficiencia - Lesión - Dermatología - Gen - Resistencia a la enfermedades.

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Temporal changes in the granulocytic responses to experimental infection of the skin of mice and sheep with *Dermatophilus congolensis*

LLOYD (D.H.), SASIAK (A.B.), KITSON (S.), McEWAN JENKINSON (D.), ELDER (H.Y.). Changements temporels des réponses granulocytaires à l'infection expérimentale de la peau de souris et de mouton avec *Dermatophilus congolensis*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 271-276

Les types de réponses cellulaires inflammatoires de la peau à l'infection avec *Dermatophilus congolensis* ont été déterminés chez des souris et des moutons à partir de prélèvements histologiques pris avant et à intervalles, après l'application topique de zoospores infectieuses sur la peau frottée à l'éther. Des cellules neutrophiles, éosinophiles, basophiles et des mastocytes ont été identifiées par coloration histochimique. Les changements temporels des cellules B, T et les populations de cellules MHC classe II⁺ dendritiques font l'objet d'un rapport distinct. Les stades filamenteux de la bactérie ont été observés dans le stratum corneum des deux espèces ; chez le mouton, ils se trouvaient également dans les couches extérieures de l'épiderme vivant. Chez les deux espèces, de grands nombres de neutrophiles et quelques lymphocytes pénétraient l'épiderme et entraient dans la zone de surface infectée. Dans le derme sous-adjacent, il y avait une accumulation de cellules dendritiques immédiatement sous l'épiderme infecté et l'on pouvait constater une dégranulation des mastocytes ; les basophiles et les éosinophiles ne semblaient pas activement impliquées. La différence frappante entre les deux espèces était la durée de l'infection et la réponse associée, qui avait une durée de 5 jours environ chez la souris contre plus de 21 jours chez le mouton. Le nombre de neutrophiles chez la souris était par exemple élevé après 12 h et avait atteint son maximum 60 h après l'infection, tandis que le maximum chez le mouton n'était atteint qu'après 120 h.

Mots clés : Ovin - Souris - *Dermatophilus congolensis* - Infection expérimentale - Histopathologie - Peau - Granulocyte - Mastocyte.

INTRODUCTION

Dermatophilus congolensis normally invades only the epidermis and provokes a predominantly neutrophilic response with epidermal hyperplasia. Recruitment of mononuclear cells occurs much later and is much less marked (8, 10). Susceptibility to infection varies substantially between species. Mice are particularly resistant, the neutrophilic response peaks early and lesions resolve in about 7 days (6, 10) whilst sheep are highly susceptible ; neutrophil responses peak at 10-12 days and healing may take as long as 38 days (1).

The reasons for the differences in resistance and the duration of lesions between species remain largely unknown. Comparative studies have shown that rabbit neutrophils are more efficient at killing *Dermatophilus* zoospores than those of sheep and guinea-pigs (10). However, no direct comparison has been made between sheep and mice to determine the reasons for the differences in their responses to infection. This study was designed to investigate and compare the cellular responses to infection in murine and ovine skin on a quantitative basis to assist in evaluation of the role of specific and non-specific immune mechanisms in protection against and recovery from dermatophilosis.

MATERIALS AND METHODS

Inoculation and sampling

Inoculum

Motile zoospores were harvested from 48 h aerobic blood agar cultures of *Dermatophitus congolensis* strain SS18C and suspended in sterile peptone water to give a concentration of 10^8 to 10^9 per ml, as described by HOW and LLOYD (3). Sterile peptone water was used as the control medium.

Mice

Groups of 4 randomly-selected, male and female BALB/c mice, aged 9-10 months, were housed separately with free access to water and a commercial rodent diet. A site (16 cm^2) on the dorsal skin of the trunk of each mouse was clipped (Oster clippers, 40 gauge blades) and swabbed with ether-soaked cotton-wool. Zoospores of *D. congolensis* were applied to each site by dipping a sterile cotton-wool swab into the inoculum and rolling it over the inoculation site for one min. Half-an-hour after inoculation all members of one group were killed by ether inhalation and a skin sample was taken from the treated skin of each of them. Skin samples were obtained in the same way from the inoculated skin of other groups of four mice at 12 h intervals thereafter, up to 120 h after inoculation. Skin samples were also obtained, after 1/2, 24, 48 and 72 h, from groups of four mice which had been clipped, ether swabbed and inoculated with sterile peptone water.

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Sheep

Ten 4-month-old Suffolk lambs were housed on straw, in two groups of five, with access *ad libitum* to water, hay and concentrates. On one group, ten sites, each 16 cm^2, were selected at random on the dorsal skin of the trunk of each animal. The sites were clipped, ether swabbed and inoculated with zoospores as described above. Skin samples were taken under local anaesthesia using a 6 mm biopsy from one site on each animal before (0h) and at 6, 12, 24, 48, 72, 120 h and at 9, 15 and 21 days after infection.

On the other group, three sites were selected and treated in the same way except that sterile peptone water was applied instead of the inoculum. Skin samples were obtained as described from each animal before and at 1/2 and 24 h, and at 9 days after treatment.

Histology

Skin samples were fixed in modified Bouin's fixative at room temperature and processed to paraffin wax using the St. Marie technique (9). Serial sections were cut at 7 µm from each block. Groups of five sections, taken at intervals of at least 50 µm, were mounted on slides pretreated with poly-1-lysine. This enabled visualisation and enumeration of each cell type under consideration, on five sections at every time interval. Neutrophils, basophils and mast cells were studied following staining with Giemsa and eosinophils after staining with carbol-chromotrope (5). Cells of dendritic morphology were counted on haemotoxylin and eosin and Giemsa stained sections.

Quantification and analysis

Cells were counted within an area of 0.021 mm^2 of dermis in all five sections from each block, a) under and b) adjacent to the lesion and the data were compared by analyses of variance.

RESULTS

Clinical observations

Mice

A faint erythematous reaction was visible on infected skin after 24 h. At 48 h raised, focal or confluent areas of scab formation, accompanied by erythema and slight swelling, were present. These scabs persisted until 84 h but were then progressively shed and at 96 h only a few foci of thin scabs could be found. At 120 h, no lesions were visible but the skin was alopecic in previously affected areas. No lesions developed at the control sites of mice treated with peptone water.

Sheep

Signs of oedema were present at inoculated sites 24 h after infection and, in some instances, a glistening exudate could be seen. Crusting and scab formation was evident at all sites by 4 days and peaked at 15 days. Scabs were firmly attached to the skin surface at that time but subsequently became progressively detached. A few detached scabs still remained attached to the growing fleece at some sites at 21 days.

Histopathology

Mice

In unstimulated skin, mast cells were prominent in the vicinity of blood vessels, particularly around hair follicles, and polymorphonuclear cells and lymphocytes were occasionally located whithin blood vessels. At 12 h the only observable change was evidence of mast cell degranulation in the zone between the sebaceous gland and the hair bulb, but mainly beneath the muscle layer (photo 1). By 24 h, scabs containing filaments of the bacterium were present at the surface. The epidermis at these locations was hypertrophied but intact, although foci of degeneration in the outer living layers were observed in places, cells of dendritic morphology were observed accumulating within the dermis immediately under the infected epidermis. There was also evidence of inflammatory cell infiltration of the dermis below the dendritic cell foci, with a predominance of neutrophils. By 36 h the subepidermal aggregation of dendritic cells was more intense and the dermal infiltration of neutrophils has increased. However, few were located within the now markedly hypertrophied epidermis, although neutrophils were present in the scabs. Mast cell degranulation was now prominent, particularly underlying the subdermal muscle. At 48 h there was marked dermal infiltration by neutrophils and some lymphocytes which accumulated under the epidermis within the zone of dendritic cells and were present in the hypertrophied epidermis, especially within vesicles which had formed in the outer *s*tratum spinosum. The larger of these vesicles, which were packed with neutrophils and contained lymphocytes, lay under extensive surface scabs within which filaments of the bacterium were prominent above a layer of neutrophils. The lower layers of the epidermis remained intact. There was still evidence of mast cell degranulation at this stage. The picture at 60 h was similar to that at 48 h although the mast cell degranulation was less evident. By 72 h, dendritic cells were still prominent under the epidermis which was still slightly hypertrophied but no longer exhibited marked vesicula-

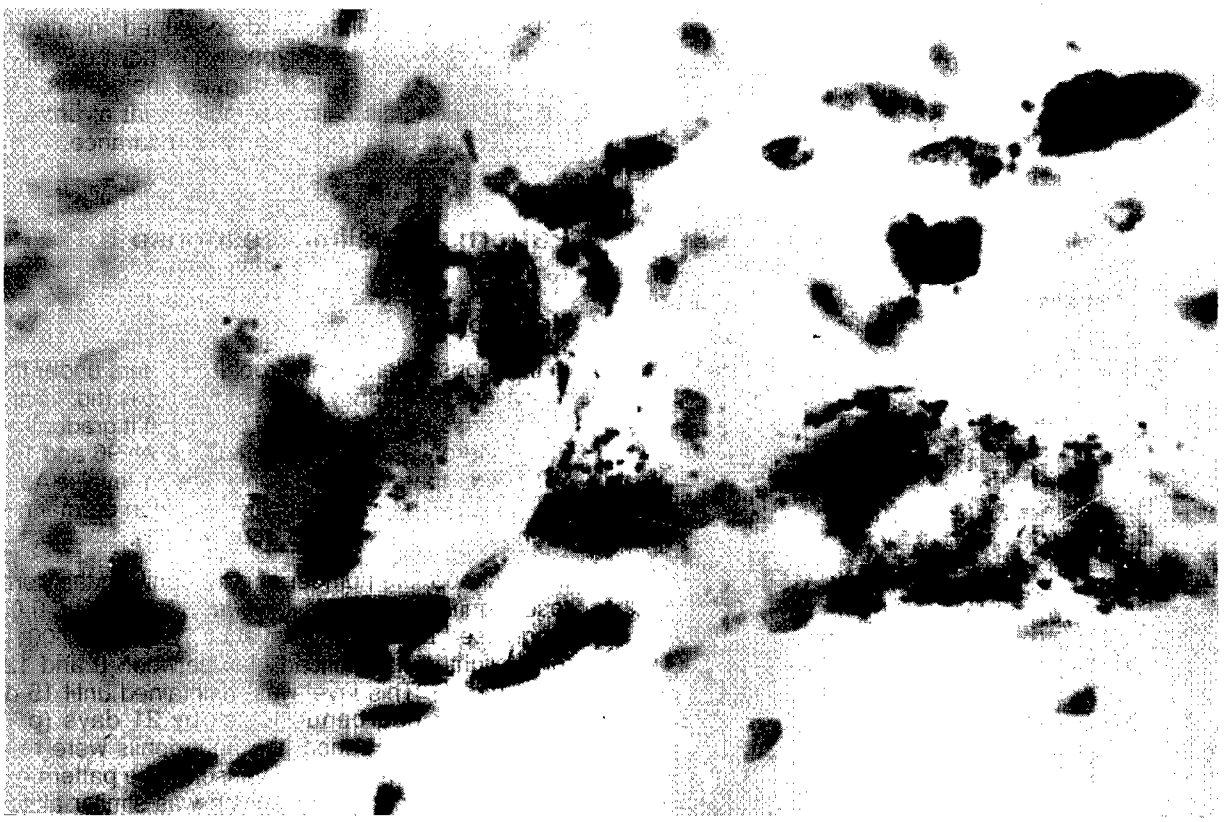

Photo 1 : Degranulating mast cells beneath the subcutaneous muscle layer of the mouse at 48 h.

tion. Scabs, each containing a zone of bacterial filaments above a layer of neutrophils, were located at the surface overlying two to three keratin layers ; in places, two layers of filaments interspersed with neutrophils were seen. Filaments were still traced within the outer layer of the stratum corneum at some locations. The dermal infiltrate was still predominantly of neutrophils, but none was traced within the epidermis. Mast cell degranulation was not observed. This situation persisted until 84 h but by 96 h, most of the skin resembled the uninfected controls, although a few foci of thicker epidermis with underlying dendritic cells and some neutrophils were still present. By 108 h only two small foci, where underlying dendritic cells and neutrophils were present, were located and by 120 h the skin was indistinguishable from uninfected controls.

Sheep

In untreated sheep skin, mast cells and dendritic cells were prominent around blood vessels and were particularly noticeable in the neighbourhood of the hair follicle units. Other cell types were only occasionally located within blood vessels. At 6 h the *bacterium* was not detected but there were signs of a host response to the challenge. There was evidence of foci of dendritic cell accumulation just under the epidermis and of mast cell degranulation. Lymphocytes had infiltrated into the dermis but few neutrophils were found outside the blood vessels. The epidermis exhibited no signs of abnormality. Between 12 and 120 h the dendritic cell accumulation at specific foci increased and neutrophil numbers within the dermis generally, but particularly at these foci, gradually rose. Lymphocytes were also present at these foci but there was no evidence of penetration of the epidermis by any of the invading cell types nor signs of the organism at the surface. Degranulating mast cells were regularly found within the dermis. At 9 days the epidermis was considerably hypertrophied and thick laminated scabs containing numerous alternating layers of bacterial filaments and densely packed neutrophils were present at the surface. In places, much of the stratum spinosum was vesiculated to the level of 1 to 2 cells above the basal lamina ; the outer vesicles were generally large and contained neutrophils and lymphocytes (photo 2) whereas those deeper within the epidermis were smaller although also full of the

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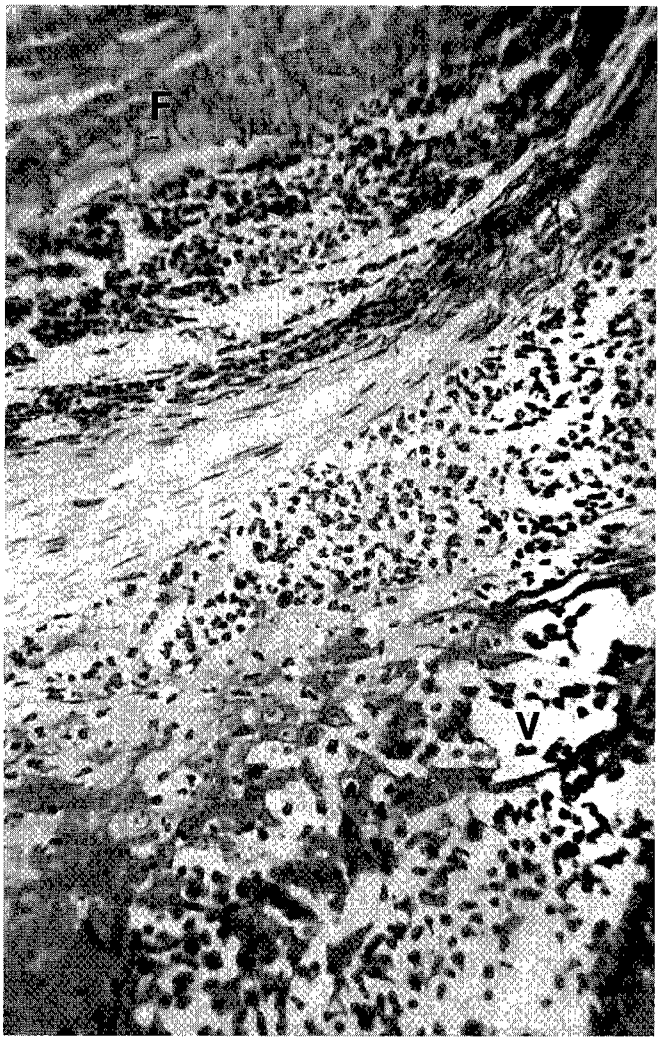

Photo 2 : Invasion of the degenerative ovine epidermis after 9 days by neutrophils in response to invasion by D. congolensis. *Filaments (F) of* D. congolensis *are present in the scab and the formation of vesicles (V) containing neutrophils and lymphocytes can be seen.*

same cell types which had generally infiltrated the epidermis. A dense dendritic cell aggregation was present immediately under the epidermis and lymphocytes and neutrophils were prominent in this region. The mast cells which were detected within the dermal infiltrate were markedly degranulated although those located between foci of infection appeared to be normal. The picture at 15 days was similar to that seen at 9 days although in places the vesicles within the epidermis extended to the basal lamina; there the dendritic cell aggregation was particularly dense. The surface scabs were often 2 to 3 times thicker than the hypertrophied epidermis. By 21 days, some laminated scabs were still detected at the skin surface, but the thickness of the epidermis had returned to control levels with the exception of a few places where slight hyperplasia was still present. The dermal blood vessels appeared to be dilated and contained neutrophils and lymphocytes; these cell types were, however, also observed outside the vessels throughout the dermis. The distribution of dendritic cells resembled that of untreated skin and the mast cells had a normal appearance.

Patterns of cellular response

Neutrophils

In the mouse, dermal neutrophil numbers under the lesion had risen significantly by 24 h ($p < 0.01$) (fig. 1) and continued to rise until 60 h ($p < 0.01$) when it gradually fell until 96 h. There was a second rise between 96 and 108 h ($p < 0.001$) compared with pre-inoculation levels. Adjacent to the lesion there was a significant rise in dermal neutrophil count between 24 and 48 h ($p < 0.001$).

In the sheep, the number of neutrophils in the dermis also rose significantly within the first 30 min ($p < 0.001$) and progressed to a plateau level by 72 h ($p < 0.001$) except for a slight, but significant, fall between 6 and 12 h ($p < 0.01$) (fig. 1). This level was maintained until 15 days but the number had begun to fall by 21 days ($p < 0.01$). During this latter period, the neutrophils were most often present in or around blood vessels. The pattern of neutrophil influx adjacent to the lesion was similar although the total numbers were only about 10 per cent of those found under the lesion.

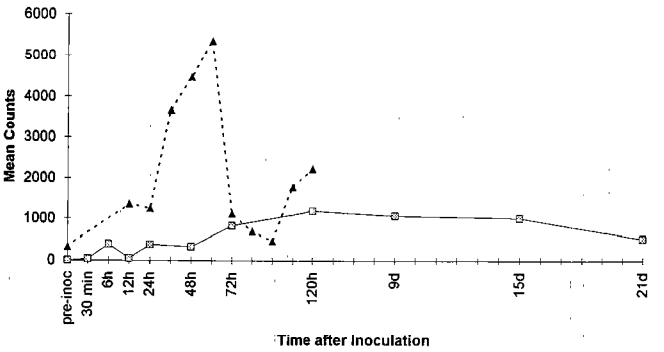

Figure 1 : Temporal changes in the number of neutrophils beneath the lesion in mice (Δ) and sheep (▲). Mean cell counts are given along the y axis and the time in hours (h) and days (d) after inoculation, along the x axis.

Eosinophils and basophils

In both species, eosinophils were only found within blood vessels and this cell type did not vary significantly in number as a result of the infection. Eosinophil number/mm² ranged from 4.90 - 44.12 in the mouse and from 1.96 - 43.14 in the sheep. Basophils were not detected in any of

the samples of mouse skin and in the sheep those present (range 1.96-21.55/mm^2) were also found only within blood vessels; ovine basophil number did not change significantly during infection. The observed variations in eosinophil and basophil number thus mainly reflected the numbers of blood vessels present in the randomly selected fields of study.

Mast cells

Mast cell number in the mouse, which ranged from 44.12-174.02/mm^2, rose significantly under the lesion between 24 and 48 h ($p < 0.001$) and remained elevated until 96 h. The number had fallen to prestimulation levels by 108 h. Between 12 and 60 h many of the cells counted exhibited signs of degranulation. Adjacent to the lesion there was no significant change in cell appearance and number. Mast cell number in the sheep ranged from 100.00-178.43/mm^2 and did not vary significantly with infection. However, mast cell degranulation was evident in the skin between 6 h and 15 days.

DISCUSSION

This work has confirmed the oft-repeated observation that neutrophils are the most abundant cells at the site of *Dermatophilus* infection (1, 10). It has also shown differences and similarities between 2 mammalian species, one resistant and one susceptible, in the amount and duration of the neutrophil influx, following similar experimental infections. The level and duration of granulocytic responses to the organism reflect the differences in lesion duration seen in the mice and sheep.

These detailed temporal studies have demonstrated for the first time the links between neutrophil recruitment to the site of infection and mast cell degranulation, which precede the appearance of clinical signs of infection, in both of the species studied.

The first phase of neutrophil recruitment to the site of infection occurred in the absence of any clinical or histological signs of infection, such as erythema or the presence of *D. congolensis* in histological specimens. This initial peak of neutrophil influx coincided with the first observations of mast cell degranulation in both species. It is not possible to state with any certainty whether the initial recruitment of neutrophils occurred because of mast cell degranulation around the blood vessels in the dermis, or whether it had already started and the involvement of mast cells led to the boost in neutrophil numbers seen later. There are inflammatory and immunologically mediated pathways which can lead to neutrophil adhesion to, and migration across, the endothelium which can be initiated by keratinocytes. Thus it is possible to speculate that damage to epidermal cells by *Dermatophilus* could cause them to release IL-1 and TNFα, which could then act directly upon endothelial cells of local blood vessels to upregulate adhesion molecule ligands such as ICAM-1, IL-8 and E-selectin leading to the trapping and recruitment of passing neutrophils. These neutrophils would then be attracted to the site of infection along a chemotactic gradient featuring IL-8 produced by the keratinocytes and by dermal fibroblasts, following IL-1 and TNFα stimulation. The degranulating mast cells around the blood vessels in the deep dermis would further contribute to neutrophil recruitment by histamine release leading to expression of other adhesion molecule ligands, such as platelet activating factor (PAF) and P-selectin on the endothelial cells (11). The mechanism which initiated the degranulation of mast cells remains unknown but may have been the generation of complement components C3a and C5a by the action of bacterial enzymes or surface components on the alternative complement pathway (7).

Such a dual-phase mechanism may help to explain the next temporal event which was a quiescent period when neutrophil numbers plateaued or fell. The end of this lag period coincided, in both species, with the clinical and histological appearance of lesions caused by *Dermatophilus*. After this, the numbers of neutrophils under the lesions rose to peaks which coincided with the peak of lesion activity in both species. As the lesions then healed, mast cell degranulation ceased to be observed and neutrophil numbers fell. The localisation under the lesion of the mast cell degranulation seen in the histological sections suggests that the phenomenon is mediated by factors which operate in tightly defined areas.

The main difference between the species was in the duration of the experimental infection and of the immune response, which was far longer in the sheep than in the mice. However, five times as many neutrophils were present under the infected sites of the mice when compared with those of the sheep and this may have been a factor in the shorter duration of lesions in the mice. The reason for the more effective recruitment of neutrophils in the mouse remains unknown.

In this study, eosinophils and basophils did not appear to have any role in the response to *D. congolensis*, this is in contrast to the situation seen in other infections involving the epidermis. Increased numbers of basophils accumulate at the site of experimental orf virus infection during the late phase of the response (4) and the role of eosinophils in the cutaneous reaction to epidermal and dermal damage from arthropod bites is well documented (2).

The most important finding from this study was the link between mast cell degranulation and neutrophil recruitment to the site of infection before any signs of clinically observable infection, either at the skin surface or in histological sections. Clearly, something is happening at a cellular level before *D. congolensis* becomes manifest in the epidermis and studies of the reactions to *Dermatophilus* which take place before 6 hours may be indicated.

The regulation of the influx of neutrophils to the site of experimental *D. congolensis* infection is only part of the story. Detailed temporal studies of the mononuclear and dendritic cell reaction to *D. congolensis* infection in sheep and mice are in progress and will be reported elsewhere.

ACKNOWLEDGEMENTS

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LLOYD (D.H.), SASIAK (A.B.), KITSON (S.), McEWAN JENKINSON (D.), ELDER (H.Y.). Temporal changes in the granulocytic responses to experimental infection of the skin of mice and sheep with *Dermatophilus congolensis*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 271-276

The patterns of dermal inflammatory cell response to infection with *Dermatophilosis congolensis* were determined in mice and sheep from histological samples taken before and at intervals after topical application of infective zoospores to ether-swabbed skin. Neutrophils, eosinophils, basophils and mast cells were identified by histochemical staining. Temporal changes in the B cell, T cell, and MHC Class II$^+$ dendritic cell populations form part of a separate report. The filamentous stages of the bacterium were observed in the stratum corneum of both species ; in the sheep they were also found in the outer layers of the living epidermis. In both species, large numbers of neutrophils and some lymphocytes penetrated the epidermis and entered the infected surface region. Within the underlying dermis there was an accumulation of dendritic cells immediately below the infected epidermis and evidence of mast cell degranulation ; the basophils and eosinophils did not appear to be actively involved. The striking difference between the two species was the duration of the infection and the associated response which, in the mouse, lasted about five days in comparison with over 21 days in the sheep. Neutrophil numbers in the mouse for example were elevated by 12 h and had peaked at 60h after infection, while in the sheep they did not peak until about 120 h.

Key words : Sheep - Mice - *Dermatophilus congolensis* - Experimental infection - Histopathology - Skin - Granulocyte - Mast cell.

LLOYD (D.H.), SASIAK (A.B.), KITSON (S.), McEWAN JENKINSON (D.), ELDER (H.Y.). Cambios temporales de la respuesta granulocítica a una infección experimental por *Dermatophilus congolensis*, en la piel de ratones y ovejas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 271-276

Se determinaron los patrones de la respuesta celular inflamatoria de la piel a *Dermatophilus congolensis* en ratones y ovejas. Se utilizaron muestras histológicas obtenidas antes y después de una aplicación tópica de zoosporas infectivas, sobre una piel previamente tratada con éter. Mediante tinciones histoquímicas se identificaron neutrófilos, eosinófilos, basófilos y mastocitos. Los cambios temporales sufridos por las células B, las células T y las poblaciones de dendritas MHC clase II$^+$, se describen en un artículo aparte. Los estadios filamentosos de la bacteria se observaron en el estrato córneo de ambas especies, observándose también en las capas superficiales de la epidermis de ovejas. En ambas especies los neutrófilos y algunos linfocitos penetraron la epidermis hasta la zona infectada. Dentro de la dermis profunda, se produjo una acumulación de células dendríticas, inmediatamente bajo la epidermis infectada, con evidencia de desgranulación de los mastocitos. Los basófilos y los eosinófilos no aparecieron activamente involucrados en este fenómeno. La gran diferencia entre las dos especies fue la duración de la infección y la respuesta asociada. En ratones tuvo una duración aproximada de cinco días, contra 21 días en la oveja. La cantidad de neutrófilos en el ratón se mantuvo elevada durante 12 horas, con un pico 60 horas post-infección, mientras que en la oveja el pico se dió a las 120 horas.

Palabras claves : Ovino - Ratón - *Dermatophilus congolensis* - Infección experimental - Histopatología - Piel - Granulocito - Célula fibrosa.

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Cellular responses in experimental chronic and acute dermatophilosis infections of sheep

POERMADJAJA (B.), AMBROSE (N.), WALKER (A.), MORROW (A.). Réponses cellulaires dans la dermatophilose expérimentale chronique et aiguë de moutons. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 277-282

Lors de lésions de la dermatophilose, le derme est infiltré par un éventail de types de cellules. Le but de cette étude était de vérifier si la composition des infiltrations cellulaires dans les lésions chroniques était différente de celle dans les lésions en voie de guérison. Des infections expérimentales de moutons par *Dermatophilus congolensis* ont été utilisées pour étudier les changements en types cellulaires qui se succèdent au cours d'infections chroniques et aiguës. Des infestations par des *Amblyomma variegatum* adultes ont été utilisées pour produire des lésions chroniques chez des moutons infectés, tandis que des infections de moutons sans tiques donnaient des lésions aiguës. Les types de cellules dans le derme des sites d'infection ont été colorés et typés par des méthodes d'histopathologie et d'immunohistochimie. Les neutrophiles étaient dominants au cours de la réponse initiale et étaient présents en plus grand nombre dans les lésions chroniques. Des cellules plasmatiques étaient présentes dans les deux types de lésions, mais persistaient dans les lésions chroniques, tandis qu'elles disparaissaient de la peau dans les sites des lésions aiguës après la guérison des lésions. Déjà à partir de 4 jours après l'infection il y avait de 2 à 3 fois plus de cellules mononucléaires dans les lésions chroniques que dans les lésions aiguës, et ces cellules persistaient dans les lésions chroniques. La population des cellules mononucléaires dans les lésions chroniques consistait en lymphocytes T auxiliaires et en lymphocytes T suppresseurs cytotoxiques dans des proportions égales, tandis que dans les lésions aiguës en voie de résolution, 14 jours après infection, le nombre de cellules T auxiliaires dépassait celui des cellules T suppresseurs cytotoxiques.

Mots clés : Ovin - Dermatophilose - *Dermatophilus congolensis* - Lésion - Tique - *Amblyomma variegatum* - Immunologie - Cellule -Lymphocyte.

INTRODUCTION

The cellular infiltrate in the dermis of ruminant skin infected with *Dermatophilus congolensis* contains a range of cell types. The dominant cells are neutrophils (2,9) and mononuclear cells (3). ODUYE (8) reviewed the histopathological studies on chronic and acute dermatophilosis, he concluded that neutrophils dominate the initial reaction and mononuclear cells dominate the reaction in the dermis of advanced chronic lesions. Neutrophils also occur in the scabs of chronic lesions. Experimental infection of ruminants with *D. congolensis* produces acute lesions which resolve without treatment in 2-4 weeks. To date there has not yet been a parallel study of cell types in experimental acute and chronic infections under controlled conditions. This information is essential for future studies on all aspects of the immunology of dermatophilosis : at present the mechanisms involved in protective responses, lesion healing responses, and pathogenic responses are not understood. However it has been suggested that skin surface antibodies may be responsible for protection (3, 6, 11) and lesion resolution (3), that increased epidermal cell turnover caused by lymphokines from mononuclear cell infiltrates may contribute to lesion resolution (13) and that a persistent mononuclear cell response may contribute to pathogenesis (1).

Recently it has been shown that chronic dermatophilosis lesions can be produced by infesting animals with adult *A. variegatum* ticks prior to infecting them with *D. congolensis* at sites far separate from the ticks (12). This paper describes the cell types present in the skin of tick infested and tick-free sheep following experimental infection with *D. congolensis*. The aim was to find out if there were differences between the cell types present in the dermis during acute and chronic dermatophilosis infections.

MATERIALS AND METHODS

Four sheep (Blackface x Suffolk ewes) were paired for weight, kept indoors at 20 °C, 75 to 95 % relative humidity and fed rations of hay and concentrates ample for health. The sheep had not been exposed to ticks before and were treated with anthelminthic (fenbendazole) before use. The autopsy material described here came from the second *A. variegatum* infestation and *D. congolensis* infection, 7 weeks after a first infestation and infection.

Adult *Amblyomma variegatum* were applied to one sheep of each pair (test sheep) at a separate site from the sites of infection with *D. congolensis*. The other sheep of each pair were without ticks (control sheep). Cotton bags to confine the ticks were glued to wool on the shoulders of test sheep. Ten male ticks were allowed to feed for at least one week then females were added to give 10 mating pairs. The timing of tick feeding was such that females had fed for approximately 5 days before *D. congolensis* was applied. Feeding proceeded until repletion or the end of that phase of the experiment at 5 weeks after the application of males.

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One flank of each sheep was sheared close to the skin but the skin was not scarified. The skin was washed with a solution of non-ionic detergent ('Tween 80'), left to dry, then degreased with a 1:1 mixture of ethanol and ether. Sites for infection with *D. congolensis* measuring 2 x 4 cm were outlined with a marker pen. The *D. congolensis* isolate was taken from a sheep in Scotland, it was cultured on Columbia agar containing 7 % calf serum. Cocci and zoospores were scraped from the agar and suspended in Hanks balanced salt solution containing 0.5 w/v pig gelatin as a thickening agent. The resulting suspension was used at a concentration of 1.25 x 10^6 per cm^2. 100 µl of *D. congolensis* suspension was applied to sites on the sheep such that at autopsy, at the end of the experiment, they had sites of ages 4, 9, 14, 18, 23 and 28 days post-infection (dp.i.). In addition an uninfected site was taken as a day 0 control and the site of infection with the highest dose from the first infection was taken to represent a 56 day old site.

At autopsy samples of the infection site from control and test sheep were taken for histology and immunohistochemistry. Samples for histology were fixed and processed for light microscopy examination using methacrylate plastic embedding. Four 2 µm thick sections from each sample were stained with Giemsa's stain, and four sections stained with pyronin and methyl green to examine plasma cells. The number of mononuclear cells, neutrophils and plasma cells were counted in 5 similar fields of view at x 1000 magnification. Fields in the upper dermis were selected to avoid major skin organelles. Median values of the cell counts were determined.

Samples of infection sites for immunohistochemistry were taken from test and control sheep at 0, 14 and 28 days post-infection, they were frozen immediately after being taken. Sections were cut at 8 µm, mounted onto coated multiwell slides and dried for 2-3 hours at 37 °C. A panel of monoclonal antibodies (MAbs) against ovine lymphocyte subsets was purchased from the Centre for Animal Biotechnology, University of Melbourne, Australia, their specificities and phenotypes are given in table I. The optimal working dilutions of the MAbs were determined by titration using the method described below. Dilutions of 1/10, 000 were selected except for CD1 which was used at 1/32,000. The MAbs were used to label lymphocytes in sections using a standard indirect immunoperoxidase technique. Briefly, the sections were fixed in acetone, endogenous peroxidase activity was blocked with hydrogen peroxide and the slides were incubated with 200 µl of an anti-ovine lymphocyte monoclonal antibody overnight at 18 °C under humidified conditions. After washing, the slides were each incubated with 200 µl of goat anti-mouse IgG peroxidase conjugate (Nordic) diluted 1/100, for 1 h at 18 °C in a humidified chamber. After washing, positive labelling was visualised using a solution of the substrate 3,3'-diaminobenzidene tetra-hydrochloride containing hydrogen peroxide. The reaction was then enhanced with osmium tetroxide. The sections were counterstained with Giemsa's stain, dehydrated and mounted in DPX. Cell counts were made as described for the histological sections except that a magnification of X 400 was used.

TABLE I List of monoclonal antibodies.

Monoclonal antibody	Antigen specificity	Cell phenotype
SBU 20.27	CD1	Antigen presenting cells
SBU 44.38	CD4	T-helper cells
SBU 38.65	CD8	T-cytotoxic/ suppressor cells
SBU 25.91	CD5	Pan T-cell marker
SBU 19.19	T-19	Gamma-delta T-cells
SBU 20.96	CD45R	B-cells

RESULTS

The *D. congolensis* infection procedure produced characteristic dermatophilosis lesions on all sheep. In the control sheep *D. congolensis* was present in lesions only up to 14 dp.i. and the lesions had resolved by 23-28 dp.i. However in tick-infested test sheep scabs were present until the end of the experiment at 56 dp.i. Histopathology showed the presence of *D. congolensis,* haemorraghia and exudation up to 56 dp.i. In both test and control sheep eosinophils and mast cells were found only in small numbers in the dermis below infection sites.

The number of neutrophils increased in both test and control sheep after infection. In control sheep their numbers peaked at 4 dp.i. and declined to preinfection levels by 14-18 dp.i. However in the test sheep they peaked at higher levels than in controls on day 9 and declined to pre-infection levels by 18-23 dp.i. (fig. 1).

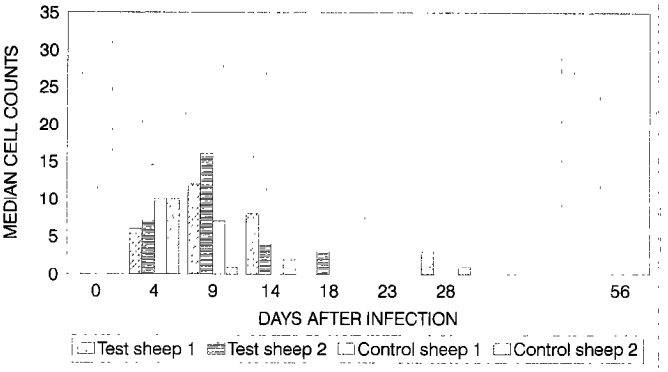

Figure 1 : Neutrophils in lesions on test and control sheep.

The number of plasma cells at infection sites increased in both test and control sheep after infection. In controls they peaked at 14 dp.i. then declined to pre-infection levels by 56 dp.i. In test sheep, the number of plasma cells peaked at 18-23 dp.i. and remained at a level above the number in pre-infection and control sheep skin until 56 dp.i. (fig. 2).

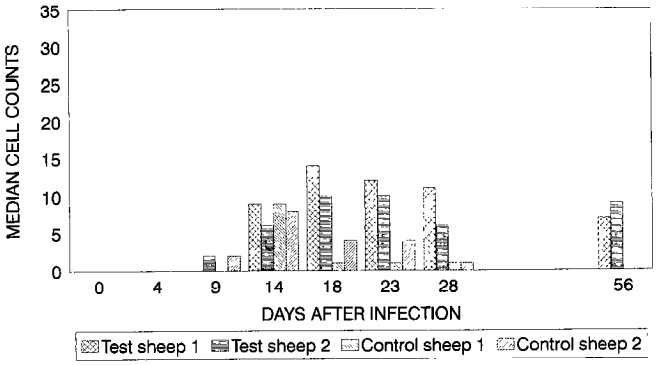

Figure 2 : Plasma cells in lesions on test and control sheep.

Mononuclear cells increased in the dermis of infection sites on control and test sheep. From 8-56 dp.i. mononuclear cells were more abundant in test than control sheep. Their numbers peaked at 14 dp.i. in all sheep, however at this time there were approximately 3 times as many in test sheep than control sheep. In the test animals mononuclear cells continued to be present at elevated levels until 56 dp.i. whereas in controls they declined between 14 and 56 dp.i. (fig. 3).

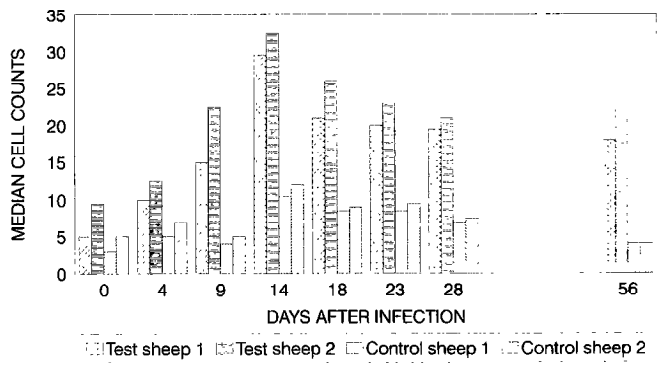

Figure 3 : Mononuclear cells in lesions on test and control sheep.

In the light of the above observations samples of skin were selected for study by immunohistochemistry. The aim was to provide more information on the differences in the composition of lymphocyte populations in the mononuclear cell infiltrate of acute and chronic lesions on control and test sheep respectively. Day 0 post-infection was selected as a pre-infection control, day 14 post-infection was selected as the time when mononuclear cell numbers were at their maximum levels and acute lesions in control sheep were beginning to heal. Day 28 post-infection was selected as the time when control skin had returned to normal, as judged by histopathological observations, whereas on test sheep lesions were persisting.

The number of CD5 positive cells increased in both test and control sheep between 0 and 14 dp.i. and then remained at elevated levels until 28 dp.i. (fig. 4). The number of CD5 cells was equal to 73 % or greater of the combined total of CD4 and CD8 in all sheep, at all times except in test sheep at 14 dp.i. In these lesions at this time CD5 positive cells accounted for only 45 % and 48 % of the CD4 plus CD8 total.

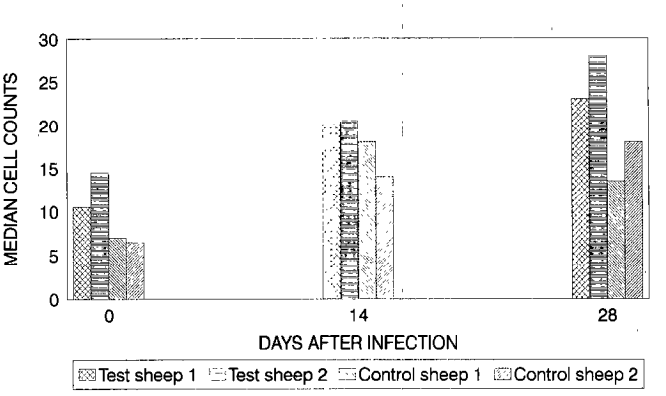

Figure 4 : CD5 positive cells in lesions on test and control sheep.

The number of CD1 positive cells (antigen presenting cells) increased in both test and control sheep between 0 and 14 dp.i., then, to 28 dp.i. they continued to increase in control sheep whereas they decreased in test sheep (fig. 5).

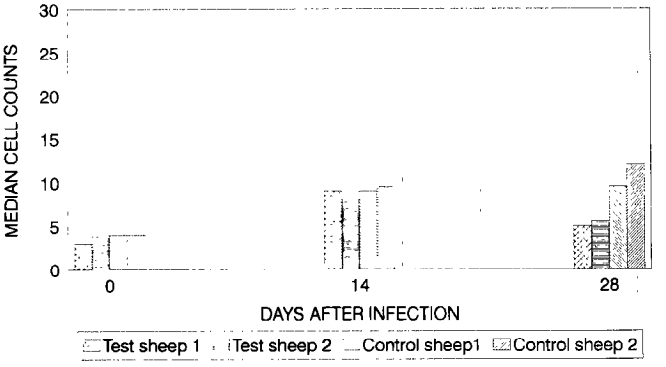

Figure 5 : CD1 positive cells in lesions in test and control sheep.

CD4 positive cells (T-helper cells) increased in number in test and control sheep between 0 and 14 dp.i., however they were more abundant in test than control sheep at 14 dp.i. (fig. 6).

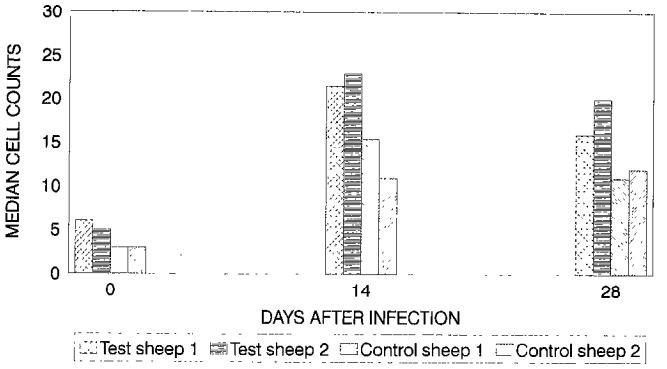

Figure 6 : CD4 positive cells in lesions on test and control sheep.

CD8 positive cells (T-cytotoxic/suppressor cells) were more abundant in the skin of test sheep at day 0. Their numbers increase from 0 to 14 dp.i. in both test and control sheep, however they were substantially more abundant in test than control sheep at 14 dp.i. (fig. 7).

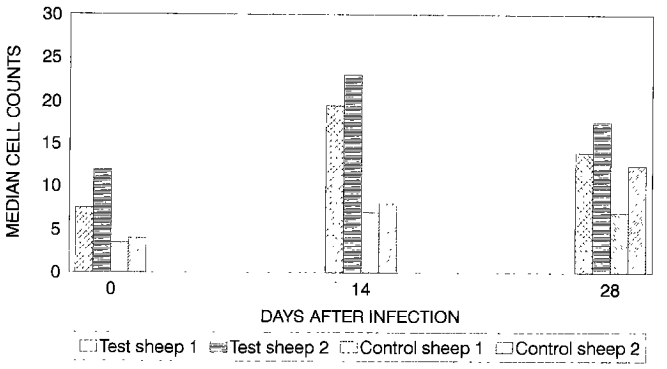

Figure 7 : CD8 positive cells in lesions on test and control sheep.

The ratio of CD4 : CD8 positive cells was 1 or less on all but three occasions ; it was substantially above 1 at 14 and 28 dp.i. in one control sheep and at 14 dp.i. in the other control sheep, these ratios were 2.2, 1.6 and 1.4 respectively.

The numbers of T-19 positive cells (gamma-delta T-cells) in both test and control sheep were small, they were slightly increased between 0 and 28 dp.i. (fig. 8).

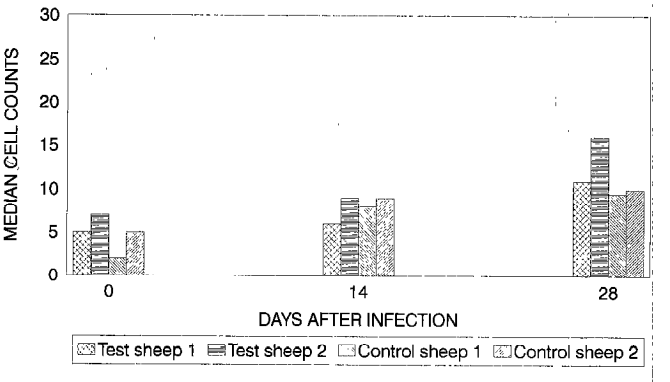

Figure 8 : T-19 positive cells in lesions on test and control sheep.

CD45R positive cells (B-cells) showed a similar pattern in test and control sheep : the numbers were small in both, from 0-14 dp.i. they increased and from 14-28 dp.i. they decreased (fig. 9).

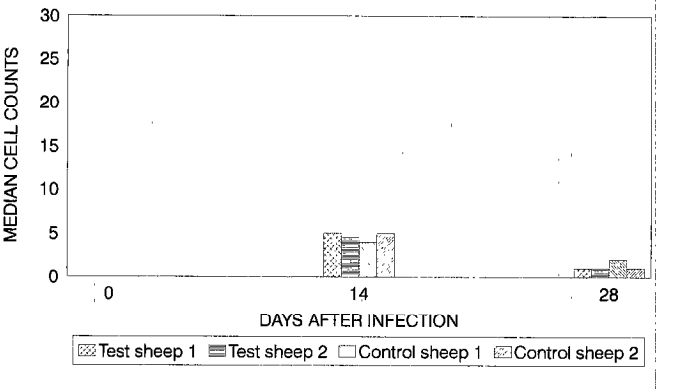

Figure 9 : CD45R positive cells in lesions on test and control sheep.

DISCUSSION

Our observations on the presence of neutrophils and mononuclear cells in the dermis following infection with *D. congolensis* are in agreement with those of other workers. Neutrophils dominate the initial reaction, this is followed by an influx of mononuclear cells into the dermis. However in the lesions which become chronic on tick-infested test sheep the peak in numbers of neutrophils was later and greater than in control sheep. ELLIS *et al.* (4) found that neutrophils from sheep with chronic dermatophilosis phagocytosed opsonized *D. congolensis* more efficiently than neutrophils from sheep that had recovered from dermatophilosis. In our study the large number of neutrophils in chronic lesions were unable to control the infection. In a transmission electron microscope study of

these lesions POERMADJAJA (unpublished observation) found that *D. congolensis* hyphae were too big to be phagocytosed by neutrophils. This result appears to contradict the suggestion of ROBERTS (10) that neutrophils contribute to lesion resolution.

Our observations on the occurrence of plasma cells are interesting in that these cells occur in larger numbers and persist for longer in chronic than acute lesions. This begs the questions : Are they producing antibodies that contribute to healing but which are ineffective for some unknown reason and, do plasma cells in chronic and acute lesions produce antibodies with different specificities ? It has been shown that following inoculation of sheep and vaccination of cattle with *D. congolensis,* skin surface antibodies are produced that are specifically directed against *D. congolensis* antigens and it was suggested that they may contribute to clearing infections (7,11). However the efficacy of these antibodies in mediating killing of *D. congolensis* and the antigen specificity of skin surface antibodies on chronically infected animals has not been investigated.

Mononuclear cells were at least twice as numerous in chronic lesions compared to acute lesions from 14-56 dp.i.. This response indicates an exaggerated reaction to *D. congolensis* as a result of the *A. variegatum* infestation and in common with the heightened neutrophil response and persistent plasma cell response, these mononuclear cells do not contribute to lesion resolution. The results from immunohistochemical studies showed that the increased number of mononuclear cells in test sheep lesions were mainly composed of CD4 and CD8 positive cells in equal proportions. It is interesting to note that in their immunohistochemical studies of experimental infections of sheep with chronic dermatophilosis and spontaneously recovered sheep, ELLIS *et al.* (4) found CD4 : CD8 ratios of one or less except in the recovered sheep at 15 dp.i.. In our study the only noticeable deviation of CD4 : CD8 above one was in acute lesions at 14 and 28 dp.i.. It seems likely that an excess of T-helper cells is required for lesions to resolve, however the immune mechanism involved in lesion resolution remains unknown.

WOODMAN *et al.* (14) showed that acute infections in rats stimulated a *D. congolensis* specific T-helper cell response and WOODMAN (13) demonstrated that culture supernatants, possibly containing lymphokines, from *D. congolensis* stimulated lymphocytes altered the kinetics of epidermal cell growth. She suggested that increased epidermal cell turnover may lead to lesion resolution. If this is correct, lymphokine production in chronic and acute lesions may be qualitatively different, as the scabs of chronic lesions are characterised by massive accumulations of keratinocytes. An alternative hypothesis is that gamma-interferon is released by T-helper cells and is bacteriocidal or bacteriostatic, thus preventing the proliferation of *D. congolensis.*

ELLIS *et al.* (4, 5) showed that numbers of CD1 positive cells in sheep were low in chronic lesions and in peripheral blood of chronically infected sheep when compared to acute lesions and blood from previously unexposed sheep. In our study there was a small difference between the number of CD1 positive cells in acute and chronic lesions at 28 dp.i., in retrospect it would have been interesting to examine the lymphocyte subsets in chronic lesions at 56 dp.i.. In a separate study AMBROSE (unpublished observation) found that chronic lesions on naturally infected cattle did not contain any CD1 positive cells although normal skin from these animals did so. Perhaps a defect in the presentation of antigens is a factor that contributes to the development of chronic dermatophilosis.

CONCLUSION

- *Amblyomma variegatum* infested and tick free sheep were used to study the cellular responses in chronic and acute dermatophilosis infections respectively.

- In this study neutrophils dominated the early reactions to *D. congolensis.* Larger numbers occurred in chronic than acute lesions.

- Mononuclear cells were more abundant in chronic than acute lesions from an early stage after infection.

- Mononuclear cells and plasma cells persisted in chronic lesions.

- Mononuclear cell responses in chronic lesions were composed of T-helper and T-cytotoxic/suppressor lymphocytes in equal proportions.

- In acute lesions at 14 days after infection, when lesions begin to resolve, T-helper were more abundant than T-cytotoxic/suppressor lymphocytes.

ACKNOWLEDGEMENTS

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POERMADJAJA (B.), AMBROSE (N.), WALKER (A.), MORROW (A.). Cellular responses in experimental chronic and acute dermatophilosis infections of sheep. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 277-282

The cellular infiltrate into the dermis in dermatophilosis lesions is composed of a range of cell types. The aim of this study was to establish if the composition of the cellular infiltrate in chronic lesions was different from that in healing lesions. Experimental *Dermatophilus congolensis* infections of sheep were used to study the sequential changes in cell types during the course of chronic and acute infections. Infestations of adult *Amblyomma variegatum* ticks were used to produce chronic lesions on infected sheep, infections of tick-free sheep provided acute lesions. Histopathology and immunohistochemistry were used to stain and label cell types in the dermis of infection sites. Neutrophils dominated the early response and were present in larger numbers in chronic lesions. Plasma cells were present in both types of lesion, however they persisted in chronic lesions but disappeared from the skin at acute lesion sites after the lesions had resolved. There were 2-3 times as many mononuclear cells in chronic than acute lesions from as early as 4 days post infection and these cells persisted in the chronic lesions. In the chronic lesions the mononuclear cell population was composed of T-helper and T-cytotoxic/suppressor lymphocytes in equal proportions whereas in acute lesions at 14 days post infection, when lesion resolution is underway, there were greater numbers of T-helper cells than T-cytotoxic/suppressor cells.

Key words : Sheep - Dermatophilosis - *Dermatophilus congolensis* - Lesion - Tick - *Amblyomma variegatum* - Immunology - Cell - Lymphocyte.

POERMADJAJA (B.), AMBROSE (N.), WALKER (A.), MORROW (A.). Respuesta celular de la dermatofilosis experimental crónica y aguda en ovejas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 277-282

El infiltrado celular en la dermis en las lesiones de dermatofilosis se compone de una gama variada de tipos celulares. El objetivo de este estudio es el de determinar si la composición del infiltrado celular en las lesiones crónicas es diferente al de las lesiones en proceso de recuperación. Se produjeron infecciones experimentales con *Dermatophilus congolensis* en ovejas, para estudiar los cambios progresivos en los tipos celulares durante el curso de infecciones tanto crónicas como agudas. Se utilizaron infestaciones con especímenes adultos de *A. variegatum* para producir las lesiones crónicas en ovejas; las infecciones de ovejas libres de garrapatas produjeron las lesiones agudas. Se utilizaron técnicas de histopatología e inmunohistoquímica para la tinción y la determinación de los tipos celulares en la dermis de las zonas infectadas. La respuesta temprana presentó una mayoría de neutrófilos, los cuales fueron mas numerosos en las lesiones crónicas. Las células plasmáticas se encontraron en ambos tipos de lesión, cabe notar que se mantuvieron en las lesiones crónicas, pero desaparecieron de la piel en las lesiones agudas después de la cura. El número de mononucleares fue 2 a 3 veces mayor en las lesiones crónicas que en las agudas y esto a partir dc 4 días post-infección, observándose también la persistencia en las lesiones crónicas. En éstas, la población de células mononucleares se compuso de linfocitos T de ayuda y T citotóxicos/supresores, en proporciones idénticas ; mientras que en las lesiones agudas, 14 días post-infección, durante la resolución de la lesión, se observó un número mayor de células T de ayuda que T citotóxicas/supresoras.

Palabras claves : Ovino - Dermatofilosis - *Dermatophilus congolensis* - Lesión - Garrapata - *Amblyomma variegatum* - Inmunologia - Célula - Linfocito.

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STVM-93

An attempt to correlate cattle breed origins and diseases associated with or transmitted by the tick *Amblyomma variegatum* in the French West Indies

MAILLARD (J.C.), KEMP, (S.J.), NAVES (M.), PALIN (C.), DEMANGEL (C.), ACCIPE (A.), MAILLARD (N.), BENSAID (A.). Tentative de corrélation de l'origine des races bovines et des maladies associées à ou transmises par la tique *Amblyomma variegatum* dans les Antilles françaises. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 283-290

A l'aide de données biologiques et de la recherche historique, on a essayé d'expliquer la différence, en ce qui concerne la résistance et la sensibilité aux maladies transmises par (cowdriose) ou associées à (dermatophilose) la tique *Amblyomma variegatum*, entre deux races bovines des Antilles françaises : la race Créole hybride de la Guadeloupe et le zébu Brahman de la Martinique. Les polymorphismes de 5 systèmes génétiques indépendants (hémoglobine érythrocytaire, albumine et transferrine du sérum, la région classe I du complexe BoLA et le gène gamma S cristallin) ont été étudiés chez différentes races, à savoir des *Bos taurus* d'Europe et d'Afrique, des *Bos indicus* d'Afrique de l'Ouest et de l'Est, le Brahman de la Martinique et le Créole de la Guadeloupe. Par comparaison des fréquences de différents allèles de ces 5 loci polymorphiques non liés et à l'aide de deux matrices mathématiques différentes de NEI et de CAVALLI-SFORZA, on a établi les distances génétiques entre ces races. Il apparaît clairement que le bovin Créole de la Guadeloupe est dans une position intermédiaire entre le *Bos taurus* N'Dama de l'Afrique de l'Ouest et deux races de zébu, *Bos indicus*, le zébu soudanais de l'Afrique de l'Ouest et le Brahman. Grâce aux études d'archives différentes dans les Caraïbes et en Europe, des preuves historiques ont pu être accumulées sur les origines géographiques et sur la chronologie de l'établissement des bovins Créole et Brahman dans les Antilles françaises. La résistance élevée des bovins Créole de la Guadeloupe aux maladies associées à ou transmises par la "tique sénégalaise", *Amblyomma variegatum*, semble due à l'héritage d'un lot de gènes de bovins de l'Afrique occidentale, en particulier de la race N'Dama. En effet, la tique *A. variegatum*, implantée dans toute l'Afrique de l'Ouest, a été introduite dans la région caraïbe avec des bovins ouest-africains. Cette tique a certainement continué à exercer une pression parasitaire, ce qui explique la capacité innée des bovins Créole à maîtriser ces maladies spontanément.

Mots clés : Bovin - Bovin Créole - Bovin N'Dama - Zébu Brahman - Zébu soudanais - Cowdriose - Dermatophilose - Tique - *Amblyomma variegatum* - Résistance aux maladies - Antilles françaises - Guadeloupe - Martinique.

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INTRODUCTION

The Brahman zebu cattle of Martinique is a crossbred population coming from a stabilized mix of different Indian zebu breeds (Gir, Nelore, Gujera) selected in the South of the USA (27, 32) and introduced in this island around 1952. These cattle are very well adapted to the tropical breeding conditions but it is to some extent susceptible to dermatophilosis (5, 11, 12, 26). As a matter of fact, before its introduction in Martinique, this cattle population had never been in contact with the tick *Amblyomma variegatum*. Also, imported "exotic" European breeds are fully susceptible to dermatophilosis and cowdriosis and are in addition not adapted to the tropical conditions.

The Creole cattle of Guadeloupe are a population highly resistant to dermatophilosis and to cowdriosis (5, 11, 43). Phenotypically, they show a more or less important hump but also several taurine features. This capability to resist diseases associated or transmitted by the "Senegalese" bont tick *Amblyomma variegatum* is an innate genetic character which is inheritable and probably maintained by a constant parasitic pressure (1, 12, 22, 29, 37, 44).

It is known for a long time that these cattle are a crossbred between *Bos taurus* and *Bos indicus* breeds, but this fact presumed by zootechnical and phenotypical observations (5) has never been proved by biological data. Furthermore the ancestral breeds of the Creole cattle have never been clearly identified. We have tried to answer several questions. On the one hand, is the *Bos taurus* type coming from European breeds, first introduced during the colonization of the West Indies (27), and/or from African taurines introduced later during trade shipping exchanges (16, 41) ? Does the *Bos indicus* type come from African and/or Indian zebu breeds ?

It is evident that if we could answer these questions, we could explain many breeding traits of this Creole population. Furthermore, it would be possible to include these breeds in comparative studies on genetic research on the improvement of disease resistance and/or zootechnical characters.

MATERIAL AND METHODS

Animals

The polymorphism of 5 genetic systems in the 9 following breeds have been compared (table I):

- 2 European taurine breeds : 139 Friesian and 212 Jersey ;

- 2 African taurine breeds, living in the West African tropical area infested by the tick *Amblyomma variegatum*, and well known to be resistant to several diseases, especially trypanosomosis. These are 1,016 shorthorn Baoule of Burkina Faso and 220 longhorn N'Dama of Guinea ;

- 167 Creole cattle of Guadeloupe ;

- 3 African zebu breeds : 106 Sudan zebus living in the Sahelian area of West Africa, 218 Boran and 393 Kapiti zebus of Kenya in East Africa ;

- 121 Brahman zebus from Martinique.

TABLE I Species, geographical origins and numbers of animals sampled in each population of the different breeds studied.

Bos t. taurus	EUROPE		139 Friesian 212 Jersey
	AFRICA	West	1016 Baoule (shorthorn) 220 N'Dama (longhorn)
Crossbreed	GUADELOUPE		167 Creole
Bos t. indicus	AFRICA	West	106 Sudan
		East	393 Kapiti 218 Boran
	MARTINIQUE		121 Brahman

Techniques

The 5 genetic systems studied were:

- the B chain of haemoglobin which is an erythrocytic protein showing 10 electrophoretic variants. The authors have considered the 2 main alleles A and B detected by electrophoresis on a cellulose acetate support, and well known to be good markers of differentiation in the *Bos genus* between the *taurus* and *indicus* species (4, 6, 8, 30, 31, 33, 35) ;

- the serum albumin displaying 7 variants by polyacrylamide gel electrophoresis (PAGE). As for haemoglobin, the authors have considered the 2 main alleles ; the F (fast) rather characteristic of *Bos taurus* breeds, and the S (slow) more frequent in *Bos indicus* breeds (3, 6, 7, 15, 19, 33, 36, 38) ;

- the serum transferrin also analysed by PAGE showing 6 electrophoretic variants : 2 of them (B and F) are known to be specific for *Bos indicus* zebu breeds (3, 6, 8, 33, 34). The Brahman population of Martinique was not typed for this system ;

- the Bovine Major Histocompatibility Complex : BoLA, highly polymorphic, shows in cattle about 50 allo specificities in the class I region (2, 9, 14, 39). These are serologically detected by the standard method of lymphocytotoxicity. Some antigens of this BoLA class I region are known to be associated with resistance or susceptibility to several diseases but also 2 of them are specific breed markers (25, 40) when considering overall breed populations.

* The KN8 specificity, isolated in Kenya, is a good *Bos indicus* breed marker (17, 25) .

* The KN18 specificity is more interesting because it is not only a *Bos taurus* marker but is above all specific for African taurines (17, 25, 42). Its frequency in European taurines and in zebu breeds is low ;

- the gamma S crystallin gene, investigated by molecular analysis (18) and showing a bi-allelic polymorphism due to a punctual G/C substitution detected at base number 1754. Indeed, after amplification by the PCR technique and separation by agarose gel electrophoresis, two DNA fragments of 128 and/or 149 bp can be detected, each DNA fragment being representative of one of the two alleles.

Mathematical models

By using the frequency differences of these 5 polymorphic loci situated on 5 different chromosomes in the bovine genome, we have established the genetic distances (21) between the 9 breeds. Calculation was based on the two mathematical matrices of NEI (28) and CAVALLI-SFORZA (10).

Historical research

We have studied different historical and commercial archives in the two French West Indies islands of Guadeloupe and Martinique and we have tried to summarize numerous specialized books and bibliographical references (16, 20, 41).

RESULTS AND CONCLUSIONS

In the B haemoglobin system, where the A allele is a marker of *Bos taurus* breeds (frequency always higher than 90 % whereas in *Bos indicus* it rarely exceeds 50 %), it was noticed (figure 1) that the Brahman frequency (55 %) is closer to the zebu average while the Creole one (71 %) indicates clearly an intermediate value between *Bos taurus* and *Bos indicus*.

In the serum albumin system, where the F allele is also a marker of *Bos taurus* breeds, it is observed here again (figure 2), an intermediate frequency of 52 % for the Creole breed whereas the Brahman value is closer to the other zebu breeds.

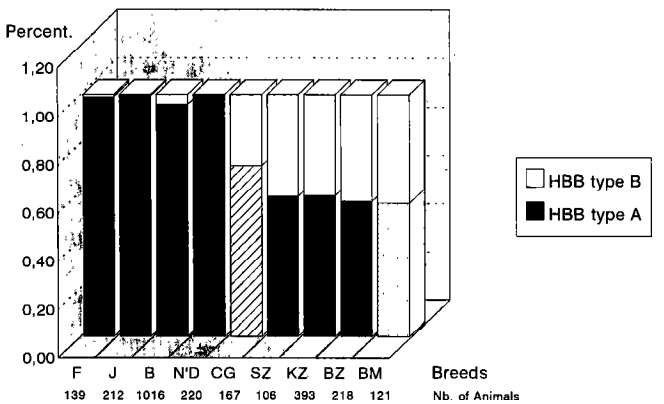

Figure 1 : Haemoglobin B type polymorphism : Allele frequencies (in percentage) in different cattle breeds : F = Friesian, J = Jersey, B = Baoule, N'D = N'Dama, CG = Creole Guadeloupe, SZ = West African Sudan Zebu, KZ = East African Kapiti Zebu, BZ = East African Boran Zebu, BM = Brahman Martinique.

The frequency of the A allele which is a taurine marker indicates in the Creole of Guadeloupe, an intermediate value (71 %) between Bos taurus *and* Bos indicus, *whereas the Brahman frequency (55 %) is closer to the zebu average. (Data sources : CIRAD-EMVT, INRA, ILRAD, AFRC/ABRO)*

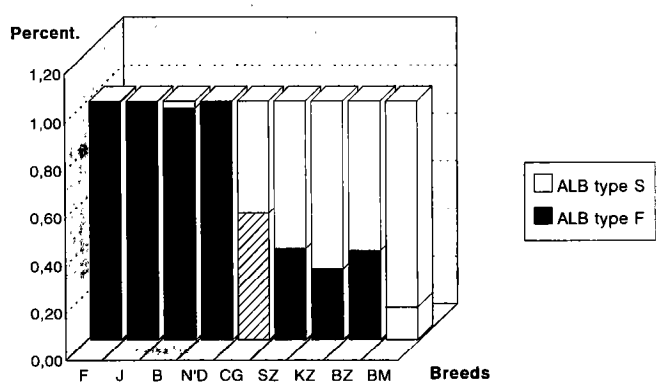

Figure 2 : Albumin type polymorphism : Allele frequencies (in percentage) in different cattle breeds (see figure 1).

The frequency of the F allele which is also a taurine marker indicates in the Creole of Guadeloupe, an intermediate value (55 %) between Bos taurus *and* Bos indicus, *whereas the Brahman frequency is 12 % . (Data sources : CIRAD-EMVT, INRA, ILRAD, AFRC/ABRO)*

The results obtained from these two systems of haemoglobin and albumin show evidence for the presence of taurine features in the Creole population of Guadeloupe.

Concerning the transferrin system :

- for the B allele, (figure 3) the Creole displays a frequency which is very similar to that of zebu breeds, whereas for the taurine breeds the allele B is completely absent ;

- for the F allele, the same phenomenon can be observed (figure 4) suggesting also the presence of *Bos indicus* blood in the Creole breed.

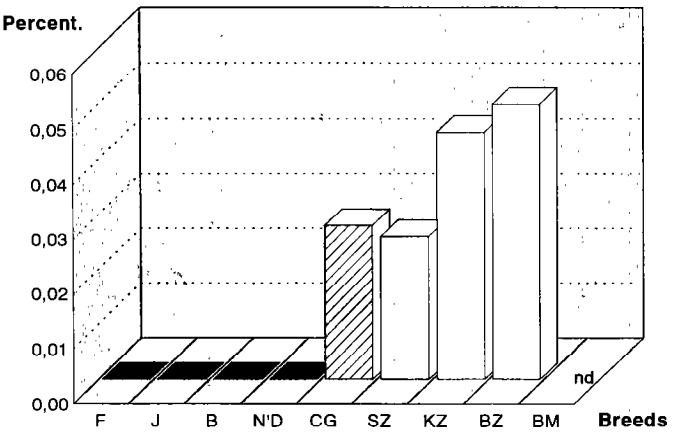

Figure 3 :Transferrin B type polymorphism : Allele frequencies (in percentage) in different cattle breeds (see figure 1) (nd = not determined).

The Creole frequency is very similar to those of zebu breeds, whereas this allele is completely absent in the taurine breeds. (Data sources : CIRAD-EMVT, CRTA, ILRAD, AFRC/ABRO)

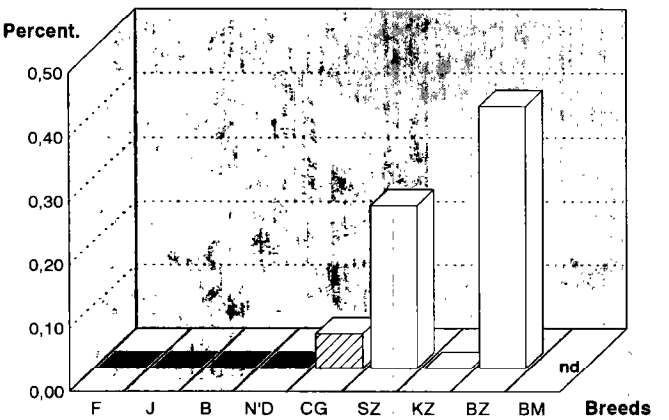

Figure 4 : Transferrin F type polymorphism : Allele frequencies (in percentage) in different cattle breeds (see figure 1) (nd = not determined).

The Creole frequency indicates an intermediate value between Bos indicus *and* Bos taurus *breeds where this allele is absent. (Data sources : CIRAD-EMVT, CRTA. ILRAD, AFRC/ABRO)*

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In the BoLA Complex:

- the frequencies of the KN8 specificity (figure 5) confirm the previous results shown with the transferrin polymorphisms, that the Creole breed possesses this zebu marker, as expected for the Brahman zebu ;

- high frequencies of the KN18 specificity (figure 6) are characteristic of West African *Bos taurus* populations whereas this specificity has only been found in a very low level in cattle from Northern Europe. For this reason, a KN18 frequency of 15 % in the Creole population is highly significative in determining the partial West African *Bos taurus* origin of the Creole breed. This result strongly suggests that Creole cattle have acquired their taurine features not only from European *Bos taurus* but also from African *Bos taurus* .

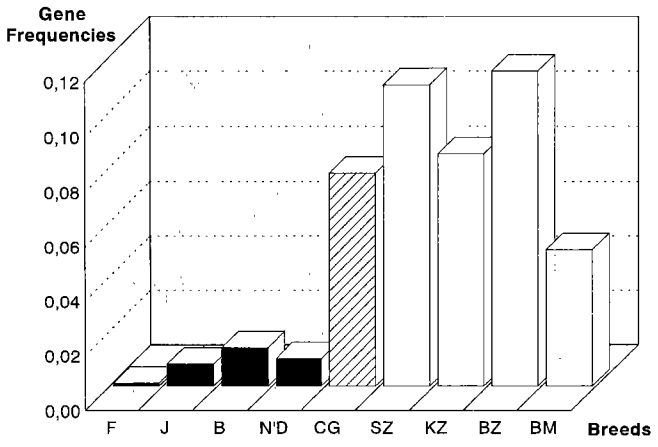


Figure 5 : Polymorphism of the KN8 specificity in the BoLA Complex : Gene frequencies in different cattle breeds (see figure 1).

The Creole frequency of this specificity is closer to those of Bos indicus *breeds including Brahman. (Data sources : CIRAD-EMVT, ILRAD)*

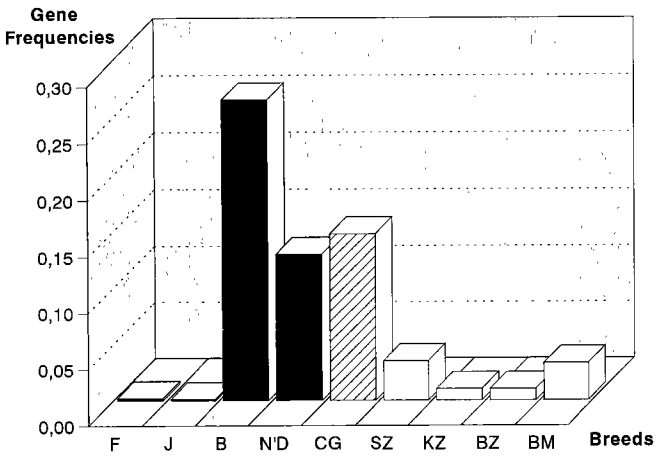


Figure 6 : Polymorphism of the KN18 specificity in the BoLA Complex: Gene frequencies in different cattle breeds (see figure 1).

The Creole frequency of this specificity is closer to those of the 2 African Bos taurus *breeds : N'Dama and Baoule, indicating its African taurine origin. Indeed, this KN18 antigen is specific to African taurine being almost absent in European taurine breeds. The frequency of the Brahman population is normally closer to those of other* Bos indicus *breeds. (Data sources : CIRAD-EMVT, ILRAD)*

For the gamma S crystallin gene polymorphism, it is found (figure 7) that the allele represented by the 128 bp fragment is monomorphic at 100 % in the Creole population. The most similar frequency of this allele is encountered in the African N'Dama (85 %). This result confirms the previous hypothesis obtained with MHC-BoLA typing, viz. that some of the Creole taurine features have been inherited from an N'Dama ancestor.

The two dendrograms obtained by the calculations of genetic distance indicate clearly (figure 8), the intermediate position of the Creole breed of Guadeloupe between the N'Dama *Bos taurus* breed of West Africa and two zebu breeds, the Sudan zebu of West Africa and the Brahman. In the NEI dendrogram the Creole is closer to the African taurine group and far from the European taurine breeds. In the CAVALLI-SFORZA dendrogram the Creole is closer to the zebu group but the N'Dama remains the nearest taurine breed. This slight difference could be explained by the existence of a close equilibrium between *Bos taurus* and *Bos indicus* types.

Regarding the historical approach, evidence have been accumulated suggesting the following chronology of the establishment of cattle in the French West Indies.

When Christopher Columbus discovered the Caribbean islands in 1492, there was no domestic livestock.

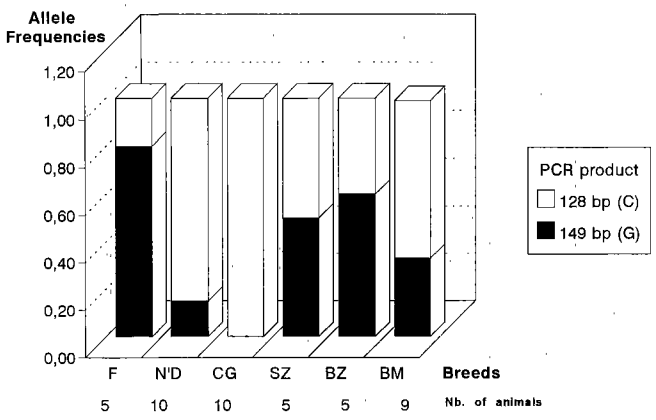


Figure 7 : G/C Bi-Allelic polymorphism of the Gamma S Crystallin gene in different cattle breeds (see figure 1).

The allele represented by a 128 bp fragment is monomorphic at 100 % in the Creole population and the most similar frequency of this allele is the one of the African N'Dama with 85 %. This indicates once again the African Bos taurus *N'Dama origin of the Creole crossbred. (Data sources : CIRAD-EMVT, ILRAD)*

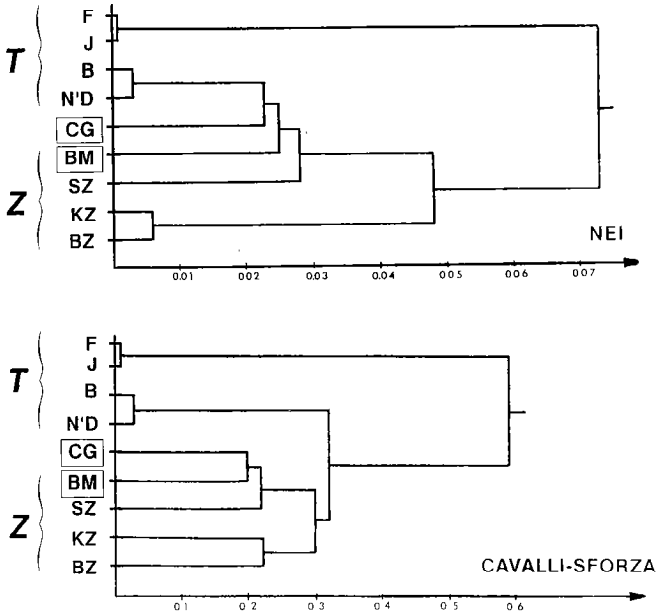
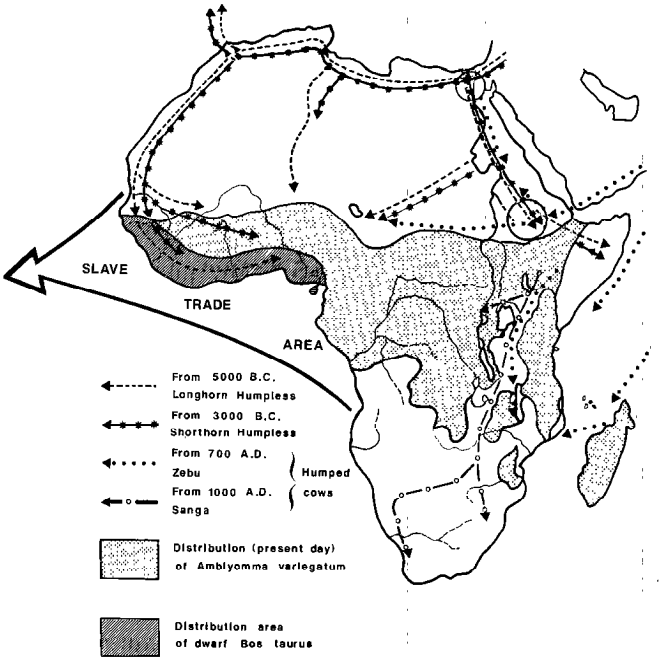


Figure 8 : Dendrograms of genetic distance are obtained by using the two different mathematical matrices of NEI and CAVALLI-SFORZA in different cattle breeds (see figure 1).

It can be seen clearly in the two dendrograms the intermediate position of the Creole breed of Guadeloupe between the N'Dama Bos taurus *breed of West Africa and two zebu breeds, the West African zebu and the Brahman. In the NEI dendrogram the Creole is closer to the African taurine group and far from the European taurine breeds. In the CAVALLI-SFORZA dendrogram the Creole is closer to the zebu group but the N'dama remains the nearest taurine breed. This slight difference could be explained by the existence of a close equilibrium between* Bos taurus *and* Bos indicus *types.*



Map 1 : Map of Africa showing the foci of origin and time of subsequent spread in indigenous breeds of African cattle. Data are based on PAYNE (1964) and EPSTEIN (1971). The distribution area of African Bos taurus *either longhorn or shorthorn lies all along the Guinea Gulf Coast and is included in the distribution area of the tick* Amblyomma variegatum. *The Spanish, Portuguese and French colonial establishments were mainly based in these areas between the South of Senegal and Angola.*

All during the colonization period from the XVIth to the XVIIIth century, the cattle which populated these islands were imported from Europe, mainly from Spain and Portugal, but a few from France. At the same time, African cattle were probably also imported from the Spanish, Portuguese and French colonial establishments of Africa, which were located essentially all along the Guinea Gulf Coast (map 1). These dwarf, humpless and shorthorn *Bos taurus* cattle have been established for 7,000 years (13) in this area infested by the tick *Amblyomma variegatum.* It was well adapted to the tropical environment and was resistant to certain tropical diseases. In this way it is possible that these cattle could be responsible for the introduction of *Amblyomma variegatum* into the Caribbean islands, since the XVIth century. Its survival during some weeks of shipping was probably possible by completing its development cycle on different hosts during the journey. Arriving in the tropical environment of the Caribbean islands, this tick was able to acclimatize.

This tick could have existed in small numbers, without visible pathologic consequences on cattle, before 1828 when its introduction is presumed to have occurred with zebu cattle of Senegal. These cattle of African origin might have resisted diseases associated or with transmitted by *Amblyomma variegatum*, whereas the cattle of European origin might have disappeared progressively.

During the XIXth century, African cattle were imported again : mainly zebus from Senegal to improve the phenotypic aspects necessary for cart traction in sugar cane cultivation, and pure or crossbred N'Dama cattle from the Gambia (24). This African zebu also originated from an area infested with *Amblyomma variegatum,* and was accustomed to the environmental conditions. It was crossbred with the taurine cattle of African origin (such as the Senepol breed of the Virgin Islands which is a crossbred population between 2 *Bos taurus* breeds: African N'Dama of Senegal and European Red Poll). The heterosis effect could improve the resistance phenomenon in the areas infested by *Amblyomma variegatum* where the constant parasitic pressure maintained this genetic predisposition.

All during the XXth century, the African zebu type was gradually replaced by Indian zebu breeds imported since, from the French establishments of India and from Venezuela. At the present time, this crossbreeding is continuing with Brahman zebu selected in the USA. Also, different attempts at crossbreeding with European breeds for zootechnical improvements were severe failures because of disease.

At present, have been studied 5 distinct polymorphic loci situated in 5 distinct chromosomes out of the 26 autosomal chromosomes constituting the bovine genome. It is therefore impossible to conclude with certainty on the exact phylogeny of the French West Indies cattle populations. Contribution of Spanish and Portuguese cattle to the taurine features of the Creole breed needs to be assessed because these cattle are the ancestors of the Criollo *Bos taurus* breed widely distributed in South America and in the Spanish Caribbean islands. Origin and genetic characteristics of European breeds have been recently studied in relation to Indian and African cattle (23, 24)). These studies performed by using molecular techniques have revealed new molecular markers, one of them associated with the Y chromosome, allowing to distinguish *Bos taurus* from *Bos indicus* genotypes.

In the light of these results, the authors plan to further investigate the genetics of the French West Indian cattle by including Spanish and Portuguese breeds in our present panel. The rationale of these studies is to define gene pools in different breeds known to be resistant or susceptible to diseases in order to preserve these gene pools in future breeding programs.

The authors also think that it could be very interesting and useful to pursue their historical investigations by studies of other archives in several countries. These could be the formerly colonized countries like Trinidad, St Domingo, Cuba and Puerto Rico in the Caribbean, and the formerly colonizing countries like Spain, Portugal and France.

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MAILLARD (J.C.), KEMP (S.J.), NAVES (M.), PALIN (C.), DEMANGEL (C.), ACCIPE (A.), MAILLARD (N.), BENSAID (A.). An attempt to correlate cattle breed origins and diseases associated with or transmitted by the tick *Amblyomma variegatum* in French West Indies. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 283-290

By using biological data and historical research, we have tried to explain the difference between resistance and susceptibility to the diseases transmitted (cowdriosis) or associated (dermatophilosis) with the tick *Amblyomma variegatum*, in two cattle breeds of the French West Indies: the Creole crossbred cattle of Guadeloupe and the Brahman zebu cattle of Martinique. Have been studied the polymorphisms of 5 independent genetic systems (erythrocytic haemoglobin, serum albumin and transferrin, the class I region of the BoLA complex and the gamma S crystallin gene) in different breeds comprising *Bos taurus* cattle of Europe and Africa, *Bos indicus* of West and East Africa, as well as the Brahman of Martinique and the Creole crossbred of Guadeloupe. By comparing the different allele frequencies of these 5 non related polymorphic loci and by using the two different mathematical matrices of NEI and of CAVALLI-SFORZA, have been established the genetic distances between these breeds. It appears clearly that the Creole cattle of Guadeloupe are in an intermediate position between the Bos taurus N'Dama breed of West Africa and two *Bos indicus* zebu breeds, namely the West African Sudan zebu and the Brahman. Thanks to studies of different archives in the Caribbean and in Europe, historical evidence have been accumulated on the geographical origins and on the chronology of the establishment of Creole and Brahman cattle in the French West Indies. The high resistance of the Creole cattle of Guadeloupe to diseases associated with or transmitted by the "Senegalese" tick *Amblyomma variegatum* seems to be due to the inheritance of a pool of genes from West African cattle and more particularly from the N'Dama breed. Indeed, the tick *Amblyomma variegatum*, endemic in all West Africa, was introduced in the Caribbean with West African cattle. Most certainly, this tick maintained a parasitic pressure, hence the innate capability of the Creole cattle to naturally control these diseases.

Key words : Cattle - Creole cattle - N'Dama cattle - Brahman zebu cattle - Sudan zebu cattle - Heartwater - Dermatophilosis - Tick - *Amblyomma variegatum* - Disease resistance - French West Indies - Guadeloupe - Martinique.

MAILLARD (J.C.), KEMP, (S.J.), NAVES (M.), PALIN (C.), DEMANGEL (C.), ACCIPE (A.), MAILLARD (N.), BENSAID (A.). Intento de correlación entre las razas bovinas y las enfermedades relacionadas o transmitidas por la garrapata *Amblyomma variegatum* en las Antillas francesas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 283-290

Mediante el uso de datos biológicos e históricos, se trata de explicar la diferencia entre la resistencia y la susceptibilidad a las enfermedades transmitidas (por ejemplo la cowdriosis) o asociadas (por ejemplo la dermatofilosis) con el ácaro *Amblyomma variegatum*, en dos razas de ganado de las Antillas Francesas : cruce autóctono (Criollo) de Guadalupe y ganado Brahman de Martinica. Se estudiaron los polimorfismos de cinco sistemas genéticos independientes (hemoglobina eritrocítica, albúmina sérica y transferrina, complejo de clase I de BoLA y gen cristalino gama S), en diferentes razas de ganado *Bos taurus* europeo y *Bos indicus* de África del Este y del Oeste, incluyendo el cruce autóctono (Criollo) de Guadalupe y el ganado Brahman de Martinica. Las distancias genéticas de estas razas, se establecieron mediante la comparación de las frecuencias alélicas de estos 5 loci polimórficos e independientes y el uso de dos matrices matemáticas diferentes, de NEI y CAVALLI-SFORZA. Parece claro que el ganado Criollo de Guadalupe se encuentra en una posición intermedia entre el *Bos taurus* N'Dama de África del Oeste y dos razas cebuínas de *Bos indicus*, el cebú Sudanés de África del Oeste y el Brahman. Gracias a los estudios de diferentes archivos, tanto en el Caribe como en Europa, logramos acumular la evidencia histórica sobre los orígenes geográficos y cronológicos del establecimiento del ganado Criollo y Brahman en las Antillas Francesas. La alta resistencia del ganado Criollo de Guadalupe a las enfermedades asociadas o transmitidas por la garrapata "senegalesa" *Amblyomma variegatum*, parece ser debida a la carga hereditaria de un "pool" de genes de ganado de África del Oeste y particularmente de la raza N'Dama. De hecho, *Amblyomma variegatum*, endémica en África del Oeste, fue introducida en el Caribe junto con el ganado del oeste africano. Aparentemente, este ácaro ha mantenido una presión parasitaria suficiente para la conservación de la capacidad natural del ganado Criollo para el control de este tipo de enfermedades.

Palabras claves : Bovino - Bovino Criollo - Bovino N'Dama - Cebú Brahman - Cebú Sudanés - Cowdriosis - Dermatofilosis - Garrapata - *Amblyomma variegatum* - Resistancia a las enfermedades - Antillas francesas - Guadalupe - (La) Martinica.

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An attempt to identify genetic markers of resistance or susceptibility to dermatophilosis in the zebu Brahman population of Martinique

MAILLARD (J.C.), PALIN (C.), TRAP (I.), BENSAID (A.). Une tentative d'identification de marqueurs génétiques de résistance ou de sensibilité à la dermatophilose dans la population de zébus Brahman de la Martinique. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 291-295

La dermatophilose est une maladie associée à la tique *Amblyomma variegatum* ; une prédisposition génétique à la manifestation des symptômes a été démontrée. En effet, les bovins Créole de la Guadeloupe constituent une population très résistante à cette maladie, tandis que les zébus Brahman de la Martinique semblent très sensibles. Néanmoins, dans cette population de Brahman il y a un gradient en ce qui concerne l'intensité des symptômes, selon les individus. Dans plusieurs troupeaux de ces zébus Brahman purs, maintenus dans les mêmes conditions, des groupes d'animaux sensibles et résistants ont été sélectionnés sur la base de la présence simultanée d'animaux cliniquement affectés par la dermatophilose et d'animaux non affectés. Plusieurs systèmes génétiques très polymorphes ont été étudiés sur ces animaux, tels que l'hémoglobine, l'albumine, le complexe BoLA (classes I et II) et le gène de gamma S crystalline. Seulement l'exon 2 du gène BoLA-DRB3, examiné par la technique de PCR-RFLP a montré un polymorphisme intéressant. Une carte génotypique a été établie qui montre au moins 4 allèles différents, dont un semble particulier à un animal sensible à la dermatophilose. Avant d'arriver à des conclusions, d'autres recherches avec plus d'échantillons d'ADN d'animaux sensibles sont nécessaires.

Mots clés : Zébu Brahman - Dermatophilose - Tique - *Amblyomma variegatum* - Résistance aux maladies - Gène - Polymorphisme génétique - Marqueur génétique - Martinique.

INTRODUCTION

Skin disease associated with the actinomycete bacterium, *Dermatophilus congolensis,* has been recognized since 1910 in Africa where it was first described in the Congo by VAN SACEGHEM (28). Since then, the disease continues to seriously limit animal production in the tropics and remains a problem in the temperate regions. It has been a particularly severe cause of losses in the Caribbean (26) where it has forced farmers in some areas to abandon ruminant husbandry. Although the epidemiology of dermatophilosis is well known, the pathogenesis of the disease is still poorly understood. No method for control, which is readily applicable in the areas of extensive husbandry where it is most needed, has yet been found. Nevertheless, recent research has begun to provide explanations for some of the factors which increase the severity of diseases (13, 14) and particularly dermatophilosis.

Resistance to infection differs considerably both between species (11), within species and between breeds (2, 3, 5, 10, 20). Individual variation within breeds is also quite significant.

Amongst cattle, the N'Dama and Muturu breeds are highly resistant (20), whilst European *Bos taurus* breeds are particularly susceptible, as are *Bos indicus* like Brahman cattle (5, 10). In the Caribbean, the Creole crossbreed cattle appear to be highly resistant (2, 3). This character seems innate and probably acquired because of its African *Bos taurus* N'Dama origin (17), maintained since its establishment in the West Indies by the constant pressure of the "Senegalese" tick *Amblyomma variegatum.* Creole cattle crossbred with susceptible breeds such as European cattle or Brahman zebus show a decrease of the resistance. The zebu Brahman population of Martinique, is mainly susceptible. It is a stabilized mix of Indian zebu breeds (Gir, Nellore, Gujera...) made in the USA (18) and introduced in Martinique around 1952. Being since in contact with the tick *Amblyomma variegatum*, there exists in these susceptible cattle a gradient in the individual susceptibility to dermatophilosis (2).

MATERIAL AND METHODS

Several herds of pure zebu Brahman were selected because of the simultaneous presence of animals affected or not by clinical dermatophilosis. In these herds, 121 non-related animals were classified as resistant or susceptible according to the absence or presence of skin lesions. Sixty nine (69) of them, highly susceptible, showed severe skin lesions whereas the 52 others never showed lesions after one year of continous survey. It is important to notice that all animals belonging to the same herd were kept under the same farming conditions.

On these animals was studied the following panel of polymorphic marker systems:

- the B chain of haemoglobin which is an erythrocytic protein showing 10 electrophoretic variants by electrophore-

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sis on cellulose acetate (1). Only the 2 main alleles A and B were detected ;

- the serum albumin, also analysed by PAGE (6), showing 7 electrophoretic variants. As for haemoglobin, only the 2 main alleles F and S were detected ;

- the bi-allelic polymorphism of the gamma S crystallin gene, investigated by molecular analysis (9). This gene shows a punctual G/C substitution detected at the base number 1754. After amplification by the PCR technique and separation by agarose gel electrophoresis, 2 DNA fragments of 128 and/or 149 bp can be detected. Each DNA fragment is representative of one of the two alleles ;

- the BoLA complex (25), highly polymorphic, shows in cattle about 50 allo specificities in the class I region (4). These are serologically detected by the standard method of lymphocytotoxicity. Some antigens of the BoLA class I region are associated with resistance or susceptibility to several diseases (21) such as eye cancer, mastitis (19, 24) or leucosis (12);

- the polymorphism of BoLA class II genes (8, 15), is investigated in the exon 2 of the BoLA-DRB3 gene (7, 22, 23), (at least 50 alleles) using the PCR-RFLP technique (16, 27). Genomic DNA of 1 susceptible and 7 resistant animals was obtained from nucleated blood cells by the standard method of phenol-chloroform extraction. After amplification by PCR, using primers surrounding the exon 2 of the BoLA-DRB3 gene, we divided the amplified product in 3 aliquots each one being separately digested by one of the following restriction enzymes *HaeIII, DpnII* and *Fnu4hI*. The DNA fragments were separated by agarose gel electrophoresis.

RESULTS AND CONCLUSIONS

No significant differences in allele frequencies between resistant and susceptible animals were found either in the polymorphic protein systems or in the gamma S crystallin gene (table I).

Only the BoLA system seems to be of interest. The W6 class I specificity showed a different frequency but the statistical significance is low (P <9 %).

On the other hand, an extensive polymorphism was detected (fig. 1) within exon 2 of the DRB3 gene.

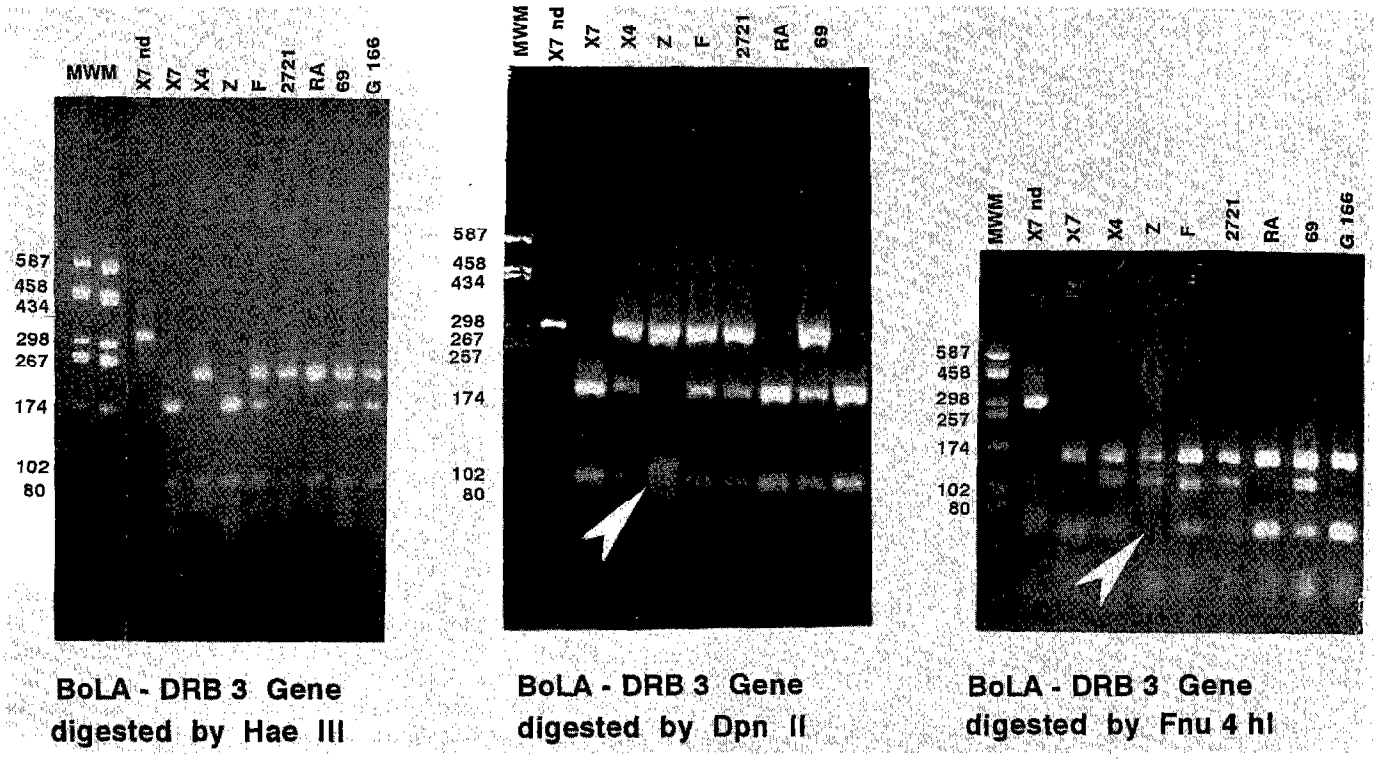

Figure 1 : Electrophoretic polymorphism of exon 2 of the BoLA DRB3 gene revealed by restriction fragment length polymorphism (RFLP). After PCR amplification, DNA of seven resistant animals (X7, X4, F, 2721, RA, G166 and 69) and one susceptible animal (Z) were subjected to restriction enzyme digestion (HaeIII, DpnII *or* Fnu4hI) *and run in a 2.5 % agarose gel. Size determination of the DNA fragment was performed by comparing samples to the products of the plasmid PUC 19 digested by* HaeIII *(MWM). Note in lane Z of the* DpnII *pattern the 3 low molecular weight DNA fragments (around 100bp) and in the* Fnu4hI *pattern, the absence of a 60bp DNA fragment characterizing this susceptible animal.*

TABLE I Allele frequencies (Fq) are expressed in percentage (%) of the Brahman population tested.

		Resistant		Susceptible		Diff.
		Nb	Fq	Nb	Fq	
HBB	A B	52	55.5 45.5	69	55 45	NS
ALB	F S	52	11.8 88.2	69	12.9 87.1	NS
G.S. CRYST.	128 bp 149 bp	9	44.4 55.6	9	55.6 44.4	NS
BoLA W6		52	4	69	10	P=0.09

Nb : number of animals tested, HBB : haemoglobin, ALB : albumin, G.S. CRYST. : gamma S crystallin, BoLA : bovine leucocyte antigens, NS : Not Significant.

By combining the analysis of the three restriction enzyme patterns, was prepared a genotypic map of exon 2 of the DRB3 gene, where 4 alleles could be fully characterized (fig. 2).

The animal Z classified as susceptible is unique by the fact that it displays the 2 particular alleles we named "b" and "d". The allele "b" is characterized by a *DpnII* site toward the 5' end of exon 2 which is absent from all other resistant animals. Furthermore, animal Z, lacks in both alleles "b" and "d" an *Fnu4hI* polymorphic site toward the 3' end of exon 2.

Before reaching any conclusion, more animals need to be tested in order to investigate if the alleles found in the animal Z correlate with the character of susceptibility to dermatophilosis.

The method described here is fast, practical and can be applied to any polymorphic region displaying restriction enzyme sites.

Thus, we plan to study the polymorphism of other genes coding for proteins implicated either in immunological processes such as interferon gamma and interleukins, or in skin proteins such as keratin.

The same method will be used in other breeds such as European breeds (mainly susceptible), in tropical breeds as taurines N'Dama and Baoule, crossbreds of Guadeloupe Creole and zebus of Cameroon and Burkina Faso.

The selection of genotypes resistant or susceptible to dermatophilosis remains another alternative to tick control and in particular in areas in which tick eradication is impossible.

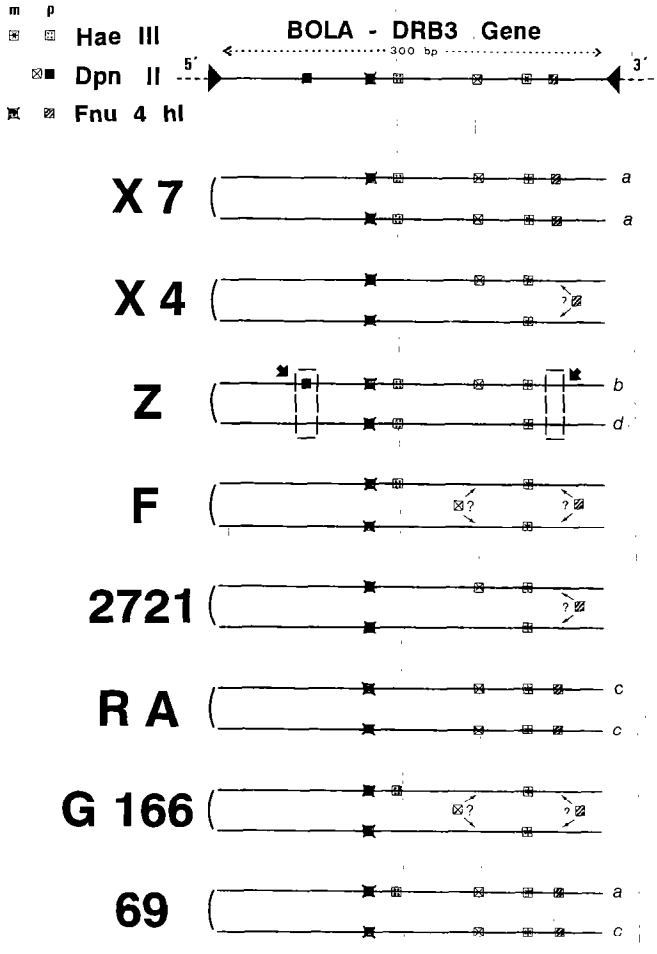

Figure 2 : Genetic map of exon 2 of the BoLA DRB3 gene encountered in the 8 Brahman cattle tested. Symbols indicate the position of restriction enzyme sites within exon 2 ; "m" and "p" design whether the site is monomorphic of polymorphic respectively. Arrows indicate the characteristics of alleles "b" and "d" differenciating the susceptible animal Z from other resistant animals. When a restriction site is ambiguous and cannot be placed in a given allele it is noted with "?".

ACKNOWLEDGEMENT

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MAILLARD (J.C.), PALIN (C.), TRAP (I.), BENSAID (A.). An attempt to identify genetic markers of resistance or susceptibility to dermatophilosis in the zebu Brahman population of Martinique. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 291-295

Dermatophilosis is a disease associated with the tick *Amblyomma variegatum*, and a genetic predisposition to the manifestation of symptoms has been demonstrated. Indeed, the Creole cattle of Guadeloupe constitute a population which is highly resistant to this disease, whereas the Brahman zebu cattle of Martinique seem very susceptible. However, in this Brahman population there is a gradient regarding the severity of symptoms depending on individuals. In several herds of these pure zebu Brahman, kept under the same farming conditions, we selected susceptible and resistant groups because of the simultaneous presence of animals affected or not by clinical dermatophilosis. In these animals we studied several highly polymorphic genetic systems such as haemoglobin, albumin, the BoLA Complex (class I and II) and the gamma S crystallin gene. Only exon 2 of the BoLA-DRB3 gene, investigated by PCR-RFLP technique, showed interesting polymorphisms. We have established a genotypic map showing at least 4 different alleles of which 1 seems particular to one animal susceptible to dermatophilosis. Before reaching any conclusion further investigations with more DNA samples of susceptible animals are needed.

Key words : Brahman Zebu cattle - Dermatophilosis - Tick - *Amblyomma variegatum* - Disease resistance - Gene - Genetic polymorphism - Genetic marker - Martinique.

MAILLARD (J.C.), PALIN (C.), TRAP (I.), BENSAID (A.). Un intento para la identificación de los marcadores genéticos de la resistencia o la susceptibilidad de la dermatofilosis en la población cebuína Brahman, en Martinica. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 291-295

La dermatofilosis es una enfermedad relacionada con el ácaro *Amblyomma variegatum*, para la cuál se ha demostrado una predisposición genética a la manifestación de los síntomas. El ganado Creole de Guadalupe es altamente resistente a esta enfermedad, mientras que el cebú Brahman de Martinica parece ser muy susceptible. Sín embargo, parece existir un gradiente en la severidad de los síntomas en los diferentes individuos de esta población de Brahman. Se seleccionaron grupos de animales resistentes, en varios hatos Brahman puros, mantenidos bajo las mismas condiciones, en base a la presencia simultánea de animales afectados o no por la dermatofilosis clínica. En estos grupos se estudiaron varios sistemas genéticos altamente polimórficos, como la hemoglobina, la albúmina, el complejo BoLA (clase I y II) y el gen cristalino gama S. El único que mostró polimorfismos interesantes fue el exon 2 del gen BoLA-DRB3, estudiado mediante la técnica PCR-RFLP. Se estableció un mapa genotípico, que muestra al menos cuatro alelos diferentes, uno de los cuales parece propio de los animales susceptibles para la dermatofilosis. Antes de emitir una conclusión, deben llevarse a cabo investigaciones con más muestras de ADN de animales susceptibles.

Palabras claves : Cebú Brahman - Dermatofilosis - Garrapata - *Amblyomma variegatum* - Resistancia a las enfermedades - Gen - Polymorfismo genético - Marcador genético - Martinica.

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Observations on a dermatophilosis outbreak in Brahman cattle in Guadeloupe. Description, epidemiological and economical aspects

NAVES (M.), VALLÉE (F.), BARRÉ (N.). Observations sur un foyer de dermatophilose sur des bovins Brahman en Guadeloupe. Description, aspects épidémiologiques et économiques. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 297-302

Un foyer de dermatophilose grave est apparue en 1985 dans un troupeau de vaches Brahman importées de la Martinique en Guadeloupe en juillet 1983. La maladie était peu connue en Guadeloupe jusqu'alors, car la race zébu locale possède une résistance naturelle élevée aux maladies transmises par les tiques ou associées à elles. Les conditions de l'apparition et du développement de la maladie ont été étudiées en rapport avec la gestion du troupeau, le climat et les traitements appliqués. Pendant 6 mois suivant l'importation, lorsque les animaux étaient à l'embouche, la maladie ne s'est pas manifestée. Les premières lésions de la dermatophilose sont apparues environ 2 mois après que les animaux ont été mis dans des pâturages infestés de tiques. Alors, 30 p. 100 du troupeau a été infecté. Le développement de l'infection a augmenté de façon dramatique à l'approche de la saison humide, et toutes les 29 vaches Brahman ont montré des lésions en juillet 1985. La maladie n'a pu être guérie que par des traitements draconiens, comprenant des antibiotiques et la désinfection locale, associés au déplacement des animaux des pâturages vers des stalles couvertes. Néanmoins, la maladie a causé la mort de 13 animaux. Des différences dans les réactions individuelles ont été notées et 7 types différents d'évolution ont été identifiés. Ces observations ont fourni des informations sur l'épidémiologie de la dermatophilose dans les conditions climatiques tropicales humides des Caraïbes. Elles ont démontré l'importance économique de cette maladie grave pour les bovins de la région, ainsi que la sensibilité des races exotiques.

Mots clés : Bovin Brahman - Dermatophilose - Tique - Conduite du troupeau - Pâturage - Antibiotique - Épidémiologie - Économie de l'élevage - Guadeloupe.

INTRODUCTION

Dermatophilosis, a bacterial skin disease due to *Dermatophilus congolensis*, is the most important infectious disease of ruminants in the Caribbean (5, 14, 15). Severe outbreaks of the disease are closely associated with the presence of the tropical bont tick *Amblyomma variegatum* (3, 5, 11, 14, 15).

In Guadeloupe, where *Amblyomma* ticks have been present for a long time, cattle breeding is based on the use of a local Creole population issued from various breeds, including African, European and Indian cattle, from taurine and zebu type. This population appears to be highly resistant to the disease (3).

The high susceptibility of Brahman cattle to dermatophilosis is well known (4, 6, 7, 9, 13), whereas several local breeds in Africa have been reported to be resistant (1, 9, 13). Also in Guadeloupe, an introduction in 1983 of a Brahman herd ended in a severe outbreak of dermatophilosis, a disease which had not yet been reported in such a critical way in our conditions. In late 1985, only 45 % of the animals were still alive, after repeated treatments and considerable efforts.

This pathological study was not the main purpose of the herd management, but the sanitary survey of the animals has been registered throughout the evolution of the disease. The present report is based on the interpretation of our observations on frequency of treatments, scabs scoring, mortality, management conditions and economic losses which could be related to the disease.

MATERIAL AND METHODS

Herd management

Twenty-nine Brahman yearling heifers were imported from Martinique in July 1983 and reared in covered pens, then in open feed-lot for a year, with a short grazing period in December 1983 and January 1984.

In July 1984, they were allowed to graze, in a single lot during the breeding season until November 1984, and then in two herds, for a comparison of animal production with cows of the local Creole breed. The herds contained : (H1) 20 Brahman and 9 Creole heifers, and (H2) : 9 Brahman and 9 Creole heifers grazing respectively 5.8 and 4 ha of *Digitaria* pasture, intensively managed with irrigation and fertilization. Both pastures were free of thorny bushes; trees offer shade in both of them, but more in (H2) than in (H1).

The first objective, a comparison of animal production, was not completely carried out since dermatophilosis occured in Brahman cows. Considering its severe impact, Brahman were removed from pastures in August 1985, firstly in a feed-lot, then in individual covered pens. The aim of this transfer was to facilitate surveillance and treatments of affected animals, and their protection against environmental agents such as ticks, sun and rain.

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Sanitary policy

Cattle on pastures were regularly sprayed against ticks, at least once a month with deltamethrin (Butox®, 0.5 ml/l), mixed with a local disinfectant (Cresyl). This treatment controlled but did not eliminate the ticks from the cattle; they remained present at a low to medium level (5 to 10 adult ticks per head) during the whole grazing period.

After their movement indoors, cattle were dipped in a solution of chlorpyriphos and toxaphene (Procibam®, 1.5 ml/l) and quaternary ammonium (0.1 ml/l), three times in the following month, in order to eliminate the ticks and disinfect the coat of the animals. Then, cows were sprayed with quaternary ammonium (0.5 and 1 ml/l), once or twice a week until November 1985, and less frequently in the following months.

Animals infected with dermatophilosis lesions were treated by intramuscular injections of antibiotics. Different preparations were successively used :

- oxytetracycline (Long Acting Terramycine®, 200 mg/ml), as a single injection (1 ml/10 kg) (LAT) ;

- spiramycine (Suanovil 20®, 0.6 x10^6 IU/ml), as a single injection (12.5 ml/100 kg) (S20) ;

- from May to July 1985, LAT or S20 treatments were replaced because of the high cost of the products (40 ml LAT cost about US $ 60) and because it was difficult to obtain in Guadeloupe. An association of penicillin and streptomycin was used, as the only formulation then available (BiPeniStreptomycine®, 5x10^6 IU/5 g), dosed 10x10^6 IU/10 g per head, every 10 to 15 days (BPS).

Interpretations

Each treatment was registered individually for each animal. On several occasions, lesion intensity was also recorded, by visual evaluation according to a four levels scale, as well as body condition and weight.

The description of the disease evolution is based on treatment procedures : frequencies at different periods, intervals between treatments and cumulative number of treatments per animal. Evolution of lesions and body condition have been related to treatments on some occasions.

Individual datas have also been analysed through factorial analysis and hierarchical classification, revealing groups of animals according to their own reactions during the outbreak. The individual data included in the analysis were :

- date of appearance of the first lesions (3 classes) ;

- season of appearance of the lesions (2 classes) ;

- number of treatments (LAT or S20) till April 1985 (3 classes) ;

- first BPS treatment in 1985 (2 classes) ;

- number of BPS treatments (2 classes) ;

- intensity of the lesions in August 1985 (3 classes).

The physical and economical incidence of the disease on the animal production has also been evaluated.

RESULTS

Evolution of the disease

The data on the frequency of the treatments express the clinical evolution of the disease. They are summarized in figure 1 for the whole period between the appearance of the first cases and the end of the worst outbreak in late 1985.

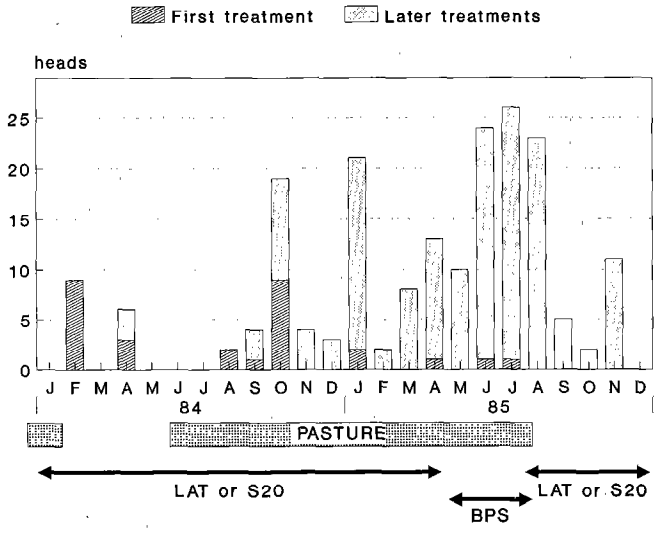

Figure 1 : Evolution of the number of animals threated per month.

Appearance of the disease

The first cases of dermatophilosis appeared two months after cattle were allowed to graze, in February 1984 on nine heifers (31 %) and on three more animals in April 1984. They were treated by LAT; for three of them, this treatment was repeated two months later. No more treatments were necessary during the three following months, neither on new cases nor on already treated animals.

Between August and October 1984, twelve new cases were recorded (41 % of the herd). By that same time, treatments had to be renewed on ten animals of the twel-

ve affected during the first months of the year. Thus, in late 1984, twenty-four (83 %) of the Brahman heifers had been affected with dermatophilosis; half of them had to be treated twice or more.

Table I presents the effect of both treatments (LAT or S20) applied in October 1984 on the intensity of the lesions : 61 % of the animals were cured two weeks later, while most of the others had only rare scabs. Both treatments appeared to have the same efficacy, and reduced significantly the body surface covered with lesions.

TABLE I Effect of treatment (October 1984)

Scab notation / Treatment	LAT	S20
	03 Oct. 1984	
1 (rare scabs)	5	3
2 - 3 (< 50 % body surface)	4	6
	17 Oct. 1984	
0 (no scabs)	6	5
1 - 2 (< 25 % body surface)	3	4

Evolution of the disease on pasture

Two phases can then be described after November 1984. During the first one until April 1985, some sporadic treatments were necessary each month, but particularly in January and April 1985 (fig. 1). The LAT or S20 treatments were periodically renewed since the beginning of the rainy season in October 1984; most animals were treated every two to four months with some differences between animals : 60 % of the treatments were renewed after two to four months, *vs* 30 % less than two months and 10 % more than four months later. No evidence of any herd management effect can be observed on the frequency of treatment in this period : 1.7 ± 0.9 (H1) *vs.* 2.0 ± 1.1 (H2).

The second phase took place from May to July 1985, during the use of BPS treatment every 2 weeks. The frequency of treatments increased rapidly. This evolution seems to be more unfavourable for the herd H1, with 60 % of cows treated since May 1985 and receiving more than 3 applications, than for H2, where all the cows were treated only in July 1985 and received less than 3 treatments. Nevertheless, no animal remained unaffected by the disease in late July 1985.

Critical stage and recovery

As the disease reached a critical stage, with the death of 6 animals, cows were moved indoors in August 1985. A new phase of treatments was initiated, with LAT or S20 antibiotics and local disinfection with quaternary ammonium. This policy combined with the protection against ticks and sun was effective as shown in table II. While 35 % of the animals were in very bad condition with severe lesions on August 23rd, the scab intensity rapidly decreased in the first week. But the recovery of the most seriously affected animals was much slower, and 7 more animals among them died. For the others, the disease was completely cured in December 1985.

TABLE II Body condition (23 August 1985) and evolution of the lesions intensity in late 1985.

Scabs notation	Body condition[1]		Evolution of the lesions			
	< 3	> = 3	23 Aug.	29 Aug.	15 Sept.	6 Nov.
No scabs				2	5	11
1 (rare scabs)	1	7	8	6	2	3
2 (< 25 % b.s.)	3	3	6	12	8	3
3 or 4 (> 25 % b.s.)	7	2	9	2	2	1

[1] *Body condition score : < 3 : weak to very weak ; > = 3 : medium to good.*

Individual reaction to dermatophilosis

Though all the animals were submitted to the same conditions, individual differences appeared. These observations were summarized in six parameters characterizing their individual reactions and reflecting their own susceptibility to the disease. A hierarchical classification following a factorial analysis of these parameters segregated seven groups of animals (table III); the issue of each animal was latter correlated to these groups.

The most important difference is in the date of first appearance of the disease (1st treatment). A second distinction between groups is based on the later evolution, on the average of the frequency of LAT or S20 treatments applied before May 1985, and particularly the incidence of BPS treatments (1st treatment and number), and the intensity of lesions in late August 1985.

This classification seems relevant with the existence of different levels of resistance or susceptibility, the main one being to the first infestation (groups 1 and 2; groups 3 to 5 and groups 6 and 7). But some modulations to the extent of the disease can be observed when this barrier is crossed. The mortality of animals is mainly explained by this distinction.

TABLE III Classification of animals according to their reactions

Groups (heads)	1 (3)	2 (2)	3 (4)	4 (5)	5 (3)	6 (5)	7 (7)
1st treatment	1985		late 1984			early 1984	
Treatment before May 1985	< = 2		< = 3	–	3	> = 4	
1st BPS treatment (Number)	July (< = 2)	May (> 2)	July (< = 2)		May (> 2)	July (< = 2)	May (> 2)
Lesions notation	0-1	> = 2	0-1	2-3	3-4	0-2	4
Dead animals	1	2	none		2	2	6

Consequences on animal production and outcome

Figure 2 presents the evolution of body weight of Brahman and Creole cows in 1985. Until May 1985, the body weight gain was quite similar in both breeds, the Brahman weighing 60 kg more than the Creole. Then, the disease had a strong effect on the weight of Brahman cattle, even on those which survived, their weight lowering until October. For the most badly affected, the effect was quite dramatic before they died.

The incidence of the outbreak on the production of the Brahman herd is summarized in table IV (per cow present in January 1985), in comparison with the Creole cows. The mortality of adults due to the disease (45 %) caused a serious loss shown by the stock variation (commercial value of the adults, including body weight gain and mortality). But the weaner production also fell down, mainly by the lower weaning rate. Thus, the economic incidence of

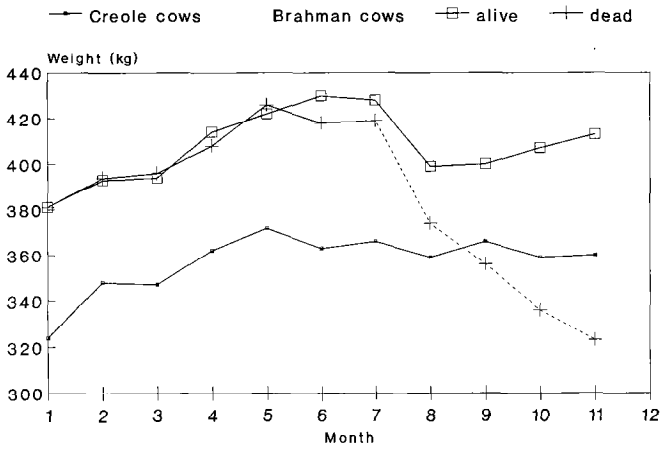

Figure 2 : Body weight evolution in 1985

TABLE IV Consequences on animal production and economic results (according to the breed ; per cow present in January 1985).

	Brahman	Creole
Body weight (kg) (1-85)	380	325
(12-85)	420	365
Mortality (Adults)	0.45	0
Weaned calves (/cow)	0.41	0.89
Weaning weight (kg) (/calf)	155	160
Stock variation (US $)	– 522	+ 122
Cost of treatments (US $)	– 200	0
Weaning production (US $)	+ 222	+ 498
Net out come (US $)	– 500	+ 620

the disease, including the cost of treatments (refered to the price of antibiotics in 1985), was particularly dramatic, with a loss of US $ 1120 per head compared to the Creole cows bred in the same conditions, but resistant to dermatophilosis.

DISCUSSION

It is interesting to notice that the disease appeared in animals, two to three months after they were allowed to graze. This could be related to the presence of ticks on pastures, which is often reported as a major cause for the induction of clinical dermatophilosis (1, 3, 6, 7, 9, 11, 13). Acaricide treatments were however carried out, but at a frequency that did not totally prevent the infestation by ticks.

On the other hand, a climatic effect could also be suspected, as the disease appeared in most of the animals (22 heads, 76 %) during the hot and moist season between

August and October 1984, and the outbreak reached its maximum in the same season in 1985. Several authors have already noticed dermatophilosis outbreaks at the start of the rainy season (1, 6, 7, 13). However, the climate in the Caribbean and particularly the constant high humidity is favorable to its development all around the year (11, 14). This can explain its incidence in the dry season, although on a few animals.

Recovery was obtained by the conjunction of several factors, as suggested by previous studies (7, 9), among which the return indoors of the animals, similarly to the effect noticed by OLOGUN *et al.* (12). This management facilitated treatments by parenteral antibiotics and external disinfection, and at the same time, reduced the incidence of the ticks, unable to live in the stable. Furthermore it suppressed the effect of sun and rain, by the disposal of a shelter. The combination of these different policies seems the only way for a sustained cure of the disease in our conditions.

The efficacy of most of the anti-infective treatments applied is in agreement with previous recommendations (2, 4, 8, 10). But the high frequency of treatments with BPS and the inadequate low dosage used had caused a lower activity by the time, already observed by ILEMOBADE (8).

The classification based on epidemiological observations is consistent with the results of immunological responses to *D. congolensis* (1, 9). Differences between individuals may be suspected concerning the role of the skin surface against infection by *D. congolensis*, as well as in the susceptibility of the animals to the extent of the disease; the methodology applied in our study could therefore be used as a tool to exemplify these differences.

The high sensitivity of Brahman cattle to dermatophilosis, already observed by many workers (3, 4, 6, 7, 9, 11, 13), is confirmed in our conditions. Its consequences on animal production, when risks and preventive policies are insufficiently assessed, are in agreement with previous reports (11, 13). In the traditional breeding systems of Guadeloupe, the use of the local breed resistant to diseases is much more justified economically than the use of exotic cattle which are more productive but highly susceptible to diseases.

CONCLUSION

These observations point out that dermatophilosis may have a great impact on animal production in the Caribbean when susceptible livestock is reared under risk conditions, meaning the presence of *Amblyomma* ticks, even if they are controlled by regular application of acaricides.

On the other hand, the local Creole cattle are of great interest, as they are obviously adapted to breeding conditions in the region. The resistance of this hardy breed to tick-borne diseases is well known, as well as its adaptation to climatic and nutritional constraints. These considerations increased the interest of the authors for the improvement of its genetic value, and selection to improve animal production in the Caribbean.

Finally, more investigations on the genetically determined susceptibility or resistance to diseases transmitted or associated with ticks are needed, as this could be a promising and sustainable way to control them.

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NAVES (M.), VALLÉE (F.), BARRÉ (N.). Observations on a dermatophilosis outbreak in Brahman cattle in Guadeloupe. Description, epidemiological and economical aspects. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 297-302

A severe outbreak of dermatophilosis occured in 1985 in a herd of Brahman cows imported from Martinique in Guadeloupe in July 1983. Little was known on this disease in Guadeloupe until then, for the local zebu breed is naturally highly resistant to diseases transmitted by ticks or associated with them. Conditions of appearance and development of the disease were studied in relation with herd management, climate and treatments applied. There was no evidence of the disease during the first 6 months following the importation, in a feedlot management. The first lesions of dermatophilosis appeared about 2 months after the animals were allowed to graze on pastures infested with ticks. Then, thirty percent of the herd became infected. Development of the infection increased dramatically as the humid season approached, and all the 29 Brahman cows showed lesions in July 1985. Only drastic treatments, including antibiotics and local desinfection, associated with the removal from pastures into covered stables allowed the recovery from the disease. Nevertheless, the disease caused the death of 13 head. Differences in individual reactions were also notest, and 7 different types of evolution were identified. These observations provided informations about the epidemiology of dermatophilosis in the climatic conditions of Caribbean humid tropics. They showed the economic importance of this severe disease for cattle in the region and the sensitivity of exotic breeds.

Key words : Brahman cattle - Dermatophilosis - Tick - Livestock management - Grazing - Antibiotics - Epidemiology - Livestock economics - Guadeloupe.

NAVES (M.), VALLÉE (F.), BARRÉ (N.). Observaciones de un brote de dermatofilosis en ganado Brahman en Guadalupe. Descripción, epidemiología y aspectos económicos. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 297-302

En 1985 se dió un brote severo de dermatofilosis en un hato de vacas Brahman, importadas de Martinica a Guadalupe, en julio de 1983. Los datos anteriores de esta enfermedad en Guadalupe son escasos, debido a que la raza cebú local posee una alta resistencia natural a las enfermedades transmitidas por garrapatas o asociadas a éstas. Las condiciones de la aparición y del desarrollo de la enfermedad se estudiaron en relación con el tipo de manejo, el clima y los tratamientos administrados al hato. En condiciones de manejo intensivo, no se encontró evidencia de la enfermedad en los 6 meses siguientes a la importación. Las primeras lesiones de dermatofilosis aparecieron al cabo de dos meses, luego que los animales fueron puestos en pastoreo en potreros infestados con garrapatas. Treinta por ciento del hato fue infectado. El desarrollo de la infección aumentó drásticamente durante la estación húmeda, con lesiones evidentes en las 29 vacas Brahman, en julio 1985. La recuperación se observó solamente en los casos en los que se aplicaron tratamientos agresivos, incluyendo antibióticos y desinfección local, junto con traslado de los animales a establos. A pesar de esto, trece de los animales sucumbieron a la infección. Se señalan también diferentes reacciones individuales, así como 7 tipos de evolución. Las observaciones proveen informaciones sobre la epidemiología de la dermatofilosis bajo las condiciones climáticas caribeñas del trópico húmedo. Se demuestra la importancia económica de esta grave enfermedad para el ganado de la región, así como la sensibilidad de las razas exóticas.

Palabras claves : Bovino Brahman - Dermatofilosis - Garrapata - Manejo del ganado - Pastoreo - Antibiótico - Epidemiología - Economía de la cría - Guadalupe.

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Study of efficacy of Lamstreptocide A & B on cases of dermatophilosis within the Caribbean

ISITOR (G.N.), NJOKU (C.O.), ADOGWA (A.O.), OYEKAN (A.O.). Étude sur l'efficacité du Lamstreptocide A et B sur des cas de dermatophilose dans les Caraïbes. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 303-308

L'efficacité du Lamstreptocide A et B a été étudiée sur 9 cas naturels de dermatophilose bovine et caprine dans 8 fermes différentes de Saint Kitts, par des méthodes histopathologiques et bactériologiques classiques. Les lésions de 5 des ruminants traités ont séché et les croûtes d'un animal gravement atteint se sont décollées considérablement, laissant apparaître un tissu érythémateux, 3 semaines après l'application du produit. En dehors de 3 cas sans gravité qui n'ont pu être suivis et qui seraient guéris, aucun des 5 animaux n'était véritablement guéri 3 semaines après le traitement, ni même après une deuxième application du produit. Un test *in vitro* sur la sensibilité au produit appliqué en surface de gélose, à des concentrations de plus de 1 p.100, a révélé un ralentissement de la croissance de *Dermatophilus congolensis*. Néanmoins, il n'y avait pas d'inhibition de la croissance de la bactérie par des disques de papier filtre imprégné.

Mots clés : Bovin - Caprin - Dermatophilose - *Dermatophilus congolensis* - Isolement - Lésion - Maladie de la peau - Bactéricide - Thérapeutique - Caraïbes - Saint-Kitts.

INTRODUCTION

Dermatophilosis is a chronic dermatitis of domesticated animals, in particular the ruminant species. The devastating effects of the condition, hide depreciation, overall decreased productivity (lowered fertility and increased culls, etc.), can hardly be ignored while pursuing policies aimed at combating the menace posed by the disease. On the contrary, past research efforts appeared skewed in the direction of multiple endless laboratory investigations with meagre contents of therapeutic procedures.

Given this background of abundant laboratory data on dermatophilosis, but with no reliable therapeutic or prophylactic regime at present, it is not surprising that in recent times there is an upsurge in attempts to formulate therapeutic products for the condition, regardless of scientific protocols. Lamstreptocide A & B represents one of such products the efficacy of which has been widely publicized by the producers at the National Veterinary Research Institute, Vom, Nigeria, but seems to have been minimally subjected to the crucible of tedious scientific testing prior to its being marketed in Nigeria.

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The producers (5) claimed an average cure rate of 93 % by the product when topically applied on cases of dermatophilosis. The product which represents a mixture of ferruginous clay and oil extracted from *Khaya* spp. was also accredited with insect repellent properties as well as efficacy on mange in rabbits, caprine fungal infections, and multiple dog skin infections.

The present investigation is a pilot attempt to verify the claim of efficacy of the product under the Caribbean climatic conditions.

MATERIALS AND METHODS

Field studies and treatment trials

Clinical evaluation studies of lesions of natural bovine and caprine dermatophilosis and the treatment trials were undertaken in 8 different animal farms on the island of St. Kitts, during the months of September and October, 1992. A total of 9 clinical cases, 7 cattle and 2 goats, was studied.

The pre-treatment lesion evaluation studies involved visual inspection of the lesion characteristics, such as the size, location and extent of scabbiness, as well as the overall animal body condition. Previous history of dermatophilosis and the prevailing husbandry practices within the farms were also evaluated. Scabs were collected from representative lesions by means of sterile forceps and stored in sterile specimen vials placed immediately in ice-cooled vacuum flasks. The vacuum flasks were further refrigerated prior to being transported to our laboratory at the School of Veterinary Medicine, University of The West Indies, Trinidad, W.I.

Treatment trials were undertaken on cases by smearing the undiluted (2.5%) Lamstreptocide A & B with the aid of paint brushes on an affected side of one half of the body or on the extremities of one side of the affected animals.

The other affected side or the opposite affected extremities were smeared with distilled water by means of another paint brush and served as control. In occasional instances where the animal was quite vicious, the brush handle was tied to a stick to facilitate application of the substance from a safe distance (photo 1).

Photo 1 : Topical application of Lamstreptocide A & B by means of a brush tied to a stick.

Laboratory studies on pre- and post-treatment specimens

Histopathologic and direct smear preparations

Portions of the scabs were fixed in 10 % buffered-neutral formalin, dehydrated in graded ethanol solution, infiltrated and embedded in paraplast, and thick sections (approximately 6 µm thick) were obtained and stained by standard Hematoxylin and Eosin procedures, and by Wright's Stain. Portions of the scabs were similarly utilized for direct smear demonstration of *Dermatophilus congolensis* by teasing in drops of sterile distilled water, and staining of the smear by Wright's method.

Bacterial isolation

This was accomplished by processing portions of the scabs according to HAALSTRA's method (2), which involved placement of scabs in sterile distilled water for 3 1/2 hours at room temperature in bijou bottles, and subsequent transfer of the bottles with loosened lids into candle jars for 15 min. Loopfuls of zoospores were obtained from the surface of the water and inoculated into blood agar plates which were incubated in 10 % CO_2 atmosphere at 37 °C for 48 h.

In vitro sensitivity tests

Different volumes of the stock preparation of Lamstreptocide A & B were mixed with a fixed volume of sheep blood agar to yield final concentrations of 0.5, 1, 2, 5 and 10 % of the drug. Loopfuls of pure colonies of *D.congolensis* were streaked on untreated (control) and lamstreptocide-treated agar plates. All experiments were carried out in triplicates. The plates were incubated in 5 % CO_2 at 37°C and the growth of *D. congolensis* was monitored for 3 to 7 days.

Lamstreptocide agar impregnated disks were prepared as follows :

0.5, 1, 2, 5 and 10 % different concentrations, of Lamstreptocide A & B were prepared in 5 mm diameter filter paper disks (Whatman No.1), sterilized at 120 °C for 15 min, and were subsequently impregnated with the various concentrations as above. The impregnated disks were placed on the first set of plates while the second set had 5 mm diameter wells cut in them and then filled with the different concentrations. All the blood agar plates were incubated at 37 °C in 5-10% CO_2 for 48 h. The growth of *D.congolensis* around the zones in which Lamstreptocide A & B were applied was measured and recorded as areas of inhibition.

RESULTS

The findings on pre-treatment clinical evaluation studies of the lesions of cases are summarized in table I. The bovine cases had lesions which were either discrete, isolated scabs, located mostly on the dorsum of the trunk (photo 2), or severe scabby encrustations mostly affecting the manus and pes (photo 3). An exceptional bovine case was a calf (MO2) with generalized, severe, confluent and exudative lesions on almost all areas of the body surface (photo 4). This calf was in overall poor body condition with marked signs of emaciation and cachexia. It had a history of previous attacks of dermatophilosis which were treated with Terramycin LA, but with subsequent relapses. In general, apart from this severely affected calf and the case shown in photo 3, both of which had a history of previous infection of dermatophilosis, most other bovine cases were first time infected cases. The 2 capri-

***TABLE I** Summary of lesion characteristics of cases.*

Lesion Type	No. of cases	Species
Mild, discrete, isolated scabs, mostly on dorsum of trunk, neck and face	5	Bovine
Severe confluent, exudative encrustations, mostly on manus and pes	1	Bovine
Very severe generalized exudative and scabby lesions ; overall emaciation and cachexia	1	Bovine
Discrete and confluent scabs on the face, pinnae, muzzle and neck	2	Caprine

Photo 2 : A pre-treatment case with discrete, isolated scabs on the dorsum of the trunk.

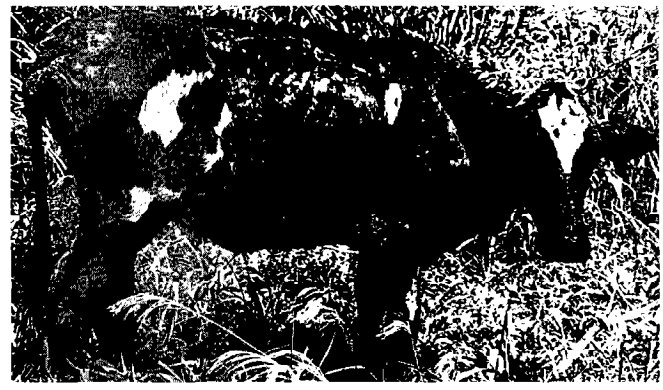

Photo 4 : A pre-treatment calf with severe, generalized confluent and exudative lesions. Note the overall poor body condition.

ne cases, MO1 and CN2, had scabby lesions on the face, pinnae and other areas of head and neck. The overall body condition of the typical caprine case was fair (photo 5).

Table II represents summaries of results obtained following treatment of cases with Lamstreptocide A & B. Pre-treatment demonstration of *D. congolensis* by histopathological and bacteriological isolation methods were positive in a total of 8 out of the 9 encountered cases. The follow-up post-treatment histopathologic and bacteriological evaluation studies undertaken at 3 weeks yielded positive results for the presence of *D.congolensis* in the lesions.

Apart from the slight changes which involved overall drying of scabs in most cases (at about 1 week post-treatment) (photo 6), and a significant peeling-off of scabs on the treated manus and pes of a case (Bk.1), exposing an

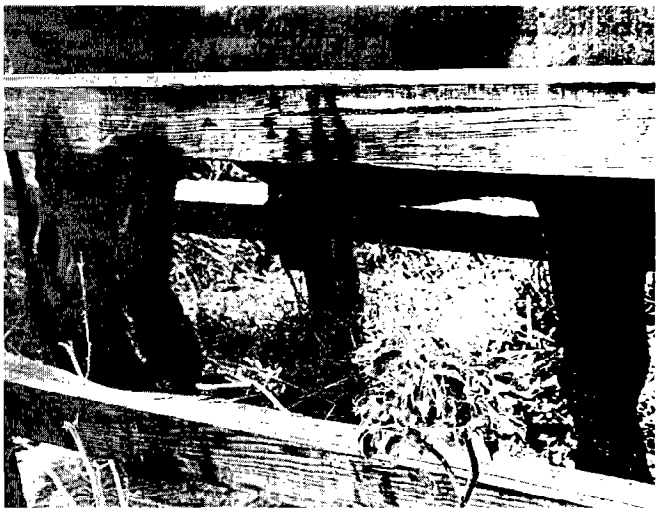

Photo 3 : A pre-treatment severely affected case, mostly involving the manus and pes.

Photo 5 : A pre-treatment caprine case showing lesions on the face, pinnae and other areas of the head and neck.

TABLE II Summary of 3 weeks. Post-treatment study.

Lesion appearance	Demonstration of *D. congolensis*		No. of cases
	Histopathology	Bacteriology	
Dried scabs on both treated and untreated body areas	+++	+++	3
Peeled-off scabs on the treated manus and pes with resultant underlying erythematous tissue	+++	+++	1
Dried-up scabs ; overall body improvement	++	+++	1
Cleared-up scabs and overall body improvement	Not done	Not done	1
Mildly improved overall body condition ; follow-up administration of Terramycin LA. ; animals subsequently slaughtered	Not done	Not done	2

Photo 6 : The dried lesions of the animal shown in photo 2 , at 3 weeks post-treatment.

erythematous underlying tissue (photo 7), there was no remarkable change of lesions between the treated and untreated body areas.

Of the remaining 3 positive pre-treatment cases, there was an observed clearing-up of the initial mild lesions of a case (Bk.1 M) which was subsequently slaughtered prior to our follow-up post-treatment study. The other 2 cases were treated with Terramycin LA according to the wish of the owners and so were not available for post-treatment follow-up studies.

A reapplication of Lamstreptocide A & B to the previously treated body areas of 3 of the 5 positive post-treatment cases did not yield any remarkable change of lesions 2 weeks later. The result of the *in vitro* sensitivity test for the antibacterial activity of Lamstreptocide A & B is shown in table III. A slowing down of growth of *D. congolensis* was observed at concentrations of Lamstreptocide A & B

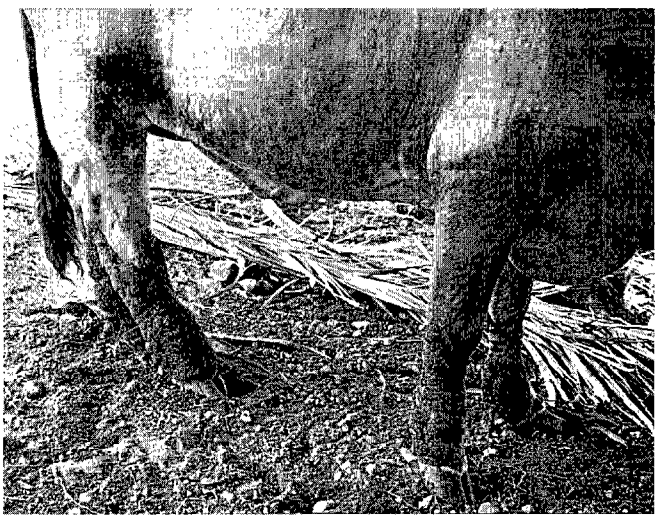

Photo 7 : The case shown in photo 3 at 3 weeks post-treatment. Note peeled-off lesions of the treated lower extremities which have underlying erythematous tissue.

TABLE III Summary of result obtained on **in vitro** *antibacterial sensitivity test of Lamstreptocide A and B.*

Method	Concentration %	Remarks
Agar-streak procedure	0.5, 1, 2, 5, 10	Slow growth of *D. congolensis* at concentration > 1% at 7 days post-inoculation
Agar-impregnated disk procedure	0.5, 1, 2, 5, 10	Growth of *D. congolensis* at 24 h post-inoculation

in excess of 1 % at 7 days post-inoculation, by the agar-streak method. There was no inhibition of growth of *D. congolensis* by Lamstreptocide A & B at any of the concentration tested, utilizing the agar-impregnated method.

DISCUSSION

As evident from this study, the efficacy of Lamstreptocide A & B on the few cases of dermatophilosis is questionable. Apart from the drying-up of scabs on most of the cases, and the peeling-off of the scabs revealing underlying erythematous tissue in a case, an outright recovery attributable to Lamstreptocide A & B was only demonstrated in 3 mild cases within the first category of lesions described in table I. Unfortunately, these mild cases were unavailable for post-treatment follow-up investigations.

One of the issues of concern pertains to the 5 cases in which the observable effect of Lamstreptocide A and B was mere drying-up and peeling-off of scabs. The major concern in this regard is the fact that the scabs of both treated and control sites were significantly positive for *D. congolensis* at 3 weeks post-treatment. Even after the reapplication of the substance to previously treated body areas of 3 of the 5 post-treatment cases, the scabs were still positive for *D. congolensis* at 2 weeks post-treatment.

Perhaps the most disturbing aspect is the *in vitro* sensitivity result in which there was no outright inhibitory activity on the growth of *D. congolensis* by Lamstreptocide A & B, employing two standard sensitivity methods. Only a slowing down of growth of *D. congolensis* at concentrations of the substance in excess of 1 % was noted, employing the agar-streak method. It may, however, be possible that the potency of the product may have been compromised as a result of transporting it over a long period and using it in a distant location (the Caribbean).

The producers had, however, indicated that "geographical variations did not seem to influence the efficacy of the product in infected cattle" (5). It is also possible that the recorded success with the product on tested cases in Nigeria, in which a cure rate of 93 % was reported (5), pertained to mild cases rather than severe cases as with some of the cases of the present investigation. In view of the continued threat of dermatophilosis on ruminant production, and of the complex etiology of the condition in which *D. congolensis* and pox viruses may play roles in the causation of the disease (1, 3, 4, 6), it is suggested that more trial studies on the efficacy of the product be undertaken in other geographic locations.

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We acknowledge The University of the West Indies for funding this research, and the Veterinary Research Laboratory, Ministry of Agriculture, Curepe, Trinidad for assistance with the laboratory processing of specimens. We are also grateful to the Chief Veterinary Officer and the Veterinary Department of the Ministry of Agriculture, St. Kitts, for cooperation in making the animals available for the project.

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G.N. Isitor C.O. Njoku A.O. Adogwa A.O. Oyekan

ISITOR (G.N.), NJOKU (C.O.), ADOGWA (A.O.), OYEKAN (A.O.). Study of efficacy of Lamstreptocide A & B on cases of dermatophilosis within the Caribbean. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 303-308

The efficacy of Lamstreptocide A & B was studied on 9 natural cases of bovine and caprine dermatophilosis in 8 different farms in St. Kitts, employing standard histopathologic and bacteriological methods. The lesions of 5 of the treated cases were dried-up, and there was marked peeling-off of scabs of a severely affected case exposing erythematous underlying tissue, at 3 weeks post-application of the product. Apart from 3 mild cases which were not available for follow-up studies and which were reported to have recovered, there was no outright recovery of the 5 animals after treatment at 3 weeks, and even after a second application of the product. An *in vitro* sensitivity test of the product revealed a slowing down of growth of *Dermatophilus congolensis* at concentrations in excess of 1 % by agar-streak method. However, there was no inhibition of growth of the bacterium by an agar-impregnated sensitivity method.

Key words : Cattle - Sheep - Dermatophilosis - *Dermatophilus congolensis* - Isolation - Lesion - Dermatology - Bactericide - Therapeutics - Caribbean - Saint Kitts.

ISITOR (G.N.), NJOKU (C.O.), ADOGWA (A.O.), OYEKAN (A.O.). Eficiencia del Lamstreptocide A y B en casos de dermatofilosis en el Caribe. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 303-308

Se estudió la eficiencia del Lamstreptocide A y B en nueve casos naturales de dermatofilosis bovina y caprina, en ocho establecimientos en San Kitts. Se utilizaron métodos histopatologicos y bacteriologicos estandard. Las lesiones de cinco rumiantes tratados fueron secadas. En un caso severo se observó la descamacion, con exposicion de tejido eritematoso tres semanas después de la aplicación del producto. Con excepción de tres casos leves, a los cuales no se les dió seguimiento, pero se reportó la cura, tres semanas después del tratamiento, no hubo recuperación en los cinco animales tratados, mismo después de una segunda aplicación del producto. Una prueba de sensibilidad *in vitro* del producto, mostró un crecimiento lento de *Dermatophilus congolensis* en concentraciones de 1% de exceso por el método de "agar-streak". Sin embargo no hubo inhibición de crecimiento bacteriano mediante el método de sensibilidad por agar impregnado.

Palabras claves : Bovino - Caprino - Dermatofilosis - *Dermatophilus congolensis* - Aislamiento - Lesión - Dermatológia - Bactericida - Terapeutica - Caribe - San Kitts.

The epidemiology and control of camel dermatophilosis

C.G. Gitao [1]

GITAO (C.G.). La dermatophilose cameline : épidemiologie et lutte. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 309-311

La dermatophilose du chameau n'a été décrite que récemment. Elle semble néanmoins plus répandue qu'on ne le croyait. Au Kenya, elle a été trouvée en général dans les régions semi-arides d'élevage du chameau dans les districts de Samburu et de Laikipia, mais n'a pas encore été mise en évidence dans les régions arides du district de Turkana. Lors d'une prospection de tiques sur 200 chameaux, aucune tique *Amblyomma variegatum* n'a été trouvée bien que de nombreuses autres tiques étaient présentes. On soupçonne *A.variegatum* de transmettre la dermatophilose à plusieurs animaux domestiques. La seule méthode de lutte contre la dermatophilose au Kenya est actuellement appliquée dans une ferme commerciale, où les chameaux sont régulièrement lavés avec une solution d'alun potassique à 1 p. 100. Les chameaux ont montré une amélioration progressive. Récemment, environ 50 chameaux importés du Pakistan ont été affectés par une infection cutanée sévère, très similaire à la dermatophilose. Tous les chameaux importés adultes ont été atteints, mais non les veaux. Etant donné qu'on n'a pas pu isoler de bactéries de ces chameaux, on pense que l'affection a été causée par une déficience en vitamine.

Mots clés : Dromadaire - Dermatophilose - *Dermatophilus congolensis* - Epidémiologie - Tique - *Amblyomma variegatum* - Kenya.

INTRODUCTION

Camels are reared in the arid and semi-arid areas of Kenya which constitute eighty per cent of the total land surface and where pastoralists derive their livelihood. The camels are particularly valuable as they survive and even thrive during the dry season while other animals die in great numbers (2). Camel dermatophilosis is a skin disease of camels recently described in one commercial farm, the Ol Maisor farm in Laikipia District in the semi-arid areas of Kenya (4).

Dermatophilosis in the bovine is described to be more prevalent in free-ranging animals rather than in well managed herds (7, 11). Bovine dermatophilosis has also been strongly associated with the tropical bont tick, *Amblyomma variegatum* (3, 8). A similar relationship has been described for goats with a severe skin infection (9). Attempts at control of dermatophilosis have been performed by dipping of cows in acaricide (8) or dusting of sheep with aluminium potassium sulphate (5).

In this study therefore, the possibility of camel dermatophilosis being present and probably more prevalent in the free-ranging camels in the semi-arid areas was examined. The presence and distribution of ticks on 200 camels in the free-ranging camels was compared to similar camels at the Ol Maisor farm. Some very severe skin lesions which developed on camels imported from Pakistan to the Ol Maisor farm were examined and the control method practised at the farm against camel dermatophilosis was studied.

MATERIALS AND METHODS

Two hundred camels kept by pastoralists in herds ranging from 5-15 camels per herd were examined. These camels are reared freely in the Samburu district which is semi arid receiving about 500 mm of rainfall annually. A similar examination of 200 camels at the Ol Maisor farm was performed. From the Pakistan camels, 30 samples were obtained from the sick camels. Skin scabs were obtained from suspicious skin lesions and processed as described before (4) and then examined for the presence of *Dermatophilus congolensis.*

Ticks were obtained from the two hundred camels reared by pastoralists and from the two hundred camels in the commercial farm. This was performed in March in the rainy season and repeated in August in the dry season. The site of attachment was noted and the ticks identified as described by Hoogstraal (6).

The application of 1 % potassium aluminium sulphate was assessed after a period of six months on eight camels which were severely affected. Four camels which were not treated were used as controls. Four sites on each camel were marked and their diameter in size and wool regeneration followed monthly.

RESULTS

Dermatophilosis was found in three pastoral households affecting twenty seven camels. Camels of different age groups were affected and the degree of skin involvement

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TABLE I Percentage tick distribution in the commercial and pastoral herds.

Tick	Pastoral		Commercial	
	March	Aug.	March	Aug.
H. dromedarii	20.8[a]	36.2	0	10.7
	16.6[b]	8.6	0	2.2
	9.4[c]	1.8	0	0
H. rufipes	24.6	32.1	3.7	9.8
	22.4	16.2	9.3	7.3
	4.3	2.0	2.6	4.5
H. truncatum	0.6	1.0	0	6.8
	0.5	0.2	0	3.8
	0	0	0	0.4
R. pulchellus	0.8	1.0	20.3	18.5
	0	0.9	18.7	11.2
			2.3	3.0
			8.6[d]	18.1
A. gemma			15.4	2.0
			15.6	1.2
			3.5	0.5
Total in sample	1604	1 518	1 050	870

[a] *males ;* [b] *females ;* [c] *engorged females ;* [d] *nymphs.*

TABLE II Attachment sites of tick species on camels in the commercial barm.

Site	No.	Tick species	% of total
Nose	110	*H. dromedarii*	70.3
		R. pulchellus	26.8
		A. gemma	2.9
Ear	17	*H. dromedarii*	92.8
		R. pulchellus	7.2
Inguinal area	50	*R. pulchellus*	44.8
		A. gemma	26.2
		H. rufipes	16.8
		H. truncatum	12.2
Fore Legs	54	*R. pulchellus*	72.4
		H. truncatum	15.3
		H. dromedarii	9.8
		A. gemma	2.5

ranged from 20 to 60 %. The most seriously affected sites were the flanks and the neck. At the Ol Maisor farm, 15 camels were affected by dermatophilosis with the degree of skin involvement varying from 10 % mostly in the adults to 60 % mostly in the calves. Similar sites of skin involvement were found as in the pastoral herds. *Dermatophilus congolensis* was not isolated from the Pakistan camels.

The predominant tick species in the commercial herd was *Rhipicephalus pulchellus* while in the pastoral herd, *Hyalomma dromedarii* and *Hyalomma rufipes* were the most important. The tick distribution on the different herds is shown on table I and the tick preference site on table II. No *Amblyomma variegatum* ticks were found on any of the camels. The tick load in both herds was higher in the rainy season than in the dry season.

There was a significant decrease in the size of lesions of the treated camels ($p<0.05$) and evidence of wool regrowth which was not evident on the untreated camels.

DISCUSSION

Camel dermatophilosis was found on animals reared in pastoral herds and it is probably more frequent than in commercial herds but this would require a wider survey. One limitation is that there is only one commercial farm keeping camels in Kenya. On the commercial farm there is regular application of acaricide grease unlike on the pastoral herds which are left to roam free with little attention. This also explains the higher tick load on pastoral herds as compared to commercial herds.

RICHARD (10) records 11 species of tick which infest camels in Ethiopia. These include *Rhipicephalus pulchellus, Rhipicephalus simus, Rhipicephalus pravus, Amblyomma variegatum, Amblyomma gemma, Amblyomma lepidum, Hyalomma excavatum, Hyalomma truncatum, Hyalomma dromedarii, Hyalomma impeltatum* and *Hyalomma rufipes*. He considered that qualitatively, *Rhipicephalus pulchellus* and *Rhipicephalus simus* were the most important. CURASSON (1) considered *Hyalomma* spp. to be the dominant species in the camel. The most important tick species probably varies according to the habitat (12), with *Hyalomma* spp. being the most prevalent in arid areas. In this study, *Hyalomma dromedarii* and *Hyalomma rufipes* were predominant in the pastoral areas and *Rhipicephalus pulchellus* predominant in the commercial herd. No *Amblyomma variegatum* ticks were found. Although *Amblyomma variegatum* ticks have been found on camels (12), they are in such low frequencies (0-1.8 %) as to be of little epidemiological value in the spread of dermatophilosis. It may be that in the dromedary unlike in the bovine, other agents like Tabanid biting flies are involved in the spread of dermatophilosis.

The severe skin lesions on the Pakistan camels were thought to be due to vitamin deficiency, since the calves which may have had enough maternal reserve were not involved.

The treatment of dermatophilosis in the camels with 1 % potassium aluminium sulphate was found to be effective. On sheep, similar treatment was found to be effective (5). The inhibitory effect of potassium aluminium sulphate is

said to be due to its ability to inhibit the motility of *D. congolensis* zoospores and so prevent re-invasion of the skin (5). Since extensive husbandry is the most practical method of camel husbandry, it is not possible to advocate improved management of camels and simple compounds which can be applied by pastoralists may be more suitable.

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GITAO (C.G.). The epidemiology and control of camel dermatophilosis. *Revue d'Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 309-311

Camel dermatophilosis was only recently described. It appears however that it is more widespread than originally thought. In Kenya it has generally been found in the main semi-arid camel rearing areas of Samburu and Laikipia districts although it has not yet been found in the arid areas of Turkana district. In an investigation of ticks on 200 camels, no *Amblyomma variegatum* ticks were found although many other ticks were present. *A. variegatum* is suspected to transmit dermatophilosis in many domestic animals. The only control method of dermatophilosis currently practised in Kenya is in one commercial farm, where camels are regularly washed with a 1 % potassium aluminium sulphate solution. The camels have shown progressive improvement. Recently, some 50 camels imported from Pakistan in this farm came down with a severe skin infection which closely resembled dermatophilosis. All imported adult camels were involved although no calves were involved. Since no bacteria were isolated from all the sick camels, it was thought to be due to vitamin deficiency.

Key words : Dromedary - Dermatophilosis - *Dermatophilus congolensis* - Epidemiology - Tick - *Amblyomma variegatum* - Kenya.

GITAO (C.G.). Epidemiología y control de la dermatofilosis en el camello. *Revue d'Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 309-311

Aunque la descripción de la dermatofilosis en el camello se hizo recientemente, la distribución de esta enfermedad es más amplia de lo que se pensó inicialmente. En Kenia, se ha encontrado generalmente en Samburu y Laikipia, principales zonas semi-aridas de producción de camellos, pero aún no se ha aislado en las zonas áridas del distrito de Turkana. En un estudio de investigación sobre 200 camellos, se encontraron varios tipos de garrapatas, pero no *A. variegatum*. Se sospecha que esta variedad de garrapata actúa como vector de la dermatofilosis en varias especies animales. El único método de control contra la dermatofilosis utilizado actualmente en Kenia, practicado en una explotacion comercial, es el baño periódico de los camellos con una solución de potasio y sulfato de aluminio al 1 p.100. Se ha observado una mejora progresiva en los animales. Recientemente, 50 camellos importados de Pakistán por este establecimiento, presentaron un brote de una enfermedad cutánea, muy similar a la dermatofilosis. Todos los adultos importados sufrieron la enfermedad, pero no los animales jóvenes. Se diagnosticó una deficiencia vitamínica, debido a que ninguna bacteria fue aislada en los animales.

Palabras claves : Dromedario - Dermatofilosis - *Dermatophilus congolensis* - Epidemiología - Garrapata- *Amblyomma variegatum* - Kenia.

The systemic effect of adult and immature *Amblyomma variegatum* ticks on the pathogenesis of dermatophilosis

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A.R. Walker[1]

LLOYD (C.M.), WALKER (A.R.). L'effet systémique des adultes et des nymphes d'*Amblyomma variegatum* sur la pathogénie de la dermatophilose. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 313-316

L'effet systémique des adultes et nymphes d'*Amblyomma variegatum* sur la pathogénie d'infections expérimentales avec *Dermatophilus congolensis* a été étudié. Trois groupes de 4 moutons ont été utilisés, tous infectés avec des doses titrées identiques de *D. congolensis*. Un des groupes a été infesté simultanément par des adultes d'*A. variegatum*, le deuxième groupe par des nymphes, et le troisième comprenait les témoins non-infestés. L'évaluation des infections indique que l'effet systémique de la tique est limité aux adultes. Des tests cutanés et sérologiques utilisant d'autres antigènes ont montré une réduction significative des réponses immunitaires cellulaires et humorales uniquement chez les moutons infestés par les tiques adultes.

Mots clés : Ovin - Dermatophilose - *Dermatophilus congolensis* - Tique - *Amblyomma variegatum* - Maladie systémique - Test ELISA - Test cutané.

INTRODUCTION

Amblyomma variegatum ticks have long been associated with chronic dermatophilosis lesions on cattle in the wet tropics (4). Until recently the association of these ticks chronic with dermatophilosis lesions has been based on field observations alone. The present report compares the effect of adult and nymphal ticks on the severity and duration of dermatophilosis on sheep. Adult ticks have been implicated as an important factor in the formation of chronic dermatophilosis lesions (5, 6). However other evidence suggests that the inflammatory and hypersensitive reactions to immature ticks and other haematophagous arthropods may predispose to dermatophilosis (1,3).

MATERIALS AND METHODS

Experimental animals

Three groups of four sheep were used with equal numbers of Blackface x Suffolk and Blackface in each group. All the sheep were kept in constant conditions throughout the experiment, 18-20 °C with 12 h light/12 dark.

Tick infestations

The ticks used in the experiment were adult or nymphal *A. variegatum* from an uninfected laboratory colony, held at 16 °C with 14 h light/10 dark at 85 % relative humidity. One group of sheep (group A) were infested with 20 adult ticks each, the second group (group B) were infested with 600 nymphal ticks and the third group (group C) were not exposed to any ticks. Equivalent numbers of adult and nymphal ticks were calculated by counting the number and size of salivary acini found in salivary glands of both life-stages.

All of the infestations were applied to cloth bags glued to the wool on the shoulders of the sheep. The wool was shaved from inside the bags and the whole area cleaned with alcohol and ether. The adult infestations consisted of 10 males and 10 females with the males being applied 7 to 10 days before the females. The nymphal infestations were applied in three batches of 200, at weekly intervals with the final batch being applied 7 days after the adult females.

Dermatophilus congolensis infections

One day after the final batch of nymphs were applied all of the sheep were experimentally infected with *D. congolensis*. The *D. congolensis* was taken from a large batch of stabilate previously cultured and frozen at -20 °C at a concentration of 1.2 x 10^7 cocci/µl. This stabilate was diluted to the required concentration of 1 x 10^7 cocci/µl in Hank's balanced salt solution with pig gelatin at 0.5 % w/v.

All 12 of the sheep were infected with identical titrated doses of *D. congolensis* consisting of 100 µl doses of seven ten-fold dilutions starting at a concentration of 1 x 10^7 cocci/µl applied to seven areas, 2 x 4 cm on the left flank of each sheep.

Before the application of the *D. congolensis* the wool was removed from the application sites and the whole area was cleaned using alcohol and ether. Each of the infection sites were marked using an indelible pen and the skin fold of each area was recorded. The *D. congolensis* was then applied, without scarification, using a bent pipette tip.

The resulting infections were assessed using a ranking system of 0 to 4 for : skin fold thickness ; percentage of each area showing signs of infection ; the severity of the

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scab, ranging from erythema to thick layers of dead, flaking epidermis ; extent of exposed dermis at the infection sites. The progression of the dermatophilosis was monitored every 3 to 4 days for 4 weeks and then weekly for another fortnight.

After an interval of several weeks the entire procedure of tick infestations and *D. congolensis* infections were repeated using the same sheep. In previous experiments differences in the infections produced on the test and control sheep were more pronounced at the second infection.

Skin testing

All 12 of the sheep were inoculated with chicken egg ovalbumin, a T lymphocyte activator. Two B lymphocyte activators were used with six of the sheep being inoculated with a polyamino acid, poly-d-glutamate-d-lysine and the remaining sheep being inoculated with freeze-dried *Brucella abortus* (Central Veterinary Laboratory, Weybridge).

Initial sensitising doses of 2.5 mg of each antigen were injected intramuscularly in an equal mixture of one T and B lymphocyte activator suspended anhydrously in Freund's incomplete adjuvant approximately one week prior to first *D. congolensis* infection. A booster dose of 1.25 mg of each antigen was injected in the same way one week before the start of the second infection.

The challenge injections of the individual antigens in phosphate buffered saline (PBS) were applied intradermally in five titrated doses to sites previously shaved and cleaned on the rump of each sheep. The ovalbumin and *B. abortus* were applied in five 100 µl doses of five-fold dilutions starting at a concentration of 2.5 mg/100 µl. The polyamino acid was also applied in 100 µl doses of five-fold dilutions, but the starting concentration was 1 mg/100 µl due to excessive viscosity of higher concentrations.

The skin test reactions were assessed 24 and 48 h after challenge by measuring the skin fold thickness and average diameter of the reactions at each of the five sites. The results were analysed using the median values obtained for the reactions at each of the challenge sites.

Serological tests

Enzyme linked immunosorbent assay (ELISA) was used to measure the humoral antibody response of the sheep to the T and B lymphocyte activating antigens. Aseptic serum was collected from all 12 sheep prior to the start of the experiment and at weekly or fortnightly intervals throughout the entire procedure. For each serum sample collected, duplicate series of five doubling dilutions, starting at 1:1,000 and 1:2,000 were tested for antibodies to ovalbumin and *B. abortus*, respectively.

The tests were carried out using 96 well ELISA plates (Immulon 1, Dynatech Laboratories). Each well was coated with 0.25 µg and 0.051 µg of ovalbumin or *B. abortus* respectively. RASh/IgG(H+L)/PO anti-ovine conjugate (Nordic Immunology-Immunoconjugate) and 3,3', 5,5'-tetramethyl-benzidine dihydrochloride in phosphate citrate buffer were used to complete the ELISA. To stop the substrate reaction 50 µl of 2M H_2SO_4 was added to each well, the optical density of each well at 450 nm was then recorded using an optical density scanner (Titertek Multiskan, Labsystems).

RESULTS

D. congolensis infections

Kruskal-Wallis test was used to compare the ranked scores of the dermatophilosis on the individual sheep in the three groups at each of the assessment days. During the first infection a significant difference between the groups was recorded only on day 27 with $P < 0.05$. The median ranked scores of the three groups on this day were 13.5 for the group infested with adult ticks and 1.5 for the sheep infested with nymphs or not exposed to ticks.

Using the Kruskal-Wallis test on the scores obtained on individual assessment days during the second infection demonstrated a very significant difference ($P < 0.01$) developing between the severity of the dermatophilosis lesions at day 27 which was maintained up to day 41 when the experiment was terminated (fig. 1).

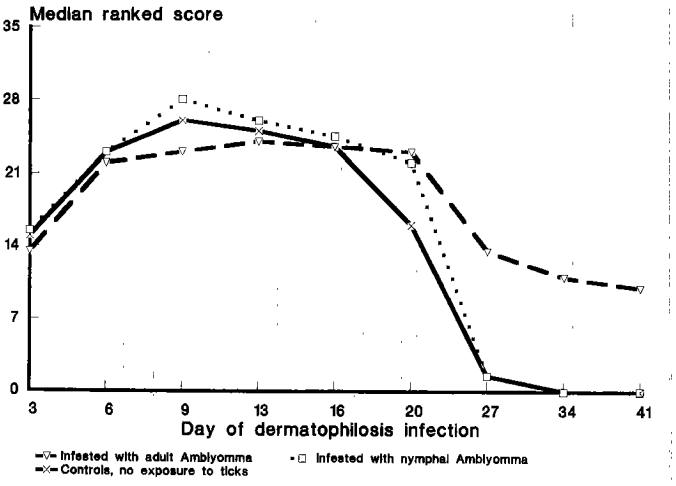

Figure 1 : Changes in the median ranked scores of secondary dermatophilosis lesions on sheep. Comparison of the effect of simultaneous infestations of adult or nymphal A. variegatum.

Skin test

Using Friedman's test on the median reactions to ovalbumin and *B. abortus* at the five individual skin test sites a very significant difference, $P < 0.01$ and $P = 0.01$ respectively, was observed between the reactions of the three groups of sheep to the antigens. Table I shows the median reactions of the three groups to both antigens.

TABLE I Skin test reactions of sheep in response to ovalbumin and **B. abortus.**

Ovalbumin

Median reactions (Skin fold x average diameter mm)			
Amount of antigen (µg/100µl)	Infested with adults	Infested with nymphs	Not tick infested
2500	102.8	369.92	246.43
500	74.64	224.57	168.74
100	25.95	91.52	64.65
20	6.6	8.2	7.65
4	4.75	6.4	5.5

B. Abortus

Median reactions Skin fold x average diameter mm)			
Amount of antigen (µg/100µl)	Infested with adults	Infested with nymphs	Not tick infested
2500	257.02	489.56	420.99
500	142.45	516.72	516.33
100	175.38	420.97	320.16
20	105.09	303.93	229.52
4	12.31	76.48	167.1

ELISA

Figures 2 and 3 show Log_{10} of 1/highest positive serum dilution of the serum samples when tested against ovalbumin and *B. abortus* antigens, respectively. Using samples collected from all 12 sheep from day thirteen after initial sensitisation until 49 days after the booster inoculation.

Using Kruskal-Wallis a significant difference, $P < 0.01$, was recorded between the immune response of the three groups of sheep to ovalbumin. Using Mann-Whitney the responses of the three groups were shown to divide into 2 very significant classes ($P < 0.01$), with the sheep infested with nymphs and the controls in one class and the sheep infested with adult ticks in another class producing a significantly lower response.

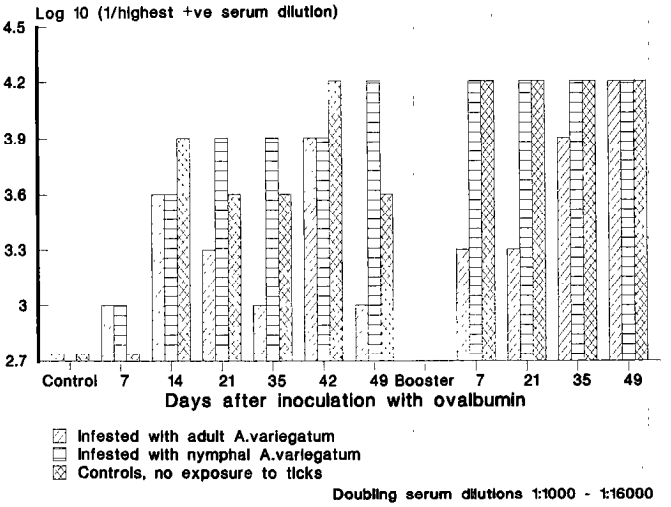

Figure 2 : Comparison of the effect of adult and nymphal A. variegatum *infestations on the antibody levels of sheep in response to ovalbumin, using ELISA.*

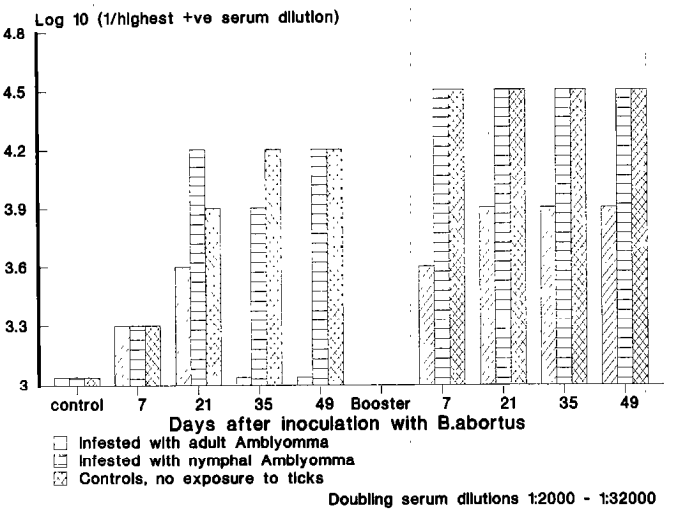

Figure 3 : Comparison of the effect of adult and nymphal A. variegatum *infestations on the antibody levels of sheep in response to* B. abortus, *using ELISA.*

Using the same analyses on the results obtained using *B. abortus* the immune responses again divided into the same significant classes as above.

DISCUSSION

This comparison of the effect of adult and nymphal *A. variegatum* follows previous work demonstrating the significant systemic effect of adult *A. variegatum* on the progression of experimental dermatophilosis on sheep (6).

In this investigation moderate chronic dermatophilosis lesions were reproduced only on sheep simultaneously infected with adult *A. variegatum* with nymphal tick feeding having no significant effect on the progression of the disease.

Evidence from the assessment of the clinical dermatophilosis of reduced immune response in the sheep infested by adult *A. variegatum* has been supported by the results obtained from the skin and serological tests.

B. abortus has been used as a sensitising antigen in previous studies to demonstrate antibody responses in sheep infected with *D. congolensis* (2). The method of application of antigen in PBS was used previously by ELLIS and SUTHERLAND (2).

Due to the experimental protocol the sheep infested with adult ticks were subjected to prolonged *D. congolensis* infections with the remaining sheep subjected to acute infections. It is possible that the significant difference in the immune reactions of the sheep may have been caused by the different levels of exposure to *D. congolensis*. However it has been concluded that the reduced immunological reactions recorded were caused by the tick feeding, this role of tick feeding causing immunosuppression in the host is well documented (4, 7).

In conclusion, the assessment of clinical dermatophilosis infections on sheep indicates that the systemic effect of *A. variegatum* is confined to the adults. A significant reduction in both the cell mediated and the humoral immune response of sheep infested with adult *A. variegatum* has been demonstrated by skin and serological testing.

ACKNOWLEDGEMENTS

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LLYOD (C.M.), WALKER (A.R.). The systemic effect of adult and immature *Amblyomma variegatum* ticks on the pathogenesis of dermatophilosis. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 313-316

The systemic effect of adult and nymphal *Amblyomma variegatum* on the pathogenesis of experimental infections of *Dermatophilus congolensis* was investigated. Three groups of four sheep were used with all 12 sheep being infected with identical titrated doses of *D. congolensis* One group of sheep was simultaneously infested with adult *A. variegatum* the second with nymphal *A. variegatum* and the third group were the controls, with no exposure to ticks. Assessment of the resulting infections indicate that the systemic effet of this tick is confined to the adults. Skin and serological tests using foreign antigens showed significantly reduced cell mediated and humoral immune response only in the sheep infested with adult *A. variegatum*.

Key words : Sheep - Dermatophilosis - *Dermatophilus congolensis* - Tick - *Amblyomma variegatum* - Systemic disease - ELISA test - Skin test.

LLOYD (C.M.), WALKER (A.R.). Efecto sistémico de estados adultos e inmaduros de garrapatas *Amblyomma variegatum* sobre la patogenicidad de la dermatofilosis. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 313-316

Se estudió el efecto sistémico de los adultos y las ninfas de *Amblyomma variagatum* sobre la patogenicidad de las infecciones experimentales de *Dermatophilus congolensis*. Se trabajó con tres grupos de cuatro ovejas cada uno. Todas las ovejas se infectaron con dosis idénticas de *D. congolensis*. Uno de los grupos se infectó simultaneamente con adultos de *A. variegatum*, el segundo con estados ninfales de *A. variegatum* y el tercer grupo se utilizó como control, por lo que no fue expuesto a ninguna garrapata. La verificación de las infecciones resultantes indica que el efecto sistémico de esta garrapata se limita a los adultos. Tests serológicos y dermatológicos, para los cuáles se utilizaron antígenos extranjeros, mostraron una respuesta inmune reducida, tanto celular como humoral, pero solamente en las ovejas infestadas con adultos de *A. variegatum*.

Palabras claves : Ovino - Dermatofilosis - *Dermatophilus congolensis* - Garrapata - *Amblyomma variegatum* - Enfermedad sistemica - Test ELISA - Prueba cutánea.

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The effect of tick control on the prevalence of dermatophilosis on indigenous cattle in Ghana

MORROW (A.N.), ARNOTT (J.L.), HERON (I.D.), KONEY (E.B.M.), WALKER (A.R.). L'effet d'une lutte contre les tiques sur la fréquence de la dermatophilose chez les bovins autochtones au Ghana. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 317-322

L'effet de trois régimes de lutte contre les tiques sur l'infestation par *Amblyomma variegatum* et sur la dermatophilose bovine a été évalué dans les plaines côtières du Ghana. Les animaux d'un kraal ont été traités tous les 15 jours avec l'amitraz très concentré pulvérisé en faible volume sur les sites préférentiels de fixation des tiques. Les animaux des deux autres kraals ont été traités par application topicale dorsale d'un acaricide à base de deltaméthrine, mensuellement pour l'un, et pour l'autre à des moments stratégiques, basés sur les augmentations saisonnières prévues du niveau de l'infestation par *A. variegatum*. Les animaux d'un quatrième kraal (groupe témoin) ont été traités par les bouviers selon les systèmes traditionnels afin d'empêcher une accumulation de tiques excessive. Le traitement effectué tous les 15 jours a réduit le niveau d'infestation par *A. variegatum* et la fréquence de la dermatophilose est tombée à un niveau bas. L'acaricide topique a réduit la fréquence de la maladie de façon semblable.

Mots clés : Bovin - Dermatophilose - Lutte antiacarien - Tique - *Amblyomma variegatum* - Acaricide - Ghana.

INTRODUCTION

Dermatophilosis is an exudative dermatitis caused by the branching filamentous actinomycete *Dermatophilus congolensis*. It is one of the main constraints to increased cattle productivity in West and Central Africa and on some of the Caribbean islands. The severe clinical form of the disease seen on cattle in parts of the tropics is usually associated with the presence of *Amblyomma variegatum* ticks (7, 9). The effectiveness of three tick control regimes on the occurrence of *A. variegatum* and dermatophilosis on indigenous cattle on the coastal plains of Ghana was investigated in this study.

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MATERIALS AND METHODS

Four herds were selected for use in the tick control study from a group of herds where the occurrence of ticks and dermatophilosis had been monitored continuously over the previous two years. Animals in these herds were kept under traditional management practices in which they were herded twice daily (dawn to 11.30 am and 14.00 to 18.00 pm) on communal unfenced land. The animals were confined in the middle of the day and at night in open unshaded kraals, fenced cattle holding areas. The four kraals were located near to Accra on the coastal plains of Ghana.

Amitraz treatment group

Animals in herd A were treated with the amidine acaricide, amitraz ("Triatix"; Pitman Moore Ltd, U.K.) at the predilection feeding sites of ticks in the axilla, groin, ventral surface, ears and under the tail every second week using a high concentration minimum volume (HCMV) technique. The acaricide, which was prepared just prior to treatments by adding one 20 g sachet of Triatix Stock Spray Powder containing 25 % amitraz as a wettable powder, to 2 l of clean water, was applied by hand spraying with a 5 l hand spraying pressure machine using 50-100 ml per animal.

A pilot study was carried out over the initial 5 months in which a group of five tagged animals were treated with amitraz HCMV spray every week to compare the level of tick control achieved under this treatment regime with that obtained following fortnightly treatments on a second group of five tagged animals of a similar age.

Deltamethrin treatment groups

Animals at two other kraals (herds B and C) were treated with the synthetic pyrethroid acaricide and insecticide deltamethrin as a pour-on containing 1 % deltamethrin in an oil base ("Spot On"; Pitman Moore Ltd, U.K.); in herd B it was applied once every month while in herd C it was used at strategic times based on expected increases in the level of infestation with *A. variegatum*. Animals were confined in a cattle race during treatments which involved the application of the deltamethrin pour-on, at a dosage rate of 1 ml per 10 kg body weight, along the dorsal mid line putting approximately one third of the treatment dose over the shoulders and a further third on the rump, using a Phillips Automatic Drencher.

The species of ticks present in a sub sample collected from animals in these kraals, before the study commenced were identified. The adult ticks present on the 10 tagged animals in the amitraz treatment group (herd A) and on a group of 5 tagged animals in each of the two deltamethrin treatment groups (herds B and C) were identified to genus and counted *in situ* before they were treated. The animals were cast and whole body counts were made of attached adult ticks of both sexes except for *Boophilus* where only partially engorged females were counted. All animals in the kraals were examined for the presence of skin lesions when they were being treated and those having exudative lesions typical of dermatophilosis were classified as positive.

Control group

Animals in the fourth kraal (herd D) were also examined each month for the presence of skin lesions and the number of adult ticks of the various types present on a group of 5 tagged animals in the herd counted. It was not possible to have a control group that remained completely untreated as this would have been incompatible with normal practices at the kraal and could have exposed the project to too high a risk. Animals at this kraal were kept under the traditional system and only received acaricide treatment to control excessive tick build-up as determined by the herdsmen. The details of the acaricide treatments that were carried out were noted during visits to the kraal.

Animals in all four kraals were of a similar breed, Ghana Sanga (West African Shorthorn/Zebu crosses) and the four groups had a similar age structure. The tagged animals in each kraal, on which tick counts were carried out, were all females aged between 9 months and one year at the beginning of the study. There were a total of 70 head of cattle in herd A, 94 in herd B, 187 in herd C and 136 in herd D.

RESULTS

The ticks found on cattle in the selected herds were identified as *Amblyomma variegatum, Hyalomma marginatum rufipes, Rhipicephalus senegalensis, Boophilus decoloratus* and *Boophilus annulatus* with A. *variegatum* being the most common species present. The numbers of adult A. *variegatum* ticks found on animals in the pilot study, to compare weekly and fortnightly treatments with amitraz HCMV spray, are shown in figure 1. Weekly treatments kept infestation levels very low. However, animals which were treated fortnightly had considerably more ticks attach during the second week compared to the first week post treatments.

The number of ticks of the various species found on animals two weeks after treatment with amitraz HCMV spray showed considerable seasonal variation with very few ticks present in January, February and early March (fig. 2). There was a very sudden and marked increase in the

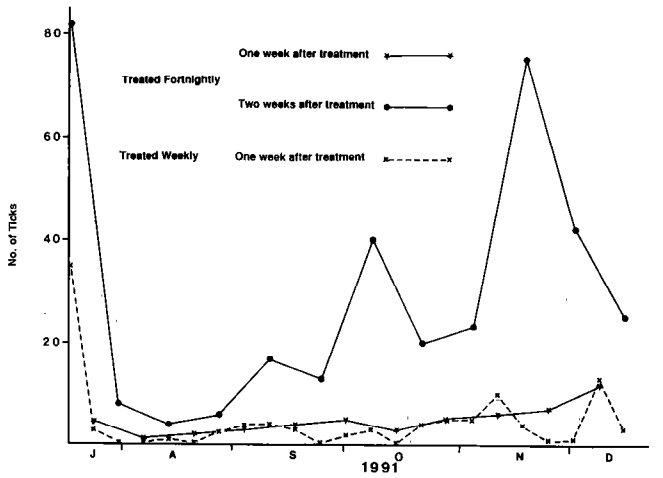

Figure 1 : Mean A. variegatum *counts on animals treated either weekly or fortnightly with amitraz HCMV.*

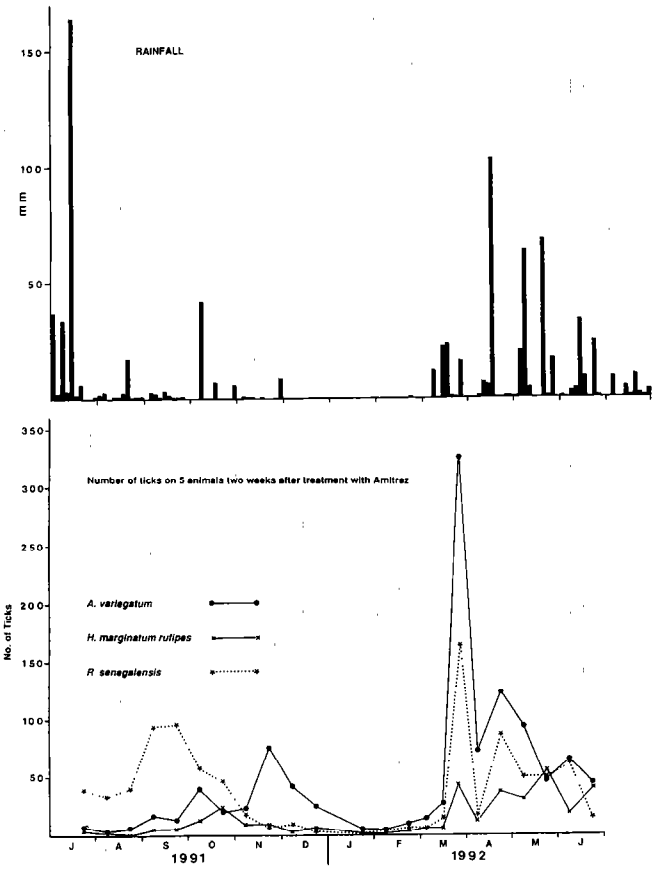

Figure 2 : Three-day rainfall totals and the number of ticks attached to a group of 5 animals two weeks after treatments with amitraz HCMV.

number of ticks, especially *A. variegatum* and *R. senegalensis* which attached between treatments in late March 1992, soon after the first heavy rainfall following a prolonged dry period (November 1991 to February 1992). The

number of *A. variegatum* which attached between fortnightly treatments declined from mid November to the end of January, remained quite low until mid March when the mean number present per animal was 5 and then suddenly increased to a mean of 65 two weeks later. The prevalence of dermatophilosis on animals in this herd fell from 11 % in August to less than 3 % in October and remained at that level until the end of the study (fig. 3). One animal had localised lesions which persisted throughout the period of the study while all new cases were mild and transient.

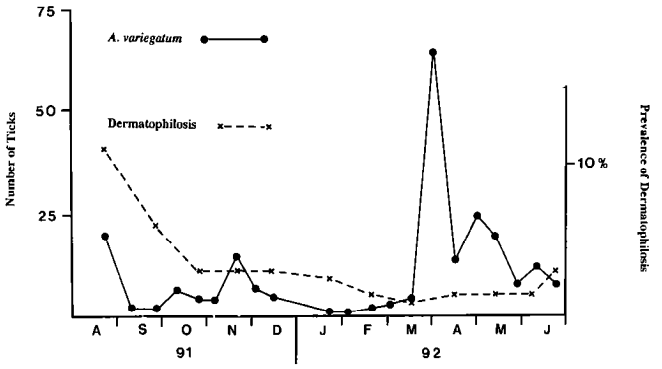

Figure 3 : A. variegatum *counts and the prevalence of dermatophilosis on animals in Herd A. The tick counts were carried out prior to fortnightly treatments with amitraz HCMV.*

The level of control of *A. variegatum* ticks obtained by monthly treatment with deltamethrin pour-on was similar but slightly better than that achieved by fortnightly spraying with amitraz (fig. 2, 4). The number of ticks present one month after treatment with delthamethrin pour-on was very low during the period from November to March and then increased suddenly during the one month period to early April. The marked increase in tick challenge which occurred during the second half of March coincided with weeks 3 and 4 post treatment when protection, which lasts for about two weeks, would have already worn off. Tick numbers on animals in all four kraals under observation increased very dramatically in this period when the first rains followed a prolonged dry period. The prevalence of dermatophilosis on animals in this herd fell from over 5 % when treatment commenced in November to less than 2 % within two months and remained at that level (fig. 4).

Restricting the application of deltamethrin on animals in kraal C to the times when it was expected that the tick challenge would increase resulted in less satisfactory tick control (fig. 5). However this was sufficient to control the occurrence of dermatophilosis, the prevalence of which fell from 5.5 % in November to less than 3 % in February,

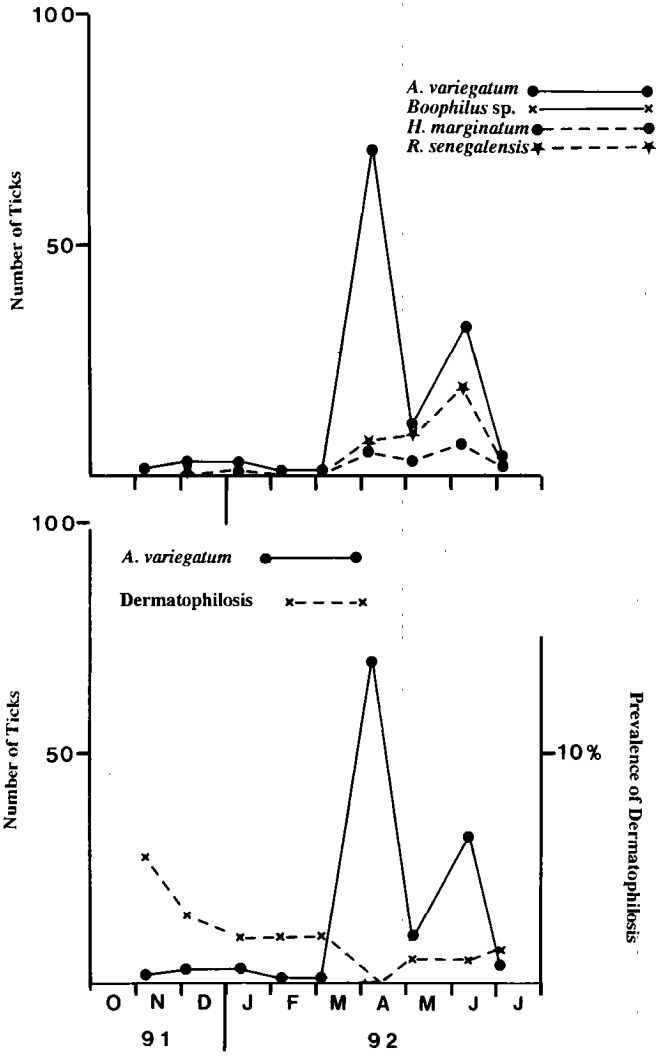

Figure 4 : Tick counts and the prevalence of dermatophilosis on animals in Herd B. The tick counts were carried out prior to monthly treatments with a deltamethrin based pour-on acaricide.

remained at a low level until June when it increased somewhat and then fell back down to less than 2 % at the end of the study.

The level of infestation on animals in the control herd where the application of acaricide was left to the discretion of the owners/herdsmen is shown in figure 6. The animals in this herd were exposed to a continuously high level of challenge with *A. variegatum.* Acaricide treatment was carried out by the herdsman by hand using lindane ("Gammatox", Coopers Animal Health Ltd, U.K.) in between the April and May tick counts and again between the May and June tick counts. The prevalence of dermatophilosis in this herd increased to above 10 % over the final four months of the study (fig. 6).

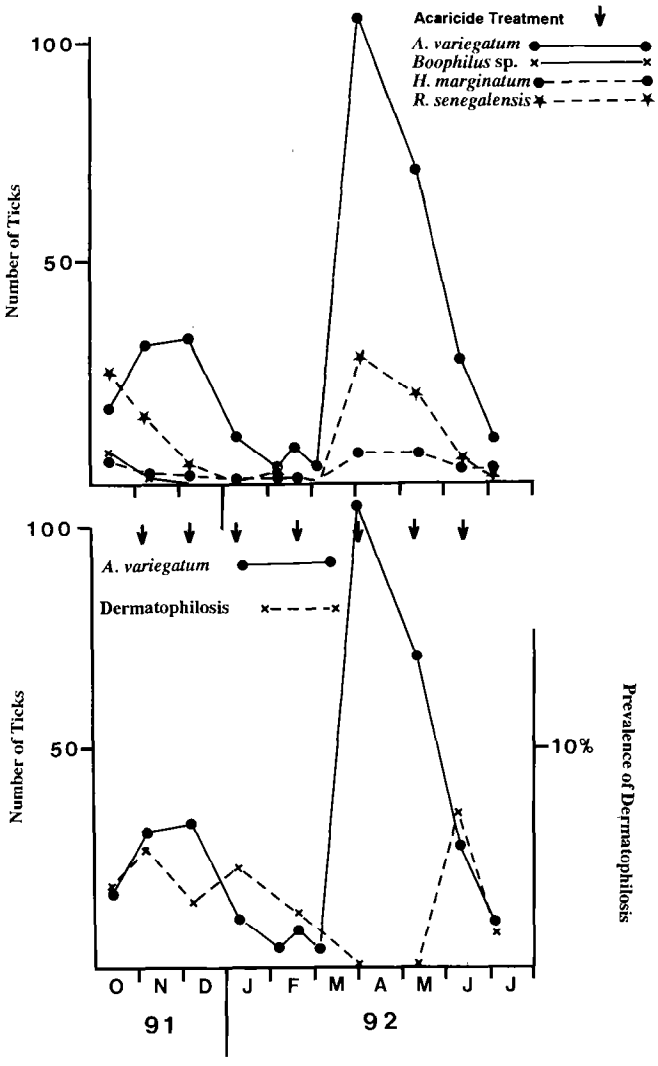

Figure 5 : Tick counts and the prevalence of dermatophilosis on animals in Herd C which were treated with a deltamethrin based pour-on acaricide at the times indicated by the vertical arrows.

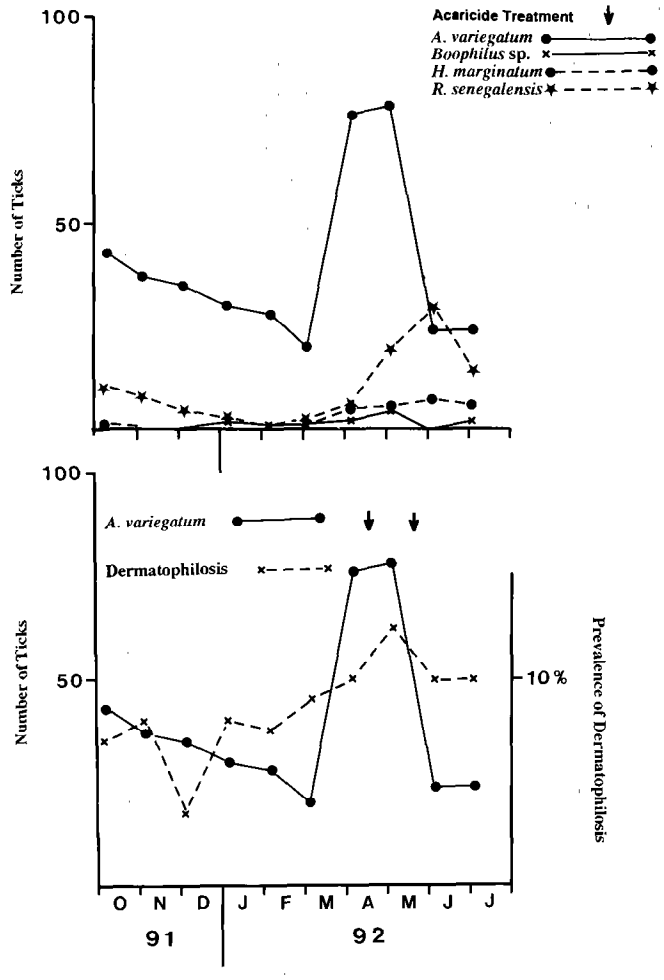

Figure 6 : Mean monthly tick counts and the prevalence of dermatophilosis in animals in Herd D (control group).

DISCUSSION

The association between the occurrence of a severe form of dermatophilosis and the presence of *A. variegatum* ticks is based on their similar geographical distribution (1, 2, 3, 6), the similar seasonal occurrence of adult *A. variegatum* ticks and dermatophilosis (7, 9) and reports that tick control measures reduce the incidence of dermatophilosis (4, 6, 9). Animals vary in the number of ticks they pick up or carry and in their susceptibility to dermatophilosis with very marked differences between certain breeds (5). The type of tick control required for the control of bovine dermatophilosis will therefore depend on the breed of animals one is dealing with. It has been shown that when susceptible Friesian cattle are maintained almost tick free they do not develop the disease whereas untreated control animals were all severely affected within 6 months of becoming tick infested (5). Ghana Sangas, which are relatively resistant to dermatophilosis are the predominant type of cattle on the Accra Plains. OPPONG (8) observed that the prevalence of dermatophilosis on cattle kept under traditional management on the Accra Plains increases from 4.9 % in the dry season to 12.8 % in July, towards the end of the early rains. The wet season prevalence mentioned by OPPONG (8) is in agreement with the findings of the present study where over 10 % of the animals in the control group were affected at that time of year. The present study showed that limited tick control on these animals is effective in reducing the prevalence of dermatophilosis. The continuing low level of

dermatophilosis in the acaricide treatment groups could be largely accounted for by the persistence of a few established cases.

Weekly treatments with amitraz HCMV at the predilection sites kept tick numbers at a low level but at fortnightly intervals the tick numbers increased in the second week post treatment indicating the relatively short residual activity of this form of acaricide and pheromone attraction when some ticks attached. Monthly treatments with deltamethrin pour-on, which has residual activity providing a high level of protection for two weeks post treatment, gave slightly better tick control than fortnightly treatment with amitraz HCMV. Using the latter dermatophilosis was reduced to less than 3 % and remained at this level until the end of the study. Treatment with deltamethrin pour-on at monthly intervals reduced the prevalence of dermatophilosis to less than 2 % whereas attempted strategic application of deltamethrin pour-on in early November, December and January, mid February, early April, mid May and mid June gave satisfactory control of *A. variegatum* and reduced the prevalence of dermatophilosis to 3 % and finally less than 2 % at the end of the study. However the data suggests that strategic tick control should be based on prevailing climatic conditions rather than expected seasonal changes in the prevalence of *A. variegatum* ticks. The sudden increase in the number of ticks on animals soon after the first rains may have had little to do with any effect which climatic conditions have on the development of the tick but rather that it was related to the availability of suitable physical conditions in the habitat for the tick to gain access to the host.

Compared to the amitraz HCMV spray deltamethrin pour-on is easier to apply, it has a longer residual effect and it kills biting flies and when extensively used in a tsetse infested area it will reduce or eliminate tsetse populations. However, the cost of treatment must be considered and it should preferably be affordable by the owners of the indigenous cattle and relate to expected financial returns. To be fully effective conventional hand spraying with acaricide requires the use of 5 to 10 l of wash per head and much of this runs off and is wasted. The HCMV technique involves application to predilection sites and tick clusters and there is little run off. Adult *A. variegatum* ticks are found feeding on animals predominantly on the udder and scrotum and in the groin and axilla and dewlap with a tendency to cluster which makes them particularly suitable for this form of treatment. Small groups could easily be treated using a hand held 500 ml or 1 l sprayer. At current prices amitraz HCMV costs £ 0.03 per treatment or £ 0.7 per annum if applied throughout the year at fortnightly intervals. The deltamethrin pour-on costs £ 0.6 per treatment or £ 7.2 if applied at monthly intervals or £ 4.2 if used 7 times strategically. The prevalence of dermatophilosis was somewhat less in the deltamethrin treatment groups compared to amitraz HCMV treatment group but the cost of the deltamethrin pour-on is such that it is difficult to see how it could be sustained in the returns expected from indigenous cattle. The owners of these cattle would be unlikely to be able to afford £ 0.6 per treatment whereas they could possibly afford £ 0.03.

CONCLUSION

Dermatophilosis on the indigenous cattle population on the Coastal Plains of Ghana can be controlled by the limited use of acaricides applied either at the predilection feeding sites of *A. variegatum* or at selected times when the level of challenge increases. However, to be effective treatments need to be carried out more frequently than is commonly practiced at present. Amitraz HCMV proved effective in the control of *A. variegatum* ticks and dermatophilosis on the indigenous type cattle used in this study and is ideally suited for use by smallholders. The deltamethrin pour-on was somewhat more effective and while considerably more convenient to use it is perhaps too costly for regular use with this type of cattle unless tsetse control is also required. The timing of strategic control is critical to its success and tactical control closely linked to short term local climatic conditions is likely to give better results.

ACKNOWLEDGEMENTS

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MORROW (A.N.), ARNOTT (J.L.), HERON (I.D.), KONEY (E.B.M.), WALKER (A.R.). The effect of tick control on the prevalence of dermatophilosis on indigenous cattle in Ghana. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 317-322

The effectiveness of three tick control regimes on the occurrence of *Amblyomma variegatum* and dermatophilosis on cattle on the coastal plains of Ghana were compared. Animals at one kraal were sprayed with amitraz at predilection feeding sites of ticks every second week using a high concentration minimum volume technique. Animals at two other kraals were treated with a deltamethrin based pour-on acaricide ; at one kraal it was applied once every month while at the other kraal it was used at strategic times based on the expected seasonal increases in the level of infestation with *A. variegatum*. Animals in a fourth kraal (control group) were treated, by the herdsmen, to control excessive tick build-up as practised under traditional management systems. Fortnightly treatment with amitraz reduced the level of infestation with *A. variegatum* and the prevalence of dermatophilosis dropped to a low level. The pour-on acaricide similarly depressed the prevalence of dermatophilosis.

Key words : Cattle - Dermatophilosis - Tick control - Tick - *Amblyomma variegatum* - Acaricide - Ghana.

MORROW (A.N.), ARNOTT (J.L.), HERON (I.D.), KONEY (E.B.M.), WALKER (A.R.). Efecto del control de garrapatas sobre la prevalencia de la dermatofilosis en ganado autóctono en Ghana. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 317-322

Se compara el éxito de los programas de control de garrapatas, principalmente *Amblyomma variegatum* y de la dermatofilosis en el ganado de las planicies litorales de Ghana. Un grupo de animales se trató por aspersión con amitraz, en los sitios predilectos de alimentación de las garrapatas, a intervalos de dos semanas y con técnicas de altas concentraciones en volúmenes mínimos. En otros dos corrales los animales se trataron por depósito ("pour-on") con un acaricida a base de deltametrina. En uno de los corrales se aplicó mensualmente, mientras que en el otro se aplicó en momentos estratégicos, de acuerdo a los aumentos estacionales esperados en el nivel de infestación con *A. variegatum*. En un cuarto corral se mantuvo un grupo control, el cual fue tratado por el finquero mediante las prácticas de manejo tradicionales, para el control de garrapatas. El tratamiento quincenal con amitraz disminuyó el grado de infestación con *A. variegatum*, así como la prevalencia de dermatofilosis. El acaricida por depósito disminuyó de manera similar la prevalencia de la dermatofilosis.

Palabras claves : Bovino - Dermatofilosis - Control de acaros - Garrapata - *Amblyomma variegatum* - Acaricide - Ghana.

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Epidemiological studies on dermatophilosis in the Caribbean

MARTINEZ (D.), AUMONT (G.), MOUTOUSSAMY (M.), GABRIEL (D.), TATAREAU (A.H.), BARRÉ (N.), VALLÉE (F.), MARI (B.). Études épidémiologiques sur la dermatophilose dans les Antilles. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 323-327

La dermatophilose est une des maladies les plus importantes des ruminants domestiques des îles caraïbes, où la maladie clinique est associée à la présence de la tique *Amblyomma variegatum*. Des études séroépidémiologiques ont été effectuées afin d'éclaircir l'épidémiologie de la maladie dans la région, en faisant particulièrement attention au rôle d'*A.variegatum*. Une banque de 1300 sérums de bovins des Petites Antilles a été examinée par ELISA pour la présence d'anticorps contre *Dermatophilus congolensis*. Il s'est avéré que des animaux séropositifs existent dans des îles non infestées par *A. variegatum*, et où la dermatophilose n'est jamais ou rarement observée. De plus, il n'y avait pas de différence significative entre la prévalence d'animaux séropositifs des zones infestées par la tique et des zones non infestées de la Martinique et de Sainte-Lucie, deux îles partiellement infestées et où la dermatophilose n'est observée que dans les parties infestées par la tique. La prévalence était basse dans les petites îles ayant un climat sec. Ceci confirme les résultats expérimentaux indiquant qu'*A. variegatum* n'est pas indispensable pour la transmission de *D. congolensis*, qui est très répandu dans la plupart des îles. Les concentrations élevées de prostaglandine E2 (entre 151 et 377 ng/ml) et de prostacycline (entre 124 et 134 ng/ml) trouvées dans la salive des femelles d'*A. variegatum*, suggèrent fortement que la tique pourrait favoriser le développement des lésions par une activité immunomodulatrice de sa salive. Néanmoins, malgré un certain succès dans la reproduction de la dermatophilose chez des chèvres simultanément infestées avec des adultes d' *A. variegatum* et scarifiées avec *Dermatophilus*, on n'a pas observé de différence entre des bovins Créole naturellement résistants et des Brahman hautement sensibles, utilisant le même modèle. Les lésions de la dermatophilose sont restées très bénignes sur les animaux des deux races. Après cette expérience, les Brahman ont développé une dermatophilose généralisée après avoir été mis au pâturage, ce qui indique que le rôle respectif des facteurs de risque identifiés comme étant d'importance majeure pour l'expression de la dermatophilose clinique, n'est pas complètement clarifié et demande d'être étudié davantage.

Mots clés : Bovin Créole - Dermatophilose - *Dermatophilus congolensis* - Epidémiologie - Enquête sérologique - Test ELISA - Infection expérimentale - *Amblyomma variegatum* - Salive - Martinique - Sainte-Lucie.

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INTRODUCTION

Dermatophilosis is an exsudative skin disease affecting numerous animal species and man caused by an Actinomycete, *Dermatophilus congolensis*. The microorganism has a worldwide distribution but is of little economic significance in temperate climates. However, severe economic losses occur in domestic ruminants and horses in the tropics (8). *D. congolenis* is not very pathogenic per se and there is no reliable method to reproduce the disease experimentally. Cofactors are necessary for the appearence of the clinical disease among which the breed, the humidity and the bite of arthropods are of primary importance (2). In the Caribbean, a close correlation was noted between the distribution of the tick *Amblyomma variegatum* and the severe extensive forms of the disease (4). In fact, it has long been noted that outbreaks are associated with the presence of this tick (17, 16). In the Antilles, outbreaks of the disease occur soon after the introduction of *A. variegatum* in a new island and in partly infected islands acute dermatophilosis is restricted to those areas where the tick is known to occur (5, 6, 12, 13, 14). In such situations the mortality in susceptible animals may exceed 50 % in the absence of treatment (15, 18) and the disease is a major pathological constraint for farmers. Since 1948, 17 new islands of the Lesser Antilles have become infected by *A. variegatum* (3), and this tick with its associated diseases represents a real threat for the American mainland (1). In the absence of a vaccine, tick control is the most effective method of controlling the disease.

Recently, by using rifampicin resistant strains of *D. congolensis*, it was experimentally shown that *A. variegatum* did not act as a vector of *D. congolensis*. Transmission occurred without the presence of ticks, and high levels of specific antibodies were elicited in goats which did not develop any lesions until adult *A. variegatum* were allowed to feed on them. An extensive dermatophilosis was thus experimentally reproduced leading to the death of experimental goats (10). The specificity of the association between acute dermatophilosis and this particular tick strongly suggests that unknown agents in the tick saliva could favour the development of skin lesions on asymptomatic carrier animals.

In this study we report the results of seroepidemiological surveys which were carried out in order to confirm experimental data on the role of *A. variegatum* in the transmission of *D. congolensis*. In addition, attempts to reproduce

dermatophilosis on cattle and preliminary investigations on the composition of *A. variegatum* saliva were carried out.

MATERIAL AND METHODS

Seroepidemiological surveys

Screening of a bank of sera

A bank of 1397 cattle sera collected during a survey on the distribution of heartwater and *Amblyomma variegatum* in the Caribbean conducted between 1983 and 1984 (4), was screened for the presence of antibodies to *D. congolensis* using an ELISA method (11).

Surveys in Martinique (1989) and in Saint Lucia (1990)

These surveys were conducted to study the relationship between the presence of antibodies to *D. congolensis* in domestic ruminant sera and the presence of clinical lesions of dermatophilosis on animals, along with their infestation by *A. variegatum*. Field studies consisted of a questionnaire-based interview with farmers about farm management, and a visual and physical inspection of animals (sex, age, breed, body score, tick count, extension and severity of dermatophilosis lesions) in selected herds. Two hundred and sixty one cattle sera were collected in 227 farms representing between 2 and 3 % of the overall number of herds in Martinique. Hundred and seventy two farms were visited in Saint Lucia in which 361 heads of cattle (3 % of the herd), 183 sheep (1 %) and 110 goats (1 %) were bled. The farms surveyed were spread all over the islands and the sera were tested by the above-mentioned ELISA method.

Attempts to reproduce extensive dermatophilosis on cattle

Fifteen Brahman and 15 Creole cattle were given 3 successive inoculations of *D. congolensis* with or without infestation with *A. variegatum* at monthly intervals. Animals were divided into 3 groups. In group 1 composed of 3 Brahman and 3 Creole, animals were infested with 30 pairs of adult *A. variegatum*. In group 2, 8 Brahman and 8 Creole zebus were simultaneously infested with 30 pairs of adult ticks and scarified with the R2 rifampicin resistant strain of *D. congolensis* (9). Group 3 consisted of 4 Brahman and 4 Creole animals scarified with *D. congolensis* in the absence of ticks. A Guadeloupe strain of *A. variegatum* reared in our laboratory on experimental goats and maintained at 25 °C and 90 % relative humidity for several generations was used for infestation of animals. Ticks were confined to hosts in bags glued to the skin of the flank with adhesive. Female ticks were applied 7 days after the fixation of males. Inoculation with *D. congolensis* was carried out by swabbing 5 ml of a broth culture containing approximately 10^7 cfu/ml on each of four 100 cm^2 skin areas of the rump previously shaved and defatted with acetone. Skin lesions were scored twice a week (10) and blood collected at weekly intervals on each animal. At the end of the experiments, animals were put into the fields in the same farm. The infestation by *A. variegatum* (tick count), the appearence and evolution of lesions of dermatophilosis were recorded each week for 3 months.

Dosage of prostaglandins in tick saliva

To confirm the hypothesis raised in previous studies (10) of an immunomodulation induced in the host by the tick bite, preliminary investigations on the presence of immunosuppressive components in the tick saliva were carried out. In particular, the presence and the amount of prostaglandin E2 and I2 which are known to have potent immunosuppressive activities, were determined in tick saliva using a competitive enzyme immunoassay (Cayman chemical). The test was performed according to the instructions of the manufacturer.

RESULTS AND DISCUSSION

The prevalence of cattle with antibodies to *D. congolensis* in relation with the presence of *A. variegatum* in the Lesser Antilles (sera from 1983-84) is summarized in table I. The seroprevalence was high in all islands infested by *A. variegatum*, but also in Cariacou, Saint Vincent and Tortola where this tick was not reported to occur. This is in agreement with the experimental results of MARTINEZ et al. (10) demonstrating the high levels of transmission of *D. congolensis* in the absence of ticks. The prevalence of seropositive cattle was low in the small dry *Amblyomma* free islands of Anegada, Jost van Dyck, Virgin Gorda, Saba and Saint Eustachius suggesting that the efficacy of transmission as quantified by the seroprevalence, was decreased in dry conditions. Martinique and Saint Lucia were selected to conduct a field study because they were only partly infested by *A. variegatum* and therefore provided a unique situation to study the association between this tick and *D. congolensis*. The percentages of seropositive animals were not significantly different between areas infested or not by *A. variegatum* (table II), confirming that this tick has no significant role in transmitting the microorganism. Moreover, the prevalence of animals with antibodies was very high, indicating that

TABLE I Seroprevalence of dermatophilosis in cattle in the Lesser Antilles (sera from Burridge et al., *1984).*

Island	Number of sera	Positive sera n (%)	*Amblyomma variegatum*
Virgin Islands :			
Anegada	19	3 (16)	–
Jost van Dyck	11	2 (18)	–
Tortola	141	48 (34)	–
Virgin Gorda	23	1 (4)	–
Anguilla	67	15 (22)	–
Saint Martin	72	54 (75)	+
Saba	27	3 (11)	–
Saint Eustachius	31	3 (10)	–
Saint Kitts	61	53 (87)	+
Antigua	199	97 (49)	+
Montserrat	155	91 (59)	+
Dominique	108	90 (83)	+*
Saint Lucia	82	54 (66)	+*
Saint Vincent	181	108 (60)	–
Cariacou	58	47 (81)	–
Barbados	108	41 (38)	+*
Les Saintes	54	1 (2)	–

* A. variegatum *is established but with limited distribution.*

TABLE II Seroprevalence of dermatophilosis on domestic ruminants in Martinique and Saint Lucia.

Saint Lucia		n	% positive sera
Cattle	non infested area	240	60[a]
	infested area	121	56[a]
	total	361	59
Sheep	non infested area	121	53[a]
	infested area	62	63[a]
	total	183	56
Goats	non infested area	75	73[b]
	infested area	35	86[b]
	total	110	77
Martinique		n	% positive sera
Cattle	non infested area	99	62[a]
	infested area	162	71[a]
	total	261	67

In the same column and the same island, numbers with different superscript letters are significantly different ($P < 0.05$).

the transmission was very efficient. However, clinical cases of dermatophilosis were reported in tick infested areas only. The possibility that the tick could favour the development of scabs through an immunomodulating activity of its saliva instead of a vector capacity, was corroborated by the high levels of PGE2 (151 to 377 ng/ml) and PGI2 (124 to 134 ng/ml) found in its saliva. These agents have potent immunomodulating activities (7) and might have influenced the course of the disease. However, their activity is restricted to those areas where they are secreted. It is thus unlikely that they have a great importance in the induction of lesions distant from the tick bite. In the West Indies, it was found that 90 % of infected cattle had lesions on the back where less than 1 % of the ticks attach (2, 14) suggesting that another mechanism than prostaglandins is involved in the development of such lesions.

The identification of the major risk factors (presence of *A. variegatum,* humidity, susceptible breed) in the development of extensive dermatophilosis and the success in reproducing the disease on Creole goats using an experimental model taking into account all these factors (10), led us to use the same model to reproduce the disease on cattle. The influence of the breed was investigated by comparing a highly susceptible (Brahman zebu) and a highly resistant Creole cattle from Guadeloupe) breed of cattle. There was no development of scabs in groups of animals inoculated with *A. variegatum* alone (group 1). In contrast, on all animals inoculated with *D. congolensis* (groups 2 and 3), scabs developed at the sites of inoculation from where the rifampicin resistant strain could be isolated. The score lesion was increased by the presence of ticks in Creole but not in Brahman cattle (figure 1). Surprisingly, Creole cattle had higher score lesions than Brahman cattle. However, the reproduction of an extensive dermatophilosis failed in both breeds : the lesions remained very mild and located at the sites of inoculation. Differences in score lesion could not be attributed to a higher infestation of Creole cattle by ticks since there was no significant difference between breeds in the percentage of female ticks which attached and engorged, except at the first infestation (figure 2). All Brahman animals having experienced a severe dermatophilosis episode several months before the experiment (15), it was decided to verify the very unlikely hypothesis that they had become protected against the disease. The 30 heads of cattle were put on an infested pasture of the farm where the experiment had been conducted. All animals become infested by *A. variegatum* a few days after their introduction on the pasture. The tick burden varied from 2 to 42 adult *A. variegatum* per animal. As expected, none of the Creole cattle developed any lesion, whereas all Brahman zebus but one started developing scabs within 2 weeks after their introduction on the pasture. The lesions became generalized within 2 to 6 weeks in 9 out of 15 animals which had to be treated with long acting Terramycin®. Five animals with only localized lesions were not treated, and one animal did not develop any lesion during the 3 months period of monitoring in the field. As in the experiments conducted on goats (10), the strain isolated from the scabs was a non rifampicin resistant field strain of *Dermatophilus.* Whether this strain was already present on the animals or was transmitted in the field was not elucidated.

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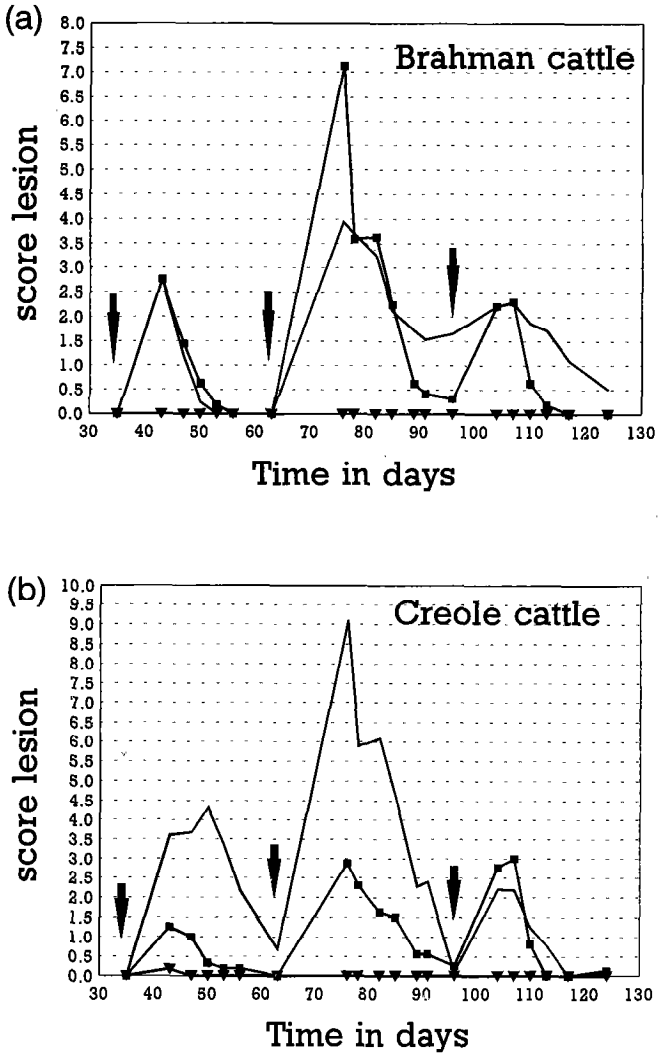

Figure 1 : Score lesion of 15 Brahman (figure a) and 15 Creole (figure b) cattle experimentally infested with 30 pairs of adult Amblyomma variegatum *without* Dermatophilus congolensis *(Δ) scarified with the R2 strain of* D. congolensis *in the absence of ticks (□), or simultaneously scarified with* D. congolensis *and infested with 30 pairs of* A. variegatum *(–). Each infestation is represented by an arrow.*

The failure to reproduce the disease could have been due to a low pathogenicity of the R2 strain, to differences between wild ticks and ticks reared in the laboratory, or to the absence of a badly identified environmental factor. Except for *Amblyomma* and *Dermatophilus* strains, the only difference in the management of animals were that cattle were maintained in shaded paddocks during the experiment and exposed to direct sunlight in the pasture. Although sunrays alone did not induce the development of scabs (the same Brahman animals maintained under the sun in tick free paddocks did not develop any lesions), they might have acted synergistically with ticks.

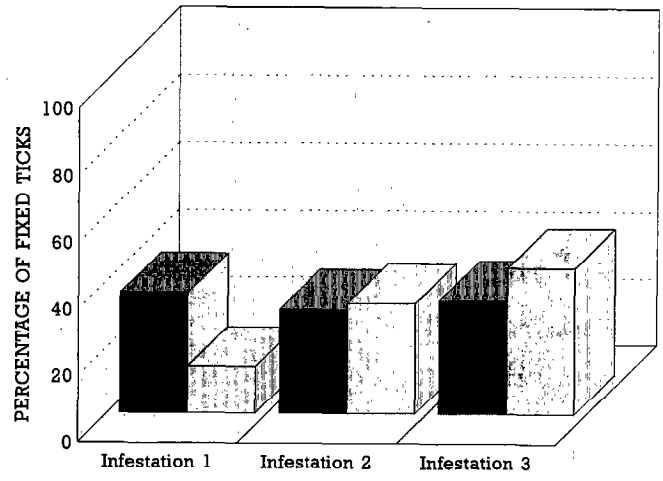

Figure 2 : Percentage of adult Amblyomma variegatum *which attached and engorged on Brahman (▒) and Creole (■) cattle during 3 successive infestations at 1 month interval. Each animal was infested with 30 pairs of adult ticks per infestation.*

In conclusion, these epidemiological data confirm that *A. variegatum* does not play a significant role in the transmission of *D. congolensis* and its major influence in the induction of scabs. However, it appeared that despite some previous success in reproducing dermatophilosis on goats experimentally, the respective role of the risk factors identified as being of major importance for the expression of clinical dermatophilosis is not clarified and needs further investigations.

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MARTINEZ (D.), AUMONT (G.), MOUTOUSSAMY (M.), GABRIEL (D.), TATAREAU (A.H.), BARRÉ (N.), VALLÉE (F.), MARI (B.). Epidemiological studies on dermatophilosis in the Caribbean. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 323-327

Dermatophilosis is one of the most important diseases of domestic ruminants in the Caribbean islands where the clinical disease has been shown to be associated with the presence of the tick *Amblyomma variegatum*. Seroepidemiological studies were conducted to clarify the epidemiology of the disease in the region with a particular attention paid to the role of A. variegatum. A bank of 1300 cattle sera from the Lesser Antilles was screened by ELISA for the presence of antibodies to *Dermatophilus congolensis*. It appeared that seropositive animals do exist in islands non infested by *A. variegatum* and where dermatophilosis is never or seldom seen. Moreover, there was no significant difference in prevalence of seropositive animals between tick-infested and non infested areas of Martinique and Saint Lucia, 2 islands partly infested by the tick, and where dermatophilosis is only seen in tick-infested areas. Prevalence was low in small islands with a dry climate. This confirms experimental data showing that *A. variegatum* is not necessary for the transmission of *D. congolensis* which is widespread in most of the islands. High concentrations of prostaglandin E2 (between 151 and 377 ng/ml) and prostacyclin (between 124 and 134 ng/ml) found in the saliva of females *A. variegatum* strongly suggest that the tick could favour the development of the lesions through an immunomodulating activity of its saliva. However, despite some success in reproducing dermatophilosis on goats simultaneously infested with adult *A. variegatum* and scarified with *Dermatophilus*, no difference was observed between naturally resistant Creole cattle and very susceptible Brahman animals using the same model. The lesions of dermatophilosis remained very mild on animals of both breeds. After the experiment, the Brahman animals put onto the field developed an extensive dermatophilosis indicating that the respective role of the risk factors identified as being of major importance for the expression of clinical dermatophilosis is not fully clarified and needs further investigations.

Key words : Creole cattle - Dermatophilosis - *Dermatophilus congolensis* - Epidemiology - Serological survey -ELISA test - Experimental infection - *Amblyomma variegatum* - Saliva - Martinique - Saint Lucia.

MARTINEZ (D.), AUMONT (G.), MOUTOUSSAMY (M.), GABRIEL (D.), TATAREAU (A.H.), BARRÉ (N.), VALLÉE (F.), MARI (B.). Estudios epidemiológicos sobre la dermatofilosis en el Caribe. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 323-327

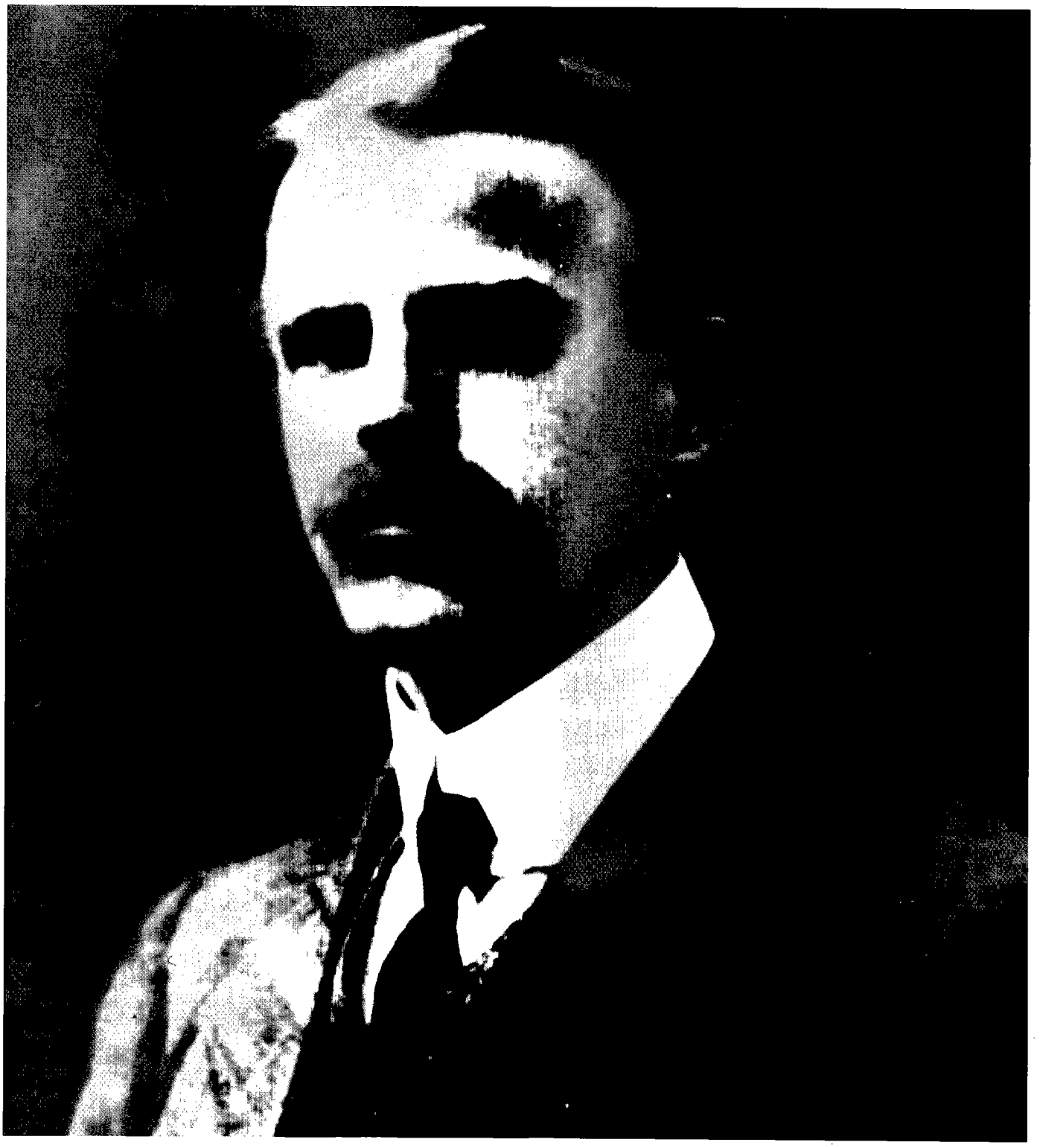
La dermatofilosis es una de las enfermedades más importantes en las Islas del Caribe, donde la enfermedad clínica se ha asociado a la presencia de garrapatas de *Amblyomma variegatum*. Se han llevado a cabo estudios sero-epidemiológicos para aclarar la epidemiología de la enfermedad en la región y en particular el papel de *A. variegatum*. Un banco de 1300 sueros bovinos provenientes de las Antillas Menores fue sometido a un monitoreo por ELISA, con el fin de detectar la presencia de anticuerpos contra *Dermatophilus congolensis*. Según los resultados, existen animales seropositivos en islas libres de A.variegatum y en las cuales la dermatofilosis se ha presentado raramente o nunca. Aún más, no se encontró diferencia significativa en la prevalencia de animales seropositivos entre las áreas infestadas con la garrapata y las no infestadas de Martinica y Santa Lucia, islas parcialmente infestadas y en donde la dermatofilosis aparece solamente en las zonas infestadas. La prevalencia fue menor en las pequeñas islas de clima seco. Esto confirma los datos experimentales, según los cuáles *A. variegatum* no es esencial para la transmisión de *D. congolensis*, que se encuentra diseminado en la mayoría de las islas. Se encontraron concentraciones elevadas de prostaglandina E2 (entre 151 y 377 ng/ml) y prostaciclina (entre 124 y 134 ng/ml) en la saliva de hembras de *A. variegatum*. Esto sugiere que la garrapata podría favorecer el desarrollo de lesiones mediante una actividad de modulación inmunológica de la saliva. Sin embargo, a pesar de un ligero éxito en la reproducción de la dermatofilosis en cabras inoculadas simultaneamente con adultos de *A. variegatum* y con *Dermatophilus*, no se encontró diferencia entre la resistencia natural del ganado "Créole" y la de los animales Brahman, altamente susceptibles. Las lesiones de dermatofilosis fueron leves en los animales de ambas razas. Después del experimento, los animales Brahman que fueron sometidos a condiciones de campo, desarrollaron una dermatofilosis extensa. Esto sugiere que el papel atribuído a los factores de riesgo catalogados como importantes para la aparición de la dermatofilosis clínica, no es aún claro y necesita investigaciones ulteriores.

Palabras claves : Bovino Creole - Dermatofilosis - *Dermatophilus congolensis* - Epidemiologia - Encuesta serologica - Test ELISA - Infección experimental - *Amblyomma variegatum* - Saliva - Martinica - Santa Lucia.

Retour au menu

Session

Amblyomma



C.P. Lounsbury in 1900 proved that heartwater is transmitted by the South African bont tick : Amblyomma hebraeum.

Retour au menu

SESSION *AMBLYOMMA* : INTRODUCTION

Amongst ticks infecting livestock, Amblyomma variegatum *is, from an economical point of view, one of the 2 or 3 most important species, in particular if we consider that it has a major role in the clinical expression of dermatophilosis. Its ability to transmit heartwater is well known. This disease which frequently ends in death, for which prevention by the use of a vaccine is not yet available, is a serious constraint, especially for selected breeds of high production potential.*

In addition, this tick is the vector of various arboviruses, rickettsiae and protozoa of less concern, but which may be of importance locally or regionally, or in particular epidemiological situations .

Moreover, A. variegatum *is one of the ticks with the widest geographical distribution and host range in Africa. This ubiquity makes this species one of the best adapted to spread in the tropics and cover an extremely wide area. The threat for the neotropics is particularly worrying.* Boophilus microplus, *a one-host tick with fairly similar climatic requirements, had invaded most tropical regions, and although* A. variegatum *for the moment has established itself outside Africa only on islands such as Madagascar, the Mascarenes, the Comoros and in the Caribbean, it also has the potential for a much wider spread. It spreads slowly but inexorably.*

In the Caribbean, the problem is of much concern if we consider the proximity of the mainland and the immensity of the territories potentially favourable to the tick, from the North of Mexico to the South of Brazil. Moreover, the phenomenon has speeded up for the last 25 years, more and more islands being infested every 2-5 years. The reason for such an increase in the propagation of the tick is not clear but there are some arguments to suppose that the cattle egret, a migrating bird recently established in the Caribbean, showing a high expansion rate and intimately associated with livestock, could be a disseminator of A. variegatum *in the Caribbean, as it is occasionally a host of the tick.*

The observations made in the Caribbean allow us to formulate some rules and recommendations to explain, predict and limit the risk of introduction and multiplication of exotic ticks :

- Ecological or climatic perturbations (deforestation, establishement of pastures, massive introduction of livestock, drought...) may render favourable for certain tick species areas previously unsuitable for them.

- Some migrating or erratic animal species, especially birds, may act when they pullulate, as new disseminators of ticks previously confined to localized areas.

- Important progress has been accomplished during these last 20 years concerning acaricides : efficiency, toxicity, residual activity, mode of application. However, constraints remain too high to obtain automatically the enthusiastic cooperation of the farmer in tick eradication programmes.

- While waiting for new progress in tick control (pheromonal baits, slow release devices, vaccine...), chemical application remains presently the only method to succeed in eradication.

- Limited foci of infestation my be easily destroyed using classical methods of application. That means an early detection of these foci, facilitated by campaigns of surveillance, information and the creation of quarantines.

- The cost and the risk of failure are proportional with the size of the foci, the number of animal owners concerned and the abundance of free ranging animals.

- It is extremely difficult (cost, organisation, regulation...) to implement a coordinated eradication campaign when the tick has invaded a vast territory covering different countries. The cost and the risks of failure discourage the donors. However, at present in the Caribbean, there is no other solution to stop the threat.

All the countries potentially threatened by the tick should be aware of the risk of propagation in order to take rapidly appropriate measures of control and eradication. Research has to be conducted on acaricides in cooperation with pharmaceutic firms to develop new technologies in order to facilitate their administration and to reduce the frequency of application.

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Ventilation in the adults of *Amblyomma hebraeum* and *A. marmoreum* (Acarina, Ixodidae), vectors of heartwater in Southern Africa

FIELDEN (L.J.), DUNCAN (F.D.), LIGHTON (J.R.B.), RECHAV (Y.). Ventilation chez les adultes d'*Amblyomma hebraeum* et *A. marmoreum* (Acarina, Ixodidae), vecteurs de la cowdriose en Afrique australe. *Revue Élev. Méd. vét. pays trop.*, 1993, **46** (1-2) : 335-338

Cette étude avait pour but de déterminer les caractéristiques principales de l'échange des gaz respiratoires chez les adultes à jeun des tiques *Amblyomma hebraeum* et *A.marmoreum*, vecteurs de la cowdriose en Afrique australe. L'émission de dioxyde de carbone a été mesurée à 25 °C par respirométrie afin de déterminer le taux de métabolisme standard (TMS) et l'évolution dans le temps de l'émission gazeuse. Le TMS était extrêmement bas pour les deux espèces et environ 100 fois moindre que celui prévisible pour un insecte d'une masse corporelle équivalente. La ventilation chez des tiques inactives était discontinue et caractérisée par des éruptions périodiques de CO_2 lors de l'ouverture des spiracles. L'avantage sélectif principal de ce type de ventilation serait une réduction de la perte d'eau respiratoire. La périodicité des émissions de CO_2 était moins fréquente chez *A.marmoreum* (toutes les 2,5 h) que chez *A.hebraeum* (toutes les 1,5 h), ce qui indiquerait que *A.marmoreum* est plus efficace pour limiter la perte d'eau respiratoire. Il est suggéré que des recherches futures sur la physiologie de l'équilibre d'eau de tiques devraient se pencher sur le rôle des profils de ventilation pour déterminer la survie des stades non-parasitaires et les associations des tiques avec leurs habitats.

Mots clés : Cowdriose - Tique - *Amblyomma hebraeum* - *Amblyomma marmoreum* - Échange gazeux - Métabolisme hydrique.

INTRODUCTION

The ability of ticks to survive for extended periods whilst of the host has important implications for the transmission of diseases. Long off-host survival not only increases the chances of finding a suitable host but also may result in ticks being the long-term reservoirs of disease in nature rather than the hosts they feed upon (6).

The long survival of ticks between blood meals is largely due to stringent use of energy and water. The mechanisms that ticks employ for water maintenance are diverse and include an integument which can dramatically restrict transpirational water loss as well as the ability to actively absorb water vapour from unsaturated air (2).

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Control of spiracular opening is also important in restricting water loss. Ticks possess only one pair of spiracles which in unfed adult ticks have been shown to open on an intermittent basis (2). These findings provide strong evidence for the occurrence of discontinuous ventilation in ticks (*i.e.* ventilation involving periodic bursts or emissions of CO_2 and intermittent uptake of O_2).

Discontinuous ventilation has been found to be wide spread in insects and its chief selective advantage is believed to lie in a reduction of respiratory water loss as a result of the loss of water vapour being restricted to discrete cyclic events (5). In ticks, however, the existence of discontinuous ventilation awaits experimental confirmation.

In this paper we confirm the existence of discontinuous ventilation in two species of ticks by direct measurement of respiratory gas exchange ; we compare the ventilatory patterns observed to those typical of insects and finally we discuss the significance of discontinuous ventilation with respect to the ability of ticks to survive for extended periods whilst off the host.

We investigated the dynamics of respiratory gas exchange in the unfed adult stages of the South African bont tick *Amblyomma hebraeum* Koch and the South African tortoise tick *Amblyomma marmoreum*. In Southern Africa, *A. hebraeum* is the main vector of the disease heartwater. *Amblyomma marmoreum* is also capable of transmitting the rickettsia but the significance of this species in the epidemiology of the disease heartwater remains unclear (9).

MATERIALS AND METHODS

Adult ticks (six-week-old) used in the experiments were from colonies maintained in the laboratory (25 °C, 85 % RH, ambient photoperiod).

Measurements of CO_2 emission were made at 25 °C using a flow through respirometry system described elsewhere (3). In brief, the system employed a Licor CO_2 analyser, respirometers of 3 ml volume, a computerized data acquisition system and flow rates of 50-100 ml. min^{-1} STPD. The incurrent air stream was scrubbed of H_2O and CO_2 by a Drierite/Ascarite column. Unfed ticks were

monitored for 8-16 h at a temporal resolution of 5-10 s. Respiratory quotients (RQ) were determined by a closed system technique (4).

RESULTS

Respiratory gas exchange in unfed adults of both species (fig. 1) was characterized by elevated and erratic CO_2 emission rates during activity and discontinuous ventilation during inactivity. Discontinuous ventilation consisted of short but clearly defined bursts of CO_2 emission (B phase) followed by much longer interburst phases where CO_2 emission was marginally above base line levels. Characteristics of discontinuous ventilation in *A. hebraeum* flat adults are summarized in table I and compared to those in *A. marmoreum*. For both species rates of burst CO_2 emission (burst VCO_2) and burst length were similar. Rate of interburst CO_2 emission in *A. marmoreum* accounted for approximately 11 % of total CO_2 emission during each discontinuous ventilation cycle (DVC). In *A. hebraeum*, CO_2 emission during the interburst period was seldom distinguishable from base line levels and hence was not measured. The major difference in ventilatory characteristics between the two species was the duration of the DVC, approximately 2.5 h in *A. marmoreum* compared to 1.5 h in *A. hebraeum*.

Standard VCO_2 ($sVCO_2$) of unfed ticks was calculated from the mean VCO_2 of the animal whilst exhibiting regular discontinuous ventilation. Measurements of $sVCO_2$ were converted to sVO_2 after determination of respiratory quotients (RQ's). Unfed adult *A. hebraeum* had a considerably lower SMR than *A. marmoreum* (table I).

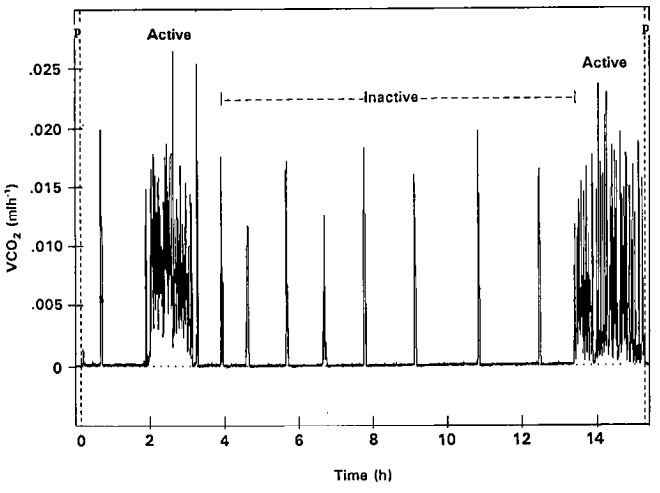

Figure 1 : CO_2 emission recording for male A. hebraeum *showing erratic and discontinuous ventilatory patterns. Baseline recording delineated by P.*

***TABLE I** Characteristics of the discontinuous ventilatory cycle in the unfed adults of two species of ticks.*

Species	*A. hebraeum*	*A. marmoreum*[1]
Mass (mg)	31.3 ± 9.9	70.2 ± 1.9
N	12 (6 M, 6 F)	5 (F)
VCO_2 (µl h^{-1})	0.416 ± 0.269	1.334 ± 0.228
VCO_2 (µl mg^{-1} h^{-1})	0.0136 ± 0.0085	0.0190 ± 0.0033
Burst VCO_2 (µl mg^{-1})	0.0262 ± 0.0104	0.0502 ± 0.0015
Burst VCO_2 (µl mg^{-1} h^{-1})	0.3288 ± 0.1701	0.419 ± 0.107
Interburst VCO_2 (µl mg^{-1})	**	0.0055 ± 0.0017
Burst length (min)	5.16 ± 1.03	6.78 ± 1.06
Burst frequency (h^{-1})	0.74 ± 0.26	0.49 ± 0.26
RQ	0.82	0.83
VO_2 (µl mg^{-1} h^{-1})	0.0158 ± 0.0097	0.0230 ± 0.0040

[1] *Values taken from Table 3 (*In *4).*
** *CO_2 emission undistinguishable from base line levels.*

DISCUSSION

In brief, the ventilatory cycle in insects consists of three stages : A closed phase (C phase) in which the spiracles are closed thus preventing any gas exchange or respiratory water loss. This stage is followed by a flutter phase (F phase) during which slight opening of the spiracles, on an intermittent basis, allows a slow ingress of O_2 but little egress of CO_2 or water vapour. Finally, in the open or burst phase (B phase), the accumulation of CO_2 from respiring tissues triggers some or all of the spiracles to open widely resulting in the rapid release of CO_2 and water vapour to the outside (5).

A continuous very low inter-burst emission of CO_2 does occur in ticks, similar to that described in insects during the constricted-spiracle (closed phase). However, unlike in insects there is no clearly defined increase in VCO_2 corresponding to the F phase occurring prior to the burst phase. This suggests that if an F phase exists in ticks, it must be characterized by extremely low rates of gaseous exchange because it cannot be clearly distinguished via diffusive loss of CO_2 through the spiracles.

It has recently been suggested (4) that ticks probably do not exhibit a fluttering-spiracle (F) phase of the kind observed in insects but instead may rely primarily on diffusive uptake of O_2 through the cuticle between burst phases. This diffusive uptake of O_2 through the cuticle appears sufficient for non-active respiration for prolonged periods, although regular release of CO_2 is still necessary. If this is the case then the standard metabolic rate of ticks can be expected to be very low compared to that of insects which are not thought to make significant use of cuticular O_2 ingress.

The allometric equation describing the relationship between insect body mass and standard metabolic rate is given below :

$$MR = 11.3M^{0.67} \quad (10)$$

where MR is metabolic rate (jh^{-1}), and M is body mass (g).

The metabolic rates of the unfed stages of ticks are much lower than that predicted on the basis of body mass from data on insects. Metabolic rates of *A. hebraeum* and *A. marmoreum* are only 0.8 and 1.4 % (2.3 %) respectively of that predicted for insects of an equivalent body mass. Considering that metabolic rates of the unfed stages of ticks are much lower than that predicted for insects of a similar size, the role of transcuticular diffusion may indeed play an important role in ixodid tick gas exchange. It should also be noted that unfed adult ixodids, due to dorso-ventral flattening, must have a large surface area to volume ratio compared to most insects of similar body size. Although this feature makes ticks more prone to desiccation, it would presumably increase their capacity for diffusive gas exchange across the integument. However, we still lack the required information on actual tick surface areas and cuticular O_2 permeabilities to test the hypothesis that transcuticular diffusion may play an important role in ixodid respiratory gas exchange.

There is little doubt that the discontinuous ventilation in ticks is important in reducing respiratory water loss during the period between moulting and feeding. Previous workers have demonstrated dramatic increases in water loss in various species of ticks in which the spiracles have been forced to remain open by exposure to high CO_2 concentrations (1,2).

Since respiratory water loss is minimal during spiracle closure (interburst phase), but high when the spiracles are open (burst phase), one can postulate that burst frequencies in any particular species of tick may give some indication as to its ability to reduce respiratory water loss. During regular discontinuous ventilation cycles, the burst frequency of *A. hebraeum* was on average once every 1.5 h which is much higher than the burst frequencies of once every 2.5 h found for *A. marmoreum*. Such low rates of spiracular opening in *A. marmoreum* would suggest that this species can more effectively reduce respiratory water loss than *A. hebraeum*.

CONCLUSION

Previous reviews of the physiological ecology of ticks have emphasized that survival between blood meals is a function of maintaining a balance between energy and water use with the maintenance of water homeostasis being considered the most critical survical factor (7). Furthermore it has been suggested that integumental permeability to water flux may be of greater value to maintenance of water balance of ixodid ticks than the critical equilibrium activity (2). In the light of our findings, we suggest that ventilatory patterns in ticks and the role they play in reducing respiratory water loss should be given more attention when trying to interpret survival potential and habitat associations of ixodid ticks. The two species under consideration in this study, *A. hebraeum* and *A. marmoreum*, are largely sympatric in their distribution in Southern Africa although A. marmoreum is found in drier areas than *A. hebraeum* (8). The apparent superior ability of *A. marmoreum* to reduce respiratory water loss may be of significance in explaining its tolerance to drier habitats than *A. hebraeum*.

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The objective of this study was to establish the major features of respiratory gas exchange in unfed adults of the ticks *Amblyomma hebraeum* and *A. marmoreum*, both vectors of heartwater in Southern Africa. Carbon dioxide emission of ticks was measured at 25 °C using flow-through respirometry in order to determine standard metabolic rate (SMR) and the temporal pattern of gaseous emission. For both species, SMR was extremely low and approximately 100 fold less than that predicted for an insect of equivalent body mass. Ventilation in inactive ticks was discontinuous and characterized by periodic bursts of CO_2 emissions during spiracular opening. The main selective advantage of this type of ventilation is believed to lie in a reduction of respiratory water loss. The periodicity of CO_2 bursts was less frequent in *A. marmoreum* (every 2.5 h) compared to *A. hebraeum* (every 1.5 h) suggesting that *A. marmoreum* is more efficient at conserving respiratory water loss. It is suggested that future research into water balance physiology of ticks should address the role of ventilatory patterns in determining off-host survival and habitat associations.

Key words : Heartwater - Tick - *Amblyomma hebraeum* - *Amblyomma marmoreum* - Gas exchange - Water metabolism.

FIELDEN (L.J.), DUNCAN (F.D.), LIGHTON (J.R.B.), RECHAV (Y.). Ventilación en los adultos de *Amblyomma hebraeum* y *A.marmoreum* (Acarina, Ixodidae), vectores de la cowdriosis en Africa del sur. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 335-338

El objetivo del presente estudio fue el establecimiento de los principales patrones respiratorios de intercambio gaseoso en adultos no alimentados de garrapatas de *A.mblyomma hebraeum* y *A. marmoreum*, ambos vectores de la cowdriosis en Africa del sur. La emisión de dióxido de carbono por parte de las garrapatas se midió a 25 °C, haciendo pasar el flujo por un respirómetro, con el fin de determinar la tasa metabólica estandard (SMR) y el patrón temporal de emisión gaseosa. En ambas especies la SMR fue extremadamente baja, aproximadamente 100 veces menor que la esperada en un insecto de masa corporal equivalente. La ventilación en las garrapatas inactivas fue discontinua y se caracterizó por picos periódicos de emisión de CO_2 durante la apertura estigmática. Se cree que la principal ventaja selectiva de este tipo de ventilación reside en una reducción de la pérdida de agua mediante la respiración. La periodicidad de los picos de CO_2 fue menos frecuente en *A. marmoreum* (cada 2,5) que en *A. hebraeum* (cada 1,5), lo que sugiere una mayor eficiencia de la primera en la conservación respiratoria de agua. Se recomienda que las investigaciones futuras sobre el balance fisiológico del agua en las garrapatas se orienten hacia el posible papel de los patrones de ventilación en la determinación de la supervivencia fuera del huesped y de las asociaciones con el medio ambiente.

Palabras claves : Cowdriosis - Garrapata - *Amblyomma hebraeum* - *Amblyomma marmoreum* - Intercambio de gases - Metabolismo del aguá.

Immunization of laboratory hosts against *Amblyomma hebraeum* and *Amblyomma marmoreum* (Acari : Ixodidae)*

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RECHAV (Y.), TEMBO (S.D.). Immunisation d'animaux de laboratoire contre *Amblyomma hebraeum* et *Amblyomma marmoreum* (Acari : Ixodidae). *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 339

Des nymphes à jeun d'*Amblyomma hebraeum* et d'*Amblyomma marmoreum* ont été inoculées, après homogénéisation, à des lapins. Les animaux ont développé une résistance aux infestations par l'espèce homologue. Le taux de gamma globulines et le nombre des éosinophiles ont augmenté de façon significative chez les animaux inoculés. La période pendant laquelle les nymphes des deux espèces se sont nourries a été influencée et le poids moyen des nymphes nourries sur des animaux inoculés était significativement plus bas que celui de nymphes nourries sur des lapins inoculés avec de l'adjuvant ou sur des lapins naïfs. Les résultats ont montré que les lapins inoculés ont acquis une résistance contre les nymphes des deux espèces. La possibilité d'utiliser des tiques homogénéisées pour l'immunisation de bovins est examinée.

RECHAV (Y.), TEMBO (S.D.). Immunization of laboratory hosts against *Amblyomma hebraeum* and *Amblyomma marmoreum* (Acari : *Ixodidae). Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 339

Homogenates prepared from unfed nymphs of *Amblyomma hebraeum* and *Amblyomma marmoreum* were used for inoculating rabbits. The recipient hosts developed resistance to homospecific infestations. The level of gamma globulins and the number of eosinophils increased significantly in the inoculated animals. The feeding periods of nymphs of both species were affected and the mean weight of nymphs fed on inoculated animals was significantly lower than that of nymps fed on adjuvant injected rabbits or naive rabbits. The results demonstrated that inoculated rabbits acquired protective immunity against nymphs of both species. The possibility of using homogenates for immmunization of cattle is discussed.

RECHAV (Y.), TEMBO (S.D.). Inmunización de animales de laboratorio contra *Amblyomma hebraeum* y *Amblyomma marmoreum* (Acari : Ixodi*dae). Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 339

Se utilizaron preparaciones homogéneas de *Amblyomma hebraeum* y *Amblyomma marmoreum*, provenientes de ninfas no alimentadas, para la inoculación de conejos. El huésped receptor desarrollo una resistencia a las infestaciones homo-específicas. El nivel de gama globulinas, así como el número de eosinófilos aumentó significativamente en los animales inoculados. Los períodos de alimentación de las ninfas de ambas especies variaron. El peso promedio de las ninfas alimentadas en animales inoculados, fue significativamente menor que aquel de las ninfas alimentadas en conejos control, inyectados con adyuvante. Los resultados demuestran que los conejos inoculados adquieren una inmunidad protectora contra las ninfas de ambas especies. Se discute la posibilidad de usar estas preparaciones para la inmunización de ganado.

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* *Seuls les résumés de cette communication sont publiés dans ce volume.*

Variability of cattle infestation by *Amblyomma variegatum* and its possible utilisation for tick control

F. Stachurski [1]

STACHURSKI (F.). Variabilité de l'infestation de bovins par *Amblyomma variegatum* et son utilisation possible pour la lutte contre cette tique. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 341-348

De grandes différences dans l'infestation individuelle par les adultes d'*Amblyomma variegatum* ont été observées chez des zébus Goudali infestés naturellement. Certains animaux (désignés comme "attractifs pour *A. variegatum*") portaient 10 à 16 fois plus de tiques que les bovins les moins parasités du troupeau (désignés comme "non-attractifs"). L'ordre des animaux, selon l'infestation par *A.variegatum*, se maintenait lors de comptages successifs de tiques. Des expériences ont été conçues afin de savoir si les animaux "non-attractifs" restent peu infestés après que les animaux "attractifs" soient retirés du troupeau. Lorsque les deux catégories de bovins ont été mises à pâturer séparément, il a été observé que les animaux "non-attractifs" portaient moins de tiques et étaient infestés plus lentement que les animaux "attractifs". Toutefois, la différence entre les deux groupes était moins grande que lorsqu'ils étaient ensemble dans un seul troupeau. La sélection d'animaux "non-attractifs", bien que ne pouvant pas être la base unique d'un programme de lutte anti-tiques, pourrait être un des composants d'une stratégie de lutte, si ce caractère est héréditaire. Une expérience en cours étudie cette question.

Mots clés : Bovin - Zébu Goudali - Tique - *Amblyomma variegatum* - Infestation - Lutte antiacarien - Cameroun.

INTRODUCTION

Australian researchers have demonstrated the heredity of cattle resistance to the one-host tick *Boophilus microplus* (3), and have subsequently selected resistant animals (1). In Africa, the majority of the most pathogenic tick species are multi-host ticks. Several authors, working in different countries with various cattle breeds, have observed the existence of important variations of individual tick infestations between animals raised in the same herd. This was noted for *Rhipicephalus appendiculatus* (4, 7), *Amblyomma variegatum* (4) and *A. hebraeum* (8). On several occasions, it was also observed that animals heavily infested by one particular tick species were equally highly parasitized by the other species present in the area (4, 10, 5). It was then suggested that tick control could be improved by culling the most infested cattle and/or selecting the resistant ones (4, 10, 11).

In the Province of Adamawa, Cameroon, the most harmful tick is *Amblyomma variegatum* (12). Preliminary observations made at the Wakwa Animal and Veterinary Research Centre (CRZV Wakwa) showed that there was an important variability of the individual infestation by *A. variegatum* in a herd, and that the infestation rank was consistent between successive tick counts. These observations are nevertheless insufficient to envisage the possibility of an eventual selection programme. Other experiments are needed to answer the following questions :

- will lightly infested cattle continue to harbour few ticks after the culling of the most infested ones ?

- is the *Amblyomma variegatum* infestation level of an animal hereditary ?

If not, selection of the least infested cattle may not have a great impact on the status of the herd, and should not be integrated in a tick control strategy. Experiments were carried out at the CRZV Wakwa to study the first point, the persistence of a low infestation of the previously lightly parasitized cattle after the removal of the most infested ones.

MATERIALS AND METHODS

The Wakwa Animal and Veterinary Research Centre is situated 10 km from Ngaoundere at an altitude of 1,200 m on the Adamawa plateau in Cameroon. The climate is characterized by a mean annual rainfall of 1,700 mm over seven and half months, from March to October, with a mean temperature of 22 °C (9). All experiments described below took place in 1990, 1991 and 1992, during the early rainy season, between May and July, which is the peak infestation period for *A. variegatum* adults (12).

The local Gudali zebu cattle breed was used. Eleven herds were involved in the preliminary trials, which allowed the demonstration of the variability of individual infestations. They comprised eight 2-3 year old males (herd O), ten 3-4 year old males (herds V and J), twenty 1-2 year old heifers (herd G) and reproductive cows older than 4 years (in seven herds containing 12 to 24 animals).

To assess the variability of the infestation by adult *A. variegatum* between animals of a herd, the protocol followed was the same for all herds. Each herd was treated against ticks with an acaricide, two times at seven to ten days interval*. Two to three weeks after the second treatment, depending on the rapidity of reinfestation (estima-

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* These trials, except those involving the reproductive females, were originally designed to determine the residual effect of the acaricides used (F.STACHURSKI and E.N.MUSONGE, unpublished data).

ted by intermediate tick counts), all the animals of the herd were caught and their infestation was determined by counting *A. variegatum* adults present on the whole body. Then, they were treated again twice at about one week interval with another acaricide, and a new trial was performed, until the infestation level of the pasture was too low to allow new studies. The infestation of each herd could be checked 2 to 4 times.

The infestation level of each animal was characterized by two criteria, the infestation rank in the herd (IR) and the infestation degree (ID). This latter parameter represents the ratio between the infestation of the animal and the mean infestation of the herd in which it was kept.

A second series of experiments was then carried out to determine whether the removal of highly infested cattle would lead to an increased infestation of previously lightly parasitized animals. Two pastures of 2.25 ha (150 m x 150 m) and two of 4.5 ha (300 m x 150 m), separated from each other by 5 meters wide passages, were established as shown in figure 1.

Two groups of animals having either a low or a high infestation degree (ID) were held separately on some of these pastures after the following 14 days preparation. They were first treated with flumethrin (Bayticol®), mean residual effect of which had been previously estimated to be 9 days. Seven days later, they were treated with amitraz (Taktic®, observed residual effect 3 to 5 days). Four days later and during four subsequent days, the animals were carefully checked every morning, and all the ticks present were manually removed. When the cattle were then put into the paddocks, they carried neither acaricide (time interval since the last treatment exceeding duration of residual effect) nor aggregation-attachment pheromone (secretion of that pheromone does not start before 3 days after the fixation of the males and its persistence on the cattle is lower than 10 days (2)).

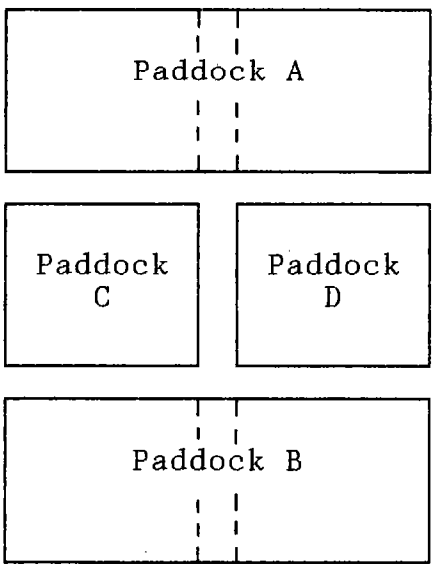

Figure 1 : Diagram of paddocks used in the second series of experiments.

The first trial was carried out with three bulls having a high ID and three with a low ID, chosen from herds V and J. It was impossible to hold the animals separately, one per paddock, as planned (in that case, paddocks A and B would have been divided into two smaller pastures). Two groups were thus constituted, containing either animals with a high ID (group H) or animals with a low ID (group L). In a first phase, during three weeks, group H animals grazed on paddock A and group L on paddock B. Then, after the two weeks preparation described above and during three subsequent weeks, group H grazed on paddock B and group L on paddock A.

Preliminary results indicated that the initial infestation of paddock B was about two times that of paddock A. To avoid interpretation difficulties due to this situation, the protocol of further trials was modified, and groups H and L were allowed to graze successively, in rotation, on the different paddocks, in the following order: A, D, B, C. They were moved from one paddock to the next each morning, and the two groups were never on the same pasture at any moment. They were thus subjected to the same "tick pressure", but they had no influence on each other.

According to this protocol, three more trials were carried out. The first was performed with the same six bulls. The second involved four of those animals (one group with two low ID zebus, the other with two high ID bulls). The third trial was carried out with 2 groups of heifers, chosen from the herd G. The first group contained three low ID females, and the second three high ID ones.

RESULTS

Magnitude of the variability of the infestation by *Amblyomma variegatum* adults between animals

The weighted mean of the successive tick counts, as well as the corresponding ID, were calculated for each animal. Table I shows this latter parameter for the animals with the higher and the lower infestation in each herd. The ratio between those two IDs indicates that it was not uncommon to find animals carrying at least 10 times more ticks than the least infested zebu of the herd. In other herds however, the differences amongst the animals were much smaller.

The percentage of the herd carrying 50 % of the tick population varied from 23 to 42 % depending on the herd. On the average, about 30 % of the animals carried half of the ticks.

TABLE I Maximum and minimum infestation degrees observed in the experimental herds ; percentage of the herd carrying half of the tick population.

Herds	Number of animals	Sex	Infestation degrees		M/m	Percentage of the herd carrying half of the ticks
			M	m		
V	10	M	1.68	0.45	3.7	33
J	10	M	1.39	0.65	2.1	42
O	8	M	2.87	0.38	7.6	26
G	20	F	1.77	0.11	16.1	35
E1	20	F	2.08	0.20	10.2	31
E2	19	F	2.18	0.19	11.5	33
E3	16	F	1.93	0.26	7.4	32
E5	16	F	2.07	0.46	4.5	35
R	12	F	1.82	0.39	4.7	33
V1	18	F	2.09	0.39	5.4	33
V3	24	F	2.80	0.28	10.0	23

Infestation degree (ID) : infestation of the animal / mean infestation of the herd.
M : maximum ID observed in the herd.
m : minimum ID observed in the herd.

Figure 2 shows the distribution of the ID of the 173 zebus observed during all trials. Although some animals had very high or very low infestations (the ratio between the two extreme IDs was 26), in general the distribution was not very skewed. Only 22 animals (13 %), infested by 4.7 % of the ticks, had an ID lower than 0.5 (that is they carried less than half the average infestation) and, on the other hand, 25 zebus (14 %) carrying 28.2 % of the tick population, had an ID higher than 1.5.

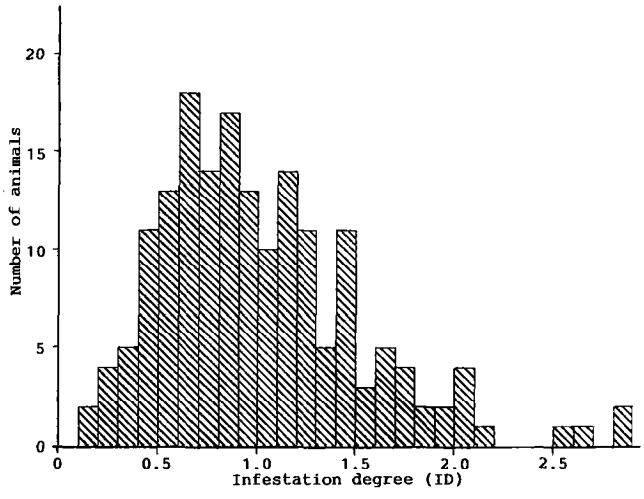

Figure 2 : Distribution of the infestation degree (ID) of the 173 cattle observed during the different trials.

Persistence of the individual tick infestation hierarchy

To assess the repeatability of the individual infestations, correlations between the infestation ranks (IR) of the successive trials were determined, by Spearman's rank correlation. Tick counts were done on four occasions for herds J and O, on three occasions for herd V and on two occasions for all the female herds. Correlations between the infestation degrees were also evaluated, by simple linear regression. The results for the male herds are presented in table II, those for the female herds in table III.

For all the herds, except one (herd J), the IRs and the IDs are correlated from one tick count to the other. The same animals are thus generally the least infested or, on the contrary, the most parasitized.

Effect of segregation of low and high ID animals on their infestation

As described above, the first experiment was carried out with two groups of three bulls : group H containing three high ID animals and group L constituted of three low ID ones. During the first phase, a tick count was done after 3 days. There were already 34 to 76 *A. variegatum* males on the animals, but only 1 to 3 females, showing that the zebus were free of pheromone when put into the paddocks. The results of the tick counts done during the two phases after 7, 14 and 21 days are reported in table IV.

TABLE II Correlations between the infestation ranks and the infestation degrees observed during the successive tick counts (TC) with the male herds.

Herds		Coefficient of correlation between					
		Infestation degrees (ID)			Infestation ranks (IR)		
		TC2	TC3	TC4	TC2	TC3	TC4
V	TC1 TC2	0.84***	0.86*** 0.86***		0.81***	0.79*** 0.83***	
J	TC1 TC2 TC3	0.88****	0.24 NS 0.51 NS	0.38 NS 0.63* 0.66*	0.87***	0.20 NS 0.49 NS	0.26 NS 0.54 NS 0.51 NS
O	TC1 TC2 TC3	0.93***	0.86*** 0.92***	0.80** 0.89*** 0.96****	0.97****	0.78** 0.74**	0.78** 0.79** 0.90***

*NS : Not significant ; * : p < 0.05 ; ** : p < 0.025 ; *** : p < 0.01 ; **** : p < 0.001.*

TABLE III Correlations between the infestation ranks and the infestation degrees observed during the two successive tick counts with the female herds.

Herds	Coefficient of correlation between	
	Infestation degrees (ID)	Infestation ranks (IR)
G	0.60***	0.52**
E1	0.72****	0.66***
E2	0.87****	0.80****
E3	0.78****	0.79****
E5	0.88****	0.85****
R	0.76***	0.67**
V1	0.74****	0.63***
V3	0.87****	0.77****

*** : p < 0.025 *** : p < 0.01 **** : p < 0.001*

It was obvious that the infestation was more important during the first phase than during the second, when the number of ticks observed was 4 to 8 times lower. It was also evident that there was an important pasture effect, the herd grazing on paddock B having consistently a higher tick burden. Because of this bias, the direct comparison of the animal infestations was impossible. To allow the comparison between the two phases, the cattle tick burden was expressed by the ratio between their own infestation and the mean infestation of the six animals, at each tick count. The results are shown in table IV, in brackets.

The trial was a 2 x 2 factorial design, the first factor being the paddock and the second the herd. Analysis of variance, done for each tick count, confirmed that there was a significant difference between the tick burden of the two paddocks ($p = 0.019$, $p < 0.001$ and $p = 0.003$ respectively for the D7 (day 7), D14 and D21 counts). On the other hand, the difference between the infestation of the two herds, significant at D7 ($p = 0.028$), later on became non significant ($p = 0.058$ at D14 and $p > 0.10$ at D21).

Figure 3 represents the evolution of the mean infestation of the two groups, H and L, during the two phases. When herd H, containing the high ID animals, was on the least infested pasture (phase 1), its infestation was, after one week, almost similar to that of herd L, which grazed on the most infested paddock. Afterwards, the difference between the two groups increased. On the other hand, when herd H was kept on the most infested pasture, its infestation was, at the beginning, 4 times higher than that of the other herd. The difference then decreased. Low ID animals were more able to limit their tick burden at the beginning of the infestation.

Because of the rapid decrease of the pasture infestation and because of the difference between the paddocks tick burden, which rendered difficult the comparison of the infestations, another protocol was used for the three further experiments (see above). The results from those trials are shown in tables V to VII. Direct comparison of the infestation of the two groups involved was possible because they were simultaneously infested and subjected to the same "tick pressure". The same following observations were made for the three trials.

TABLE IV **Amblyomma variegatum** *adults infestation (in brackets, expressed by the ratio between the animal infestation and the mean infestation of the 6 cattle) observed 7, 14 and 21 days after the beginning of the experiment in the groups H and L, containing respectively high ID bulls and low ID ones. The two herds grazed successively on the two paddocks.*

			Animals	Infestation by *A. variegatum* adults		
				D7	D14	D21
First phase	Paddock A	Group H	10 19 29	132 (1.01) 139 (1.06) 111 (0.85)	158 (0.73) 175 (0.81) 172 (0.80)	178 (0.69) 192 (0.75) 201 (0.78)
	Paddock B	Group L	13 15 16	85 (0.65) 183 (1.39) 138 (1.05)	207 (0.96) 315 (1.46) 266 (1.23)	253 (0.98) 406 (1.58) 313 (1.22)
Second phase	Paddock B	Group H	10 19 29	30 (1.88) 14 (0.88) 33 (2.06)	55 (1.31) 58 (1.39) 64 (1.53)	70 (1.08) 79 (1.22) 114 (1.76)
	Paddock A	Group L	13 15 16	7 (0.44) 5 (0.31) 7 (0.44)	21 (0.50) 30 (0.72) 23 (0.55)	35 (0.54) 55 (0.85) 35 (0.54)

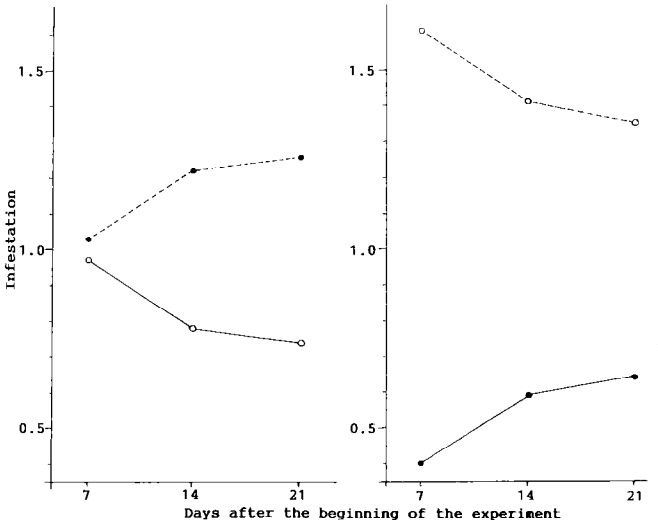

Figure 3 : Evolution of the mean infestation of the L (•) and H (o) groups, grazing on paddock A (-) or B (--), during the first (left) and second (right) phases. The infestation of each animal is expressed by the ratio between the individual tick burden and the mean infestation of the 6 zebus.

The infestation of group L, which always contained the low ID animals, remained lower than that of group H. Because of the small number of animals involved, the difference between the mean infestation of the two groups is significant only when the standard deviations of the means are low. In the second trial for example (table VI), the difference between the groups was higher at D31 than at D28, but it was less significant because of the higher standard deviation.

TABLE V **Amblyomma variegatum** *adults infestation (infestation degree — ID — calculated in comparison with the mean infestation of the 6 animals) observed 7, 14 and 21 days after the beginning of the experiment in the group H and L, containing respectively high ID bulls and low ID ones. The herds grazed in rotation on all the paddocks.*

Animals		Infestation by *Amblyomma variegatum* adults		
		D7	D14	D21
Group H	10 19 29	18 (1.17) 18 (1.17) 18 (1.17)	40 (1.17) 48 (1.40) 37 (1.08)	40 (0.92) 51 (1.18) 47 (1.08)
Group L	13 15 16	12 (0.78) 17 (1.11) 9 (0.59)	22 (0.64) 36 (1.05) 22 (0.64)	40 (0.92) 48 (1.11) 34 (0.78)
Means comparison		p = 0.084	p = 0.058	p > 0.10

The difference between the mean infestation of the two groups is lower than that observed when the animals were held in the same herd. Thus, the mean IDs observed during the last trial (table VII) were 1.30 for group H and 0.70 for group L, although they were respectively 1.55 and 0.39 when the animals were together in herd G. The same observation was made with the males involved in the other trials.

TABLE VI **Amblyomma variegatum** *adults infestation (infestation degree — ID — calculated in comparison with the mean infestation of the 4 animals) observed during the course of the experiment in the group H and L, containing respectively high ID bulls and low ID ones. The herds grazed in rotation on all the paddocks.*

Animals		Infestation by *Amblyomma variegatum* adults								
		D6	D9	D12	D15	D18	D21	D25	D28	D31
Group H	19	59	76	106	122	139	142	161	159	182
	29	44	80	92	108	134	144	157	160	160
Group L	13	24	49	74	98	92	114	118	126	118
	16	35	48	86	100	112	120	124	128	124
Means comparison		p > 0.10	p = 0.003	p > 0.10	p > 0.10	p = 0.078	p = 0.011	p = 0.006	p < 0.001	p = 0.046

TABLE VII **Amblyomma variegatum** *adults infestation (infestation degree — ID — calculated with the mean infestation of the 6 animals) observed during the course of the experiment in the group H and L, containing respectively high ID heifers and low ID ones. The herds grazed in rotation on all the paddocks.*

Animals		Infestation by *Amblyomma variegatum* adults						
		D6	D12	D18	D21	D24	D28	D31
Group H	60	5	15	16	17	20	23	24
	72	6	14	17	19	22	24	25
	78	3	7	12	18	24	27	24
Group L	59	2	3	10	11	17	16	15
	68	1	4	8	10	10	13	11
	76	3	3	7	14	18	22	18
Means comparison		p = 0.060	p = 0.027	p = 0.018	p = 0.009	p = 0.066	p = 0.058	p = 0.009

There was a tendency, as illustrated in figure 4, for the difference between the mean IDs of the two groups to decrease during the course of the experiment. This difference was always higher during the first two weeks of the trials than during the subsequent weeks.

DISCUSSION

Because of the sometimes very variable pasture tick burden, it is impossible to directly compare the infestation levels of animals kept in various herds on different pastures. The use of the infestation degree (ID) allows such a comparison, although it is certainly not a perfect criterion, because there is no proof that the mean infestation of different herds would reach the same level on the same pasture. However, this criterion allows the identification of lightly and highly infested animals.

The variability of the infestation between animals was very high in some herds and very limited in others. The percentage of the herds carrying half of the tick population (30 %) was higher than the 25 % observed in Uganda by KAISER *et al.* (4) with the same tick species (*A.variegatum*). Nevertheless, the ID range was very large, several zebus harbouring at least 10 times more ticks than their partners, and the ratio between the two extreme IDs being 26. As soon as the variability of the individual infestation in a herd is high enough, correlations between infestation ranks and infestation degrees were observed between successive tick counts. The absence of correlation in herd J is thus in relation with the low differences observed between the individual infestations in that herd: the ratio between the two extreme IDs was only 2.1, and half of the tick population was found on 42 % of the herd.

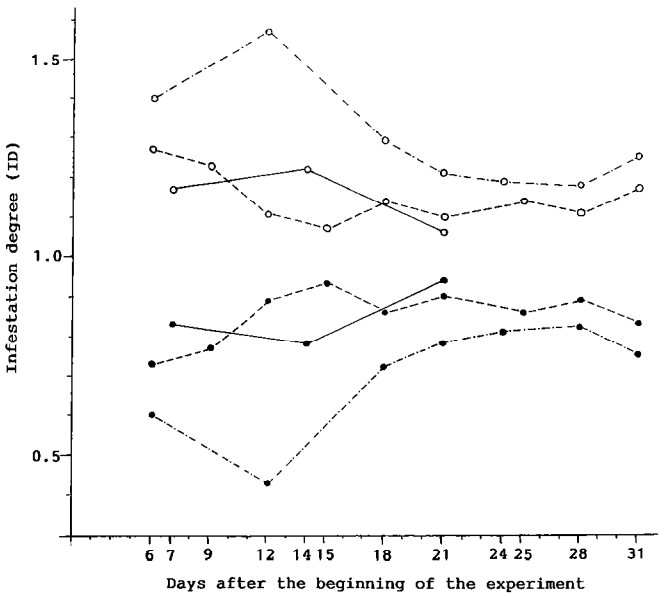

Figure 4 : Evolution of the mean degree (ID) of the L (•) and H (o) groups during the first (-), second (--) and third (-.-.-) trials, realized following the modified protocol, the herds grazing on all the pastures in rotation.

These observations (great variability of the individual infestations and consistency of the infestation levels) are favourable for the possibility of a selection procedure to be undertaken, as already stated by KAISER *et al.* (4), PEGRAM *et al.* (10) and SPICKETT *et al.* (11). However, the consistency in infestation hierarchy observed in the present experiments may be explained by other reasons.

Tick counts were actually always done after an acaricidal treament. Although the active components were various (amitraz and pyrethroids like flumethrin, deltamethrin, cypermethrin), it is possible that the residual effect of those acaricides may be more important on certain skin types, *i.e.* on certain animals, which may have always carried fewer ticks.

Similarly, since the trials followed one another rapidly, it is possible that the pheromone produced by male *A. variegatum* attached during a trial, may be still present when the next trial started. Assuming that the attractive power of the pheromone depends on its quantity, this could explain why the most infested animals were always the same.

A third reason could be the cause of the persistence of the infestation hierarchy. NEWSON *et al.* (6) observed that the hierarchical position of an animal in a herd, in particular during grazing, influenced the number of *Rhipicephalus appendiculatus* that it carried, cattle being in the front of the herd harbouring the most ticks. The same may be true of *A. variegatum*. In that case, after the removal of the most infested zebus, other animals would graze at the front of the herd and would be in turn the most infested. The selection of the lightly parasitized animals would then be useless.

To examine these possibilities, experiments involving a group of low ID animals and a group of high ID ones were designed. Because of the 14 days preparation that the animals under-went, they were free of pheromone and of acaricide when put into the paddocks. Since they grazed on different pastures, there was no competition between the two groups, and the eventual previous hierarchical positions in the herd were modified. With these precautions, it was observed that the low ID group remained less infested than the high ID one.

But, during the experiments carried out with the two groups of three bulls, it was observed that, in group L, it was always the same animal (n°15) which harboured the highest number of ticks (tables IV and V). It was even more infested than animals of H group on certain occasions. This animal certainly had the highest initial ID of the three low ID animals. Nevertheless, the fact that it behaved like a high ID animal, after the removal of the latter, indicates that there may be an environmental component (or behavioural) to the infestation level of the animals. It also could lead one to think that the selection of lightly infested animals would not entail a great decrease of the herd infestation. Nevertheless, after the removal of that bull (table VI) and during the last trial (table VII), the infestation of L animals remained lower, showing that the infestation level also has an innate component.

It seemed that the low ID cattle were more able to limit their infestation, even on a very infested paddock, at the beginning of the experiments, and that this possibility decreased after the fixation of the first *A. variegatum* males. Once these latter produce the aggregation-attachment pheromone, low ID cattle seemed to be less able to avoid the fixation of further ticks. For this reason, the most infested cattle, those with a high ID, are called "attractive for *Amblyomma variegatum*", and the others, with a low ID, "non-attractive for *A. variegatum*".

The difference between the infestation of L and H groups was less important than that observed when the animals grazed together. But the higher the difference between the IDs of animals when they were in the same herd, the higher it remained when the animals were kept separately. For this reason, and despite the above reservations, selection of cattle "non-attractive for *A. variegatum*" should be useful for tick control.

CONCLUSION

It was shown that the cattle "non-attractive for *A. variegatum*" remained less infested than the "attractive" ones when they grazed separately. The difference between the infestation of the two groups was, however, lower than

that observed when the animals grazed together. In addition, the ability of the "non-attractive" cattle to limit their infestation decreased during the course of the experiments. For these reasons, a tick control programme could probably not be based exclusively on the selection of such "non-attractive" cattle. Nevertheless, their selection and their breeding could be part of a tick control strategy. For example, the slower reinfestation of such zebus after acaricidal treatment would allow the increase of the interval between treatments, and would lead to less expensive tick control. In addition, it was observed during all these studies (unpublished data) that the "non-attractive" animals are less often affected by dermatophilosis lesions. Their selection would thus facilitate the control of this important disease.

But, before such a selection programme can be implemented, a last point has to be explored. Is the "non-attractivity for *Amblyomma variegatum*" a hereditary characteristic ? A study involving some of the low ID and high ID reproductive cows and bulls mentioned above has just started, and an answer to that question is expected in 1994.

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STACHURSKI (F.). Variability of cattle infestation by *Amblyomma variegatum* and its possible utilisation for tick control. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 341-348

A great variability of the individual infestation by *Amblyomma variegatum* adults was observed on naturally infested Gudali zebus. Some of the animals (called "attractive for *A. variegatum*") had a tick burden 10 to 16 times higher than that of the least parasitized cattle of the herd (called "non-attractive"). Ranking of the animals based on *A. variegatum* infestation was correlated for successive tick counts. Experiments were designed to determine if the "non-attractive" cattle remained lightly infested when the "attractive" ones are removed from the herd. When these two types of cattle grazed separately, it was observed that the "non-attractive" animals had a lower tick burden and that their infestation occurred more slowly than that of the "attractive" ones. The difference between the two groups was nevertheless smaller than that existing when the animals were in the same herd. The selection of the "non-attractive" cattle, on which a tick control programme should not exclusively be based, could however be used as a component of a tick control strategy, if this characteristic is hereditary. An experiment in progress will study the question.

Key words : Cattle - Gudali zebu cattle - Tick - *Amblyomma variegatum* - Infestation - Tick control - Cameroon.

STACHURSKI (F.). Variabilidad de la infestación del ganado con *Amblyomma variegatum* y su posible utilización para el control de garrapatas. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 341-348

Se observó una gran variabilidad en cuanto a la infestación individual con adultos de *Amblyomma variegatum* en cebués Gudali infestados en forma natural. Algunos de los animales (llamados "atractivos para *A. variegatum*") presentaron una carga parasitaria 10 a 16 veces superior que la de los animales de menor parasitosis en el hato (llamados "no atractivos"). Se encontró una correlación entre la clasificación de los animales, basada en la infestación con *A. variegatum* y los números sucesivos de garrapatas. Los experimentos se diseñaron para determinar si el ganado "no atractivo" permaneció ligeramente infestado cuando aquellos animales "atractivos" fueron eliminados del hato. Cuando estos dos grupos de animales estuvieron en grupos de pastoreo separados, se observó que los animales no atractivos presentaron una carga parasitaria menor y que la infestación se dió más lentamente que en los animales atractivos. Sin embargo, la diferencia entre estos dos grupos fue menor que la existente cuando estos se encontraban reunidos en un solo grupo de pastoreo. La selección del ganado no atractivo, para el cual no es necesario poner en marcha un programa específico de control de garrapatas, podría sin embargo utilizarse como una estrategia de control, siempre y cuando esta característica sea hereditaria. Este punto se estudia actualmente en otro proyecto.

Palabras claves : Bovino - Cebú Gudali - Garrapata - *Amblyomma variegatum* - Infestación - Control de garrapata - Camerún.

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Acaricides for eradication of the tick *Amblyomma variegatum* in the Caribbean

BARRÉ (N.), GARRIS (G.), APRELON (R.). Acaricides pour l'éradication de la tique *Amblyomma variegatum* dans les Antilles. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 349-354

Le succès d'une campagne d'éradication de la tique *Amblyomma variegatum* dans les Caraïbes impose l'utilisation d'acaricides efficaces, si possible rémanents, faciles d'emploi, ne nécessitant pas le recours à de l'eau pour la préparation de liquides dilués, ni à des équipements coûteux pour le transport et l'application. Des tests de sensibilité *in vitro* conduits sur des souches de Porto Rico et de Guadeloupe ainsi que l'observation de l'impact des campagnes de détiquage conduites dans les Caraïbes semblent indiquer qu'il n'y a pas dans la région de problèmes de résistance aux acaricides. Les pyréthrinoïdes ont l'avantage d'être actifs à très faible concentration et d'être peu toxiques pour l'environnement. Certains sont rémanents. La formulation "pour-on" (application topicale dorsale) permet une application rapide et une diffusion du produit sur tout le corps. Il n'y a pas de délai d'attente. Cependant, des améliorations doivent être apportées pour faciliter l'application chez les bovins qui, aux Antilles, sont en majorité élevés à l'attache. Par exemple, l'application en "spot-on" est plus adaptée qu'en "pour-on". Pour les petits ruminants et les chiens, mais aussi pour les bovins, des dispositifs à diffusion lente imprégnés d'acaricide permettraient de réduire la fréquence d'intervention. Cependant, des essais sur des chèvres avec des colliers à la fluméthrine indiquent une activité de moins de 55 jours, insuffisante pour justifier leur emploi à grande échelle dans une campagne d'éradication.

Mots clés : Tique - *Amblyomma variegatum* - Lutte antiacarien - Acaricide - Pyréthrinoïde - Antilles - Guadeloupe.

Amblyomma variegatum, the tropical bont tick, was introduced into the Caribbean from West Africa in 1830 (11). This tick species is an efficient vector of *Cowdria ruminantium* and is associated with acute dermatophilosis, two diseases of ruminants which cause considerable economic loss in the Caribbean (10, 22).

This tick is spreading in the Caribbean. For nearly one hundred years, the tick remained distributed only on Guadeloupe, Antigua and Marie-Galante. In 1948, the tick was found in Martinique and in the last 20 to 30 years, the tick has been found on 14 other islands in the Caribbean region (1, 17, 21, 22). Considering the economic losses caused mainly from dermatophilosis when the tick was initially introduced, and the threat of the establishment on the American mainland (1, 4, 7, 21), recommendations were formulated to eradicate this tick from the Caribbean (21, 23). Research on the biology and ecology of the tick were conducted in Puerto Rico (12) and Guadeloupe (3, 6, 8) to develop the knowledge required to eradicate the tick. Studies to identify effective acaricides for use in eradication programs were also carried out. In this paper, we will summarize the research conducted in Guadeloupe on the efficacy of different acaricides, and on the efficiency of different methods of application for use in eradication programs and suited to particular farming conditions of the Lesser Antilles.

CHOICE OF ACARICIDES

Acaricide susceptibility to commercially available acaricides

In vitro tests of susceptibility of Puerto Rican strains of *A. variegatum* to various organophosphates, amidines and pyrethroids (13) indicate that ticks from Puerto Rico are as susceptible as African *A. variegatum* populations to the acaricides tested (15, 16). In the case of Guadeloupe, larvae and, to a lesser extent nymphs, are even more susceptible than African ticks to the acaricides tested with the exception of ethion. Ticks exposed to ethion showed a high level of tolerance to this acaricide (LC50 from 0,01 to 0,04 depending of the geographical origin of the strain (12).

Another approach is to evaluate the efficiency of acaricides in the field. In Guadeloupe, the acaricides most often used are : ethion (Rhodiacide®) which has been used for more than 20 years ; a mixture of chlorpyriphos and toxaphene (Procibam®) which has been used for about 10 years, deltamethrin (Butox®) and amitraz (Taktik®) which both have been in use for about 5 years. They are used in the government control program, alternatively without consideration for management for acaricide resistance. No resistance has been demonstrated to these acaricides in Guadeloupe. However, use of ethion has resulted in animal owner complaints and some concern that resistance may be developing. This observation seems to point to a tolerance of the ticks in the French Antilles to ethion. Only one other report of acaricide resistance in the Caribbean occurs in the literature, that of MOREL (1967) with gamma HCH (Tigal ®) on *Boophilus microplus* in Guadeloupe.

To our knowledge, there is no acaricide resistance in populations of the tropical bont tick in Guadeloupe, Martinique, Marie-Galante, La Désirade and St-Martin, to

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coumaphos, amitraz, and the commercially available pyrethroids. Consequently, there is a wide choice of acaricides available for use in an eradication program.

Advantages of the use of synthetic pyrethroids

Low concentration efficiency

In vitro acaricide susceptibility of larvae and nymphs of *A. variegatum* from Guadeloupe has been tested. For larvae, the LC50 of deltamethrin is about 6 times less than for amitraz, 40 times less than for Procibam ®, 70 times less than for coumaphos, 540 times less than for toxaphene and as much as 1200 times less than for ethion (12). These ratios are approximately the same for nymphs.

Low toxicity

One advantage of the use of synthetic pyrethroids is that small concentrations of the acaricide are highly effective in controlling populations of ticks. Secondly, these acaricides are theoretically less damaging to the environment because they are used in such small quantities. In addition, these acaricides do not have a withdrawal period before slaughter for meat or a withdrawal period before milking. It is a significant advantage in countries like those in the Caribbean islands where analysis of residues in animal meat and milk products is not carried out.

Also, it seems unlikely that there is a host reaction to use of pyrethroids on different hosts, which would facilitate the organisation of an eradication campain where the same acaricide could be used on all livestock to be treated.

Length of residual effectiveness

Residual activity of liquid formulations

Different experiments have been conducted in Guadeloupe to determine the length of time of residual activity to adults of *A. variegatum*.

In one experiment (5), flumethrin was used as a pour-on, spray or dip on Friesian cattle ranged on pasture which was naturally infested with ticks. In this study, the first engorged female was observed to detach and was able to lay viable eggs 21 days after acaricide treatment. From this experiment a residual activity of 6-9 days was estimated with dip and 9-18 days with pour-on and spray formulations of flumethrin (fig. 1).

In a second test, flumethrin and deltamethrin pour-on and spray formulations were used. In this study, 4 goats in each test were infested with adults ticks by placing the ticks in attached bags glued to the animal. The goats were infested with adults at increasing intervals. The same experiment was also carried out on cattle treated with flumethrin pour-on and spray and coumaphos spray. Results from these studies are summarized in figure 1. With the spray or pour-on formulations of flumethrin used on goats the period of residual effectiveness ranged from 12 to 15 days and 6 to 9 days, respectively. For cattle, both formulations of flumethrin have a residual period of 6-9 days. The residual period for deltamethrin spray formulation used on goats was 3 to 6 days and for the pour-on formulation less than 3 days.

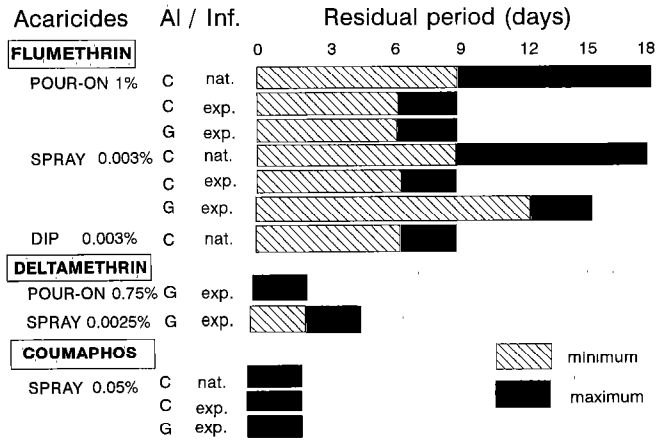

Figure 1 : Residual period of three acaricides after experimental (exp.) or natural (nat.) infestation of cattle (C) and goats (G). (Unpublished, BARRÉ et al., 1987, BARRÉ and GARRIS, 1992).

The residual period of 6 to 9 days for flumethrin pour-on is substantially shorter than the residual period obtained by HAMEL and VAN AMELSFOORT (1985) for *A. hebraeum* which was 12-14 days. Although the residual activity of flumethrin pour-on is less than that found for *A. hebraeum*, it is sufficient to effect eradication, when used at fourteen day intervals in the field in Guadeloupe (9) and in other Caribbean islands (2).

Lenght of effectiveness of slow - release devices

In a third experiment, collars impregnated with flumethrin at 2,25 % were placed on each of 18 goats. Before infestation with female ticks the goats were allowed to graze on a pasture in a humid zone of Guadeloupe (about 3 000 mm rainfall annually).

One month before placement of the flumethrin treated collars on the goats, each of three goats were artificially infested with 15 males. Six days after the males were placed on each of the three goats, the goats were infested with 15 females. At intervals of 1, 15, 35, 55 and 75 days after the placement of the flumethrin collars, the infestation procedure described above was repeated. When

each goat was infested with either males or females, the goats were held in a large bag for two hours and the ticks were introduced into these bags. This procedure was used to improve the attachment success of the artificially infested goats.

For collection of ticks from the treated goats, each goat was placed in a specially constructed stanchion built over slatted floors which would allow for the ticks to normally detach and be easily collected.

The results from this study are presented in figure 2. A few males and females were able to attach to some of the goats soon after the ticks had been exposed to the flumethrin in the collars. However, the first females were observed to engorge, detach and lay viable eggs only when placed on the goats with males attached 55 days after the placement of collars. Moreover, in another group of 45 goats on which collars were placed and allowed to graze on pastures, 12 (27 %) had lost their collar after 1 month.

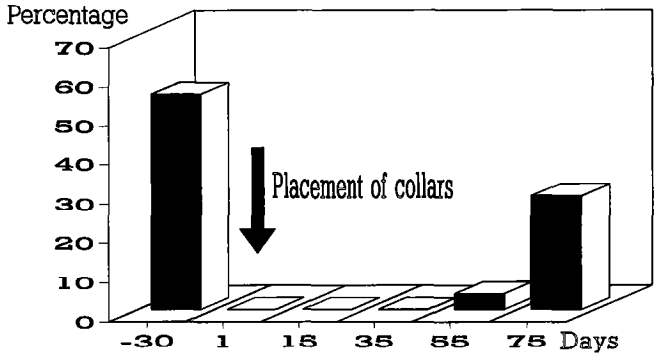

Figure 2 : Percentage of females engorged and detached depending on the delay between collar placement (impregnated with flumethrin 2.25 %) and the release of males on the goats (15 males, then 6 days later 15 females per goats, 3 goats per test).

Because female ticks were observed to engorge and reproduce, and because goats were able to cut off or pull out their collars, the use of the flumethrin collars on stray goats or sheep whose capture for replacement of expired collars would be difficult would not be a reasonable approach in an eradication campaign. In an eradication program, all hosts must be accessible for treatment with an acaricide or these non accessible animals will produce new generations of ticks and thus, prevent a successful eradication.

However, the collars tested were formulated for control of the ticks and fleas on dogs and therefore the concentration of active ingredient may be insufficient for control of *A. variegatum* on goats over a long period of time.

METHOD OF ACARICIDE APPLICATION

Of importance to an eradication program would be the method of application of an acaricide. Dips were constructed in Guadeloupe in the fifties (16 in Grande-Terre, 10 in Basse-Terre, 3 in Marie-Galante) (19). However and due to the dissemination of livestock in the country, dips were never used.

Mobile spray equipment has been used in these islands starting in 1963. The use of spray formulations of an acaricide in contrast to pour-on formulations requires expensive equipment and it must be mixed with a carrier, in most cases water. Adequate supplies of water in remote areas in some of the Caribbean countries are limited, making use of the spray formulation impractical.

Also it takes quite a long time to spray one animal (3 min per bovine) and the liquid may not reach ticks hidden in some remote places of the body. It pollutes the environment, for we can estimate that at least 1 liter of solution is wasted, sprayed on the surrounding pasture, for each animal treated.

Since some spray formulations are mixed with water as a carrier, these acaricides are at disadvantage when used in the tropical areas during the rainy season.

Furthermore, access to the remote areas in some of the countries is also limited and in some cases, there is no road. In some remote areas, walking is the only means of getting to the animals which need to be treated.

A pour-on or spot-on formulation comes from the manufacturer as a premixed complete acaricide which could be used in the field without dilution. Not having to dilute the acaricide in the field should reduce the probability, if used over long periods of time, of development of acaricide resistance in the target tick population. In addition, a premixed acaricide already has the level of the active ingredient in the acaricide set by the manufacturer and thus, testing to determine the level of active ingredient in the field is reduced.

Pour-on is also very easily and rapidly applied (a few seconds per bovine) on animals restrained in a coral. Moreover, some pour-ons spread rapidly and completely on the whole body (20), reaching in less than one hour even those ticks attached to hidden sites.

The pour-on or spot-on fomulations contain oil-based ingredients that are less miscible in water and therefore, may be used even during rainy periods.

In field studies in Guadeloupe (9), pour-on formulations of flumethrin or deltamethrin applied to cattle completely eliminated all *A. variegatum* ticks on the treated animals three months after treatments began. The pour-on acaricides were applied every 14 days to the test cattle for one year. By the end of the study, it was apparent that the

pour-on formulations of the tested acaricides were highly effective in controlling the ticks and that these acaricides showed promise for use in an eradication program.

However, the pour-on method application requires that the animal being treated be approached to within a meter so that the acaricide can be poured onto the backline of that animal. Because about 90 % of the livestock in the Caribbean region are tethered and are of the zebu breed, many of these animals are difficult to approach. A study was conducted to determine if the pour-on acaricide, flumethrin, would be as effective when applied as a spot-on in controlling the tropical bont tick.

In the study, the acaricide was applied to two groups of 15 tick infested cattle each group every 14 days for a period of 6 months. One group of 15 cattle received the normal pour-on method of application of the acaricide at the manufacturer recommended rate of 1 ml/10 kg body weight. Flumethrin as a pour-on material comes as a 1 % W/v formulation (Bayticol®). To simulate a spot-on method of application using the same acaricide (flumethrin pour-on) at the same concentration, the acaricide was applied to a second group of 15 animals in three jets propelled from a distance of 1.5 to 2 meters onto the upper part of one side of each study animal using a drench gun (Instrument supplies Ltd. Hamilton, New Zealand). As was found in another study (9) all ticks on the pour-on formulation treated group of animals were eliminated by the third month of treatment (table I). No adult ticks were found on the pour-on treaated animals three months after the end of the study.

In the spot-on simulation study group (table I) some male and semi-engorged female ticks were found on the treated animals during the months after the start of the treatment. These results suggest that the spot-on method is not as effective as the pour-on technique of acaricide application. However, no fully engorged female ticks were found on the spot-on treated animals after the start of treatment. Further research is needed : site of application, dose, viscosity of the carrier, etc., to reach the efficiency of pour-on.

CONCLUSION

A wide spectrum of acaricides is usable in a campaign of eradication of the tropical bont tick in the Caribbean. Most of them are active against this tick. However, it is obvious that the most appropriate are the pyrethroids : they are efficient at a very low concentration, the toxicity to mammals is low, no withdrawal period is necessary after their administration, some of them have a long residual activity, and most of them come as a pour-on formulation which is ready for use and which does not need expensive equipment or a supply of water for mixing.

However, the deposit of the acaricide on the back line as a pour-on is almost impossible in the rearing conditions of the Caribbean where more than 90 % of the cattle are tethered and are quite mobile at the end of their chain even if it is shortened at the time of treatment. The administration of the compound by spot jets with a gun from a short distance may considerably simplify the application and minimize the constraints imposed on cattle owners. Research however must be implemented to increase the efficiency of this method.

TABLE I Mean numbers and standard deviation (SD) of male (M) and female (F) **A. variegatum** *attached to one side of cattle (n = 15) treated with flumethrin applied as a pour-on or a spot-on, every 14 days (from T1 the 16 april 1992 to T12 the 17 September 1992) at 1 ml per 10 kg body weight.*

Date	Ttmt	FLUMETHRIN POUR-ON					FLUMETHRIN SPOT-ON				
		M	SD	F	SD	%C	M	SD	F	SD	%C
6 Feb		8.6	3.7	6	3.6		10.5	6.7	8.5	6.1	
18 Feb		18.1	7.9	8.8	4.8		7.5	4.6	4.4	2.3	
18 Mar		18.9	11.4	3.1	2		15.4	13.1	4.5	4.8	
16 Apr	T1	22.4	14.8	4.2	3.2		20.5	16.1	1.9	1.5	
14 May	T3	0.6	0.7	0.1	0.4	97	2	1.8	0.1	0.4	90
12 Jun	T5	1.3	1.9	0.3	0.6	94	1.6	1.1	0.6	0.9	90
9 Jul	T7	0.2	0.4	0		99	0.3	0.6	0.3	1	97
6 Aug	T9	0.1	0.3	0		99	0.5	0.6	0		98
3 Sep	T11	0		0		100	0.3	0.6	0		99
2 Oct		0		0		100	0		0		100
29 Oct		0		0		100	0.2	0.4	0.1	0.3	99
25 Nov		0.1	0.4	0		99	0.7	1	0.1	0.4	96
21 Dec		0.1	0.3	0		99	0.7	1.2	0.3	0.5	95
20 Jan		0		0		100	1.2	1.6	0.3	0.6	93

% C = percentage of control.

Also, experiments should be continued with impregnated slow release devices for each animal species in order to obtain the longest possible period of activity and consequently to reduce the frequency of acaricide application on animals by other means.

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BARRÉ (N.), GARRIS (G.), APRELON (R.). Acaricides for eradication on the tick *Amblyomma variegatum* in the Caribbean. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 349-354

The success of an eradication campaign against the tropical bont tick in the Caribbean imposes the use of active acaricide compounds, if possible with residual activity, easy to apply and requiring few or no accessible water supplies and expensive application equipment. Tests of *in vitro* susceptibility of tick strains from Puerto Rico and Guadeloupe as well as observations of the impact of the current tick control campaigns conducted in some Caribbean islands, seem to indicate that there is no problem of resistance to acaricides. Pyrethroid acaricides have an advantage since they are active at very low concentration levels and have a low toxicity for mammals and to the environment. Some of them are in a pour-on formulation which allows for rapid application and complete coverage of the whole body of the animal. A withdrawal period is not necessary. However, improvements must be found to facilitate the application onto the back of cattle that, for the majority in the Caribbean, are tethered and not perfectly restrained. A spot-on application method with a drench gun seems more adapted to tethered animals than the pour-on. For small ruminants and dogs but also for cattle, slow release devices impregnated with acaricides may be useful in reducing the frequency of animal treatments. However, experiments on goats with collars impregnated with flumethrin indicate an efficiency of less than 55 days, insufficient to justify their use on a large scale in an eradication program.

Key words : Tick - *Amblyomma variegatum* - Tick control - Acaricid - Pyrethroid - West Indies - Guadeloupe.

BARRÉ (N.), GARRIS (G.), APRELON (R.). Acaricidas utilizados para la erradicación de *Amblyomma variegatum* en el Caribe. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 349-354

El éxito de las campañas de erradicación contra la garrapata tropical en el Caribe, exige el uso de compuestos activos de acaricidas, que de preferencia no provoquen residuos, de fácil aplicación y que requieran poca o ninguna utilización de agua o equipo sofisticado. Los tests de susceptibilidad *in vitro* de las cepas de garrapatas originarias de Puerto Rico y Guadalupe, así como las observaciones sobre el impacto de las campañas de control de garrapatas llevadas a cabo en algunas islas del Caribe, no parecen indicar la existencia de problemas de resistencia a los acaricidas. Los piretroides son recomendables, debido a que actúan a concentraciones muy bajas y presentan un grado mínimo de toxicidad para los mamíferos y para el medio ambiente. Algunos de ellos se presentan en forma de "depósito" (*pour-on*), lo que permite una aplicación rápida, con buena cobertura del cuerpo del animal. Además, el período de ensayo no es necesario. Sin embargo, debe mejorarse el sistema de aplicación sobre el dorso del ganado, ya que por lo general, en las islas del Caribe éste se encuentra atado y no estabulado. La administración por *spot*, con una pistola de aplicación, parece más adaptada a los animales atados que el sistema de depósito. Para pequeños rumiantes, perros e incluso bovinos, los métodos de liberación lenta impregnados con acaricidas, pueden ser útiles en la reducción de la frecuencia de los tratamientos. Sin embargo, los experimentos realizados en cabras con collares impregnados de flumetrina, indican una eficiencia en la duración menor a 55 días, lo cual no justifica el uso de éstos a gran escala en los programas de erradicación.

Palabras claves : Garrapata - *Amblyomma variegatum* - Control de garrapatas - Acaricida - Piretroide - Antillas - Guadalupe.

B.H. Bokma [1]

J.L. Shaw [1]

Eradication of a new focus of *Amblyomma variegatum* in Puerto Rico

BOKMA (B.H.), SHAW (J.L.). Éradication d'un nouveau foyer d'*Amblyomma variegatum* à Porto Rico. *Revue Élev. Méd. vét. Pays top.*, 1993, **46** (1-2) : 355-358

Une infestation par *Amblyomma variegatum* est apparue en mai 1992 dans un petit troupeau d'élevage de génisses laitières à Camuy, un site tout à fait nouveau près de la côte nord-ouest de Porto Rico. Cette constatation a été faite après environ trois années sans infestation à Porto Rico et dans les Iles Vierges américaines, et l'infestation ne serait pas associée à une des infestations précédentes. Les méthodes pour éradiquer la tique comprennent un traitement de tout le bétail, à intervalles de 2 semaines, par pulvérisation avec de l'amitraz à 0,025 p. 100 et de la perméthrine à 0,055 p. 100. Les chiens et les poules sont également inspectés et les propriétaires sont encouragés à réduire le nombre de ces espèces. Tout le bétail des fermes limitrophes est inspecté et traité fréquemment. Il n'y a pas eu de signe d'extension et l'infestation devrait être éliminée dans les 18 mois à venir.

Mots clés : Bovin - Chien - Poule - *Amblyomma variegatum* -Tique - Lutte antiacarien - Acaricide - Pulvérisation - Porto Rico - Saint-Croix - Iles vierges américaines.

INTRODUCTION

The 3-host African-origin tropical bont tick (TBT), *Amblyomma variegatum* (Fabricius), has been present in the Caribbean since the early 1800's (1, 10, 13). The TBT spread from early established infestations on Guadeloupe and later Antigua and St. Kitts to many different islands. This spread included St. Croix (U. S. Virgin Islands, late 1960's), Puerto Rico (1970's), and its island municipalities, Vieques (early 1980's) and Culebra (mid 1980's) (1, 2, 6, 11, 13)[b*]. The general biology, mechanisms of spread, and the peculiarity the TBT presents in the Caribbean are described by other authors (4, 5, 8)[c].

In Puerto Rico and the U. S. Virgin Islands, infestations of ruminants, particularly cattle, have been associated with high mortality due to the cutaneous dermatophilosis, an infection caused by the aerobic actinomycete, *Dermatophilus congolensis* (3,12)[a,e]. The disease heartwater, an infection caused by a rickettsia, *Cowdria ruminantium*, has not been identified in ruminants in Puerto Rico and the U. S. Virgin Islands[e].

In addition to the infestations with the TBT, in 1978 Puerto Rico became reinfested with *Boophilus microplus* (Canestrini), the 1-host southern cattle tick, after a period of some 24 years of free status following its earlier eradication in 1954. An active eradication program has ensued since. Both Puerto Rico and the U. S. Virgin Islands are currently under Federal quarantine for *Boophilus microplus* [d,e].

Eradication efforts in St. Croix in the late 1960's and early 1970's, in Puerto Rico and its island municipalities during the late 1970's and the 1980's, and an additional infestation in St. Croix during the 1980's, have been described by several authors (2,6,7,9,11)[b]. The TBT has been repeatedly eradicated from individual foci of infestations. No active infestations of the TBT existed subsequent to elimination on St. Croix (December 1987) and Culebra island (April 1989). The last incidental findings of the male tick was in the Lares municipality and in August of the same year, two male ticks were found in the Rincon municipality[e].

The new active infestation was detected in Puerto Rico during May 1992.

The intent of this paper is to review surveillance for the TBT in Puerto Rico and to present and discuss the eradication procedures used in the most recent infestation.

MATERIALS AND METHODS

Surveillance and detection of infestation

As part of the *Boophilus microplus* eradication program, all active livestock premises are visited routinely by surveillance teams made up of livestock inspectors. These teams inspect cattle for ticks, both visually and by manual palpation or scratching. All cattle farms not known to be infested are inspected on a random basis but at least annually. Those farms considered to be at a higher risk of becoming infested (larger herds, multiple sites, dealers of cattle, adjacent to known infested farms) are inspected more frequently. In addition, as part of the *Boophilus microplus* eradication program, movement permit teams visit farms in order to allow movement of non-infested cattle. After preventive treatment with acaricide, cattle found to be free of tick infestations are permitted movement. Approximately 14,000 of the islands's 21,000 active cattle farms are considered non-infested (either "post-treatment" or "free.")

1. USDA, APHIS, VS, PO Box 71355, San Juan, Porto Rico 00936-8455.

* See "Other sources" at the end of the article.

Eradication procedures

The treatment programs used for TBT eradication in St. Croix and Puerto Rico have been described previously (2,6,7,9)[b,d].

Generally the program consists of quarantine and bangle tag identification of all animals on affected pastures; intensive surveillance for TBTs on all host animals; a reduction in the number of free-roaming dogs and poultry; a minimal 18-month program of biweekly inspections and spray treatments of all ruminants and equines with acaricides (in cattle and goats, 0.025 % amitraz, with occasional use of 0.055 % permethrin, and in sheep and equines, 0.055 % permethrin); the biweekly treatment of, and tick surveillance on, all domestic mammalian host animals on the immediately adjacent premises; increased area surveillance for ticks within a mile or so of the fencelines, and an increased island-wide surveillance for TBT.

Infested herds are maintained under treatment for at least 18 months and until such time that no ticks are collected for at least six months. In the event that several herds are involved, quarantine releases are effected in blocks of herds, after all herds have completed the minimal requirements.

Additional measures that can be used but are not currently a part of this effort are brush clearing and ground treatment with an acaricide and the trapping of, inspection for ticks on, and elimination of free-roaming animals, especially dogs and poultry, but also mongoose and wild deer (St. Croix and Culebra). These latter two species have not delayed eradication in our experience.

Herds found to be infested with *Boophilus microplus* are placed under individual farm quarantine and all cattle are whole body spray treated with amitraz at 0.025 %. Treatments are generally given every three weeks. The treatment cycles last for 231 days. If the farm is found positive at the end of the treatment cycle then the treatments are extended for at least 63 days or until no ticks have been found on pre-release scratch. Other species of livestock have not been found to present persistent infestations and are not now being placed under systematic treatment.

RESULTS

Surveillance and detection of infestation

On 18 May 1992 during a routine movement permit inspection, one herd was found to be infested with the TBT. The infested farm is located in the Quebrada barrio (ward) of the Camuy municipality. The farm consists of 18.2 ha and is in karst hill-lands typical of Puerto Rico's northern coast, about 16 km inland from the north coast. The farm is surrounded by karst hills and many adjacent cattle herds. The herd is located in an area where the Camuy River runs underground.

The cattle population of this farm on 19 May 1992 was determined to be 33 animals, including 30 dairy replacement heifers and three bulls. The owner typically raises heifers from about three months of age until they are bred and ready for sale as replacement heifers. Cattle were generally of Holstein Friesian breeding with some Brown Swiss or Creole breeding evident. Twenty-nine animals were found to be infested with adult and nymphal stages of the TBT. About 300 specimens of *Amblyomma variegatum* were collected during two days of inspections and many specimens were submitted for laboratory confirmation. Interestingly, this herd did not show signs of cutaneous dermatophilosis.

A very active effort to detect further cases of TBT infestations began immediately after the detection on the infested farm. Five immediately adjacent premises were surveyed for host animals, including cattle, horses, goats, sheep, dogs, chickens, and other fowl. These animals were all inspected for TBTs. Approximately 100 total sites were visited on the first round of proactive surveillance. A total of three rounds of surveillance involving 78 premises in the immediate vicinity have been accomplished during the period May 1992 to January 1993.

Island-wide surveillance for the TBT was increased via a publicity campaign for program employees and livestock owners.

The augmented surveillance for the TBT has not resulted in any additional findings. One entirely circumstantial result of this augmented surveillance has been the detection of a lone male *Amblyomma cajennense* (Fabricius) tick during routine surveillance of a dairy herd in the Rincon municipality, located at the extreme western end of Puerto Rico.

Intensive surveillance in the area also resulted in detecting a number of herds infested with *Boophilus microplus*, reinforcing in program inspectors the continued need for surveillance activities.

Eradication procedures

Quarantine and treatment procedures specific for the TBT were begun on the infested farm as described above. The infested herd has continued under a biweekly schedule of treatment and scratching and has been free of all signs of infestation since June 1992.

Movement restrictions do not allow movement from the infested farm unless repeated examinations before treatments demonstrate no ticks on any animals. Follow-up of animals moved from the infested farms to dairy herds has been negative.

All livestock herds immediately adjacent to the infested farm were also treated biweekly as described above. After five biweekly treatments, adjacent *Boophilus microplus* infested herds were changed to routine treatments on a 21-day schedule. The treatments are to be discontinued after 231 days of treatment, after a successful negative scratch for all ticks, provided the TBT-infested farm remains free of ticks.

DISCUSSION

Surveillance and detection of infestation

Over the years, surveillance for the TBT in our area has depended primarily on the physical presence and activities of governmental livestock inspectors, animal health technicians, and veterinarians on farms. In addition, private veterinarians, livestock owners, extension and University personnel, and abattoir inspectors have occasionally had important roles in detecting and reporting the TBT.

In Puerto Rico, surveillance for tick infestations has depended markedly on the inspectors in the *Boophilus microplus* eradication program. These employees see thousands of the preferred hosts in their routine activities. The level of coverage has increased to the point that almost all cattle herds are examined yearly. Many non-infested herds are inspected as frequently as every three months. All cattle in infested herds are treated routinely every three weeks. Vieques island remains free of TBT, and except for cattle movement permits it is not currently covered by the eradication program. Culebra island has been cleared of both ticks, and except for movement permits is not under any treatment.

The risk of spread of the TBT has been reduced significantly. This is evidenced by the relative lack of findings subsequent to the eradication in Puerto Rico, St. Croix, Vieques, and Culebra. In contrast, once established in focal areas, such as occurred in Puerto Rico during the 1970's, the TBT successfully demonstrated its ability to infest new areas (2).

The Camuy herd is located in an area of Puerto Rico that has not before had TBT infestations. Camuy is at least thirty miles from any previous focus and well over one hundred miles away from most recent areas of infestation. These areas, Culebra and St. Croix, are separate islands with very little movement of livestock or other animals to Puerto Rico.

In the case reported here, after extensive surveying house-to-house, there is no evidence of movements, legal or illegal, of livestock, poultry, dogs, and cats. Domestic animal movements are therefore an unlikely mechanism for TBT spread.

The occasional findings of isolated male TBTs and this particular active but limited infestation, in areas widely separated from the known foci of infestation, are presumably due to the movement of TBTs on migratory or emigrating birds, such as the cattle egret. An additional possibility is small domestic mammals. Studies on migration patterns of the cattle egret have been reported at the October 1992 meeting of the United States Animal Health Association (Louisville, Kentucky) by Dr. Victor NETTLES (Southeastern Cooperative Wildlife Study Group, Athens, Georgia) and Dr. Glen GARRIS (World Health Organization, Bridgetown, Barbados). Marked cattle egrets from Guadeloupe or from Antigua have been encountered as far away as in Florida (marked in Guadeloupe).

This infested farm described in this report is a preferred area for cattle egrets, *Bubulcus ibis* (L.), perhaps due to a freeflowing spring and lush grassy conditions. Due to the lack of any other known link to any active focus of infestation, we presume that cattle egrets transmitted this infestation from a neighboring island.

Mentioned previously was the finding in June 1992 of a male *Amblyomma cajennense* tick in Rincon. Of interest is that in August 1983, a single semi-engorged female *Amblyomma cajennense* was collected from a bull in Aguada, a neighboring municipality. We speculate that the spread of this species from countries that have this tick could also occur on birds, similar to the TBT. Another method that has been proposed is via infested materials brought in either legally or illegally.

A concern regarding the effective surveillance for TBT is that active treatment of herds for *Boophilus microplus*, done on a 21-day schedule, may mask TBT infestations. Established low level infestations should become evident after treatments are halted. A benefit of continual treatments could be the elimination of TBT infestations before they become infested.

The most previous examination of this particular herd was in November 1990. The herd had not been scratched in over 18 months. While this particular herd was not found to be infested with *Boophilus microplus,* several adjacent herds were infested. The resulting increased emphasis on surveillance has greatly benefitted the tick program.

Eradication procedures

The eradication techniques described here were developed based on investigative work completed in Puerto Rico by the USDA (5, 6). The costs of TBT eradication have been presented previously (6). Due to the mechanics and specific costs involved in delivering spray-application pesticide to animals on farms, Puerto Rico's tick eradication program would benefit enormously by the availability of pour-on formulations of efficacious acaricides, such as flumethrin or amitraz.

From our perspective, the eradication of the TBT from different infested islands is highly desirable. This will certainly depend on the availability of adequate resources, infrastructure, and governmental or industry authority, as well as governmental and industry resolve.

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BOKMA (B.H.), SHAW (J.L.). Eradication of a new focus of *Amblyomma variegatum* in Puerto Rico. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 355-358

In May 1992 an infestation with the tropical bont tick appeared in a small dairy heifer replacement herd in Camuy, Puerto Rico, a completely new site located near Puerto Rico's northwest coast. This finding occurred after some three years of no infestation in either Puerto Rico or the U.S. Virgin Islands and is not suspected of being associated with any previous infestations. The methods used to eradicate this tick include spray treatment at a two-week interval of all domestic livestock, with amitraz at 0.025 % and permethrin at 0.055 %. Dogs and chickens are also inspected. Owners are encouraged to reduce numbers of these species. All livestock on adjoining farms are inspected and treated on a frequent basis. There has been no evidence of any spread and the infestation is expected to be eliminated within 18 months.

Key words : Cattle - Dog - Hen - *Amblyomma variegatum* - Tick - Tick control - Acaricide - Spraying - Puerto Rico - Saint Croix - U.S. Virgin Islands.

BOKMA (B.H.), SHAW (J.L.). Erradicación de un foco de *Amblyomma variegatum* en Puerto Rico. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 355-358

En mayo 1992 apareció un brote de garrapatas en un pequeño hato lechero de novillas de reemplazo en Camuy, región indemne en la costa noroeste de Puerto Rico. Este hallazgo se dió tres años después de la última aparición en Puerto Rico o en las Islas Vírgenes de los E.U.A. y no se sospecha que tenga relación alguna con infestaciones anteriores. Los métodos utilizados para la erradicación de la garrapata incluyeron el tratamiento con aerosol de amitraz al 0,025 p. 100 y permethrin al 0,055 p. 100, a dos semanas de intervalo para todos los animales domésticos. En la inspección se incluyeron perros y gallinas, recomendándose la reducción en número de estas especies. Todos los animales domésticos de fincas contiguas fueron inspeccionados y tratados frecuentemente. Hasta el momento no han habido muestras de diseminación y se espera obtener la eliminación del brote en 18 meses.

Palabras claves : Bovino - Perro - Gallina - *Amblyomma variegatum* - Garrapata - Control de Garrapata - Acaricida - Pulverización - Puerto Rico - Santa Cruz - Islas Vírgenes de los E.U.A.

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Progress towards a program for the eradication of *Amblyomma variegatum* from the Caribbean

GARRIS (G.I.), BARRÉ (N.), CAMUS (E.), WILSON (D.D.). Progrès vers un programme d'éradication d'*Amblyomma variegatum* des Caraïbes. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 359-362

***Amblyomma variegatum* (Fabricius), la tique tropicale bigarrée, est maintenant largement répartie dans les Caraïbes. Dix-huit îles sont actuellement infestées ou l'étaient récemment. Afin d'arrêter la propagation de cette tique vers d'autres îles non infestées et vers le continent d'Amérique du Sud, Centrale et du Nord, un programme régional d'éradication a été proposé et approuvé par les gouvernements de toutes les îles infestées, y compris les îles françaises et les pays membres ou membres associés de CARICOM. Au nom de ses pays membres et membres associés, CARICOM a demandé à l'Organisation des Nations unies pour l'alimentation et l'agriculture (FAO) : de développer des propositions d'éradication ; d'assister les membres de CARICOM à maintenir les programmes existants de lutte contre *Amblyomma* ; de conseiller et assister à rédiger la législation nécessaire à la réalisation d'un programme d'éradication ; d'aider à identifier les fonds pour exécuter le programme d'éradication et, si les fonds sont obtenus, de coordonner la campagne d'éradication contre la tique sur toutes les îles infestées. La répartition actuelle de la tique et la situation concernant le programme d'éradication proposé sont examinées.**

Mots clés : Tique - *Amblyomma variegatum* - Lutte antiacarien - Projet de recherche - CARICOM - FAO - Caraïbes.

The tropical bont tick, *Amblyomma variegatum*, is a serious parasite of domestic livestock and wildlife in the Caribbean. This tick transmits heartwater, caused by *Cowdria ruminantium*, and is associated with acute dermatophilosis, a skin disease of livestock caused by the bacterium, *Dermatophilus congolensis*. Wherever the tick and its associated diseases are found, livestock producers experience severe economic losses. Mortality due to acute dermatophilosis is especially severe in susceptible livestock imported into the islands in order to increase production of needed sources of animal protein and products for human consumption. Additional economic losses occur because producers must treat their animals with acaricides to control ticks and with antibiotics to try to reduce mortality due to the diseases, especially dermatophilosis.

Heartwater was identified in clinical cases in Guadeloupe in 1980 and since 1980 it has been confirmed as endemic in three islands, Marie Galante, Guadeloupe and Antigua (5,10). Because heartwater was found in the Western Hemisphere and because its vector, *Amblyomma variegatum* was found in Puerto Rico and St. Croix, considerable interest in the distribution of the disease and tick and in the eradication of these from the hemisphere was expressed by a number of individuals and institutions during the early 1980's. A flood of research activities were funded during this period (5,10). It was predicted that at least one new island would become infested with the tick per year (1) and this prediction has essentially held true since about 1988.

In the last ten years, the tick has been found infesting livestock on 18 islands in the Caribbean and is continuing to spread to new locations. Evidence recently found seems to indicate that migratory birds, especially the cattle egret, may play an important role in the spread of the tick in the Caribbean. Cattle egrets appeared in the Caribbean in the late 1950's and became well established as breeding colonies on a number of islands during the 1960's and 70's. The tick began to spread to a number of islands during the 1970's which circumstantially (9) suggests that cattle egrets may play an important role in the spread of the tick in the Caribbean. Recent data indicate that not only do cattle egrets migrate frequently, but may do so for long distances - one bird captured live and marked in Guadeloupe was sighted in Layton, Long Key Island, Florida (USA) , some 1152 miles (1920 km) to the north while another bird marked in Guadeloupe was sighted about 240 miles (400 km) to the south in Grenada (6).

To stop the spread of *A. variegatum* and its associated diseases, it has been proposed that a regional eradication program be initiated. The technology needed to successfully eradicate the tick from a given island is available (3). Furthermore, successful eradication programs have been carried out on St. Croix (8) and Puerto Rico and Vieques (7) and Culebra (U.S. Department of Agriculture (USDA), APHIS-VS, personal communication 1990). This technology involves the efficient delivery of an effective acaricide (4) to all domestic hosts every 14 days for a perod of two years. The primary goal of the eradication technique is to prevent adult ticks from mating on the host and thus, prevent the production of offspring (3,7).

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DEVELOPMENT OF A PLAN OF ACTION

Because of the importance of the tick and its associated diseases, heartwater and dermatophilosis, and because of the seriousness of the threat to the U.S. and other mainland countries in North, Central and South America, recommendations in regard to a regional eradication program against the tick were being discussed. Furthermore and in direct response to recommendations made by Chief Veterinary Officers of the Caribbean Community Secretariat (CARICOM), member and associate member countries, Ministers of Agriculture of CARICOM countries, National Cattlemen's Association (USA) and the U.S. Animal Health Association, as well as the Inter-American Institute for Cooperation on Agriculture (IICA) sponsored a task force which met and outlined a feasibility study proposal for the management of the tick and its associated diseases. The task force produced an outline of the feasibility proposal, identified members of a study group needed to produce the proposal and developed budgetary requirements to accomplish the task. Funding for the study group was provided by U.S. Agency for International Development (USAID), U.S. Department of Agriculture (USDA), CIRAD-EMVT, IICA, and Food and Agriculture Organization (FAO) of the United Nations.

The study group met on several occasions between May and October 1986 before discussing its findings in November 1986 with a larger group of interested parties representing countries, national and international organizations including CARICOM, and invited consultants. The feasibility proposal presented for discussion the following :

- an up-to-date picture of the known distribution (1986) of the tick and of heartwater and dermatophilosis in the Caribbean ;

- a review of the existing veterinary/animal health infrastructures, laws and regulations affecting animal health ;

- an economic evaluation of the tick and its associated diseases, the consequences of its possible spread and the benefits derived from its eradication ;

- research activities necessary to provide information required for the management (eradication) of the problem ;

- an eradication strategy, including economic estimates associated with managing the problem ;

- an organizational framework that could be used to coordinate and implement the eradication campaign.

This report was presented in March 1987 to a "Technical workshop on the tropical bont tick *A. variegatum*" which was organized by CARICOM and attended by representatives of Caribbean countries/islands and international organizations.

PROGRESS TOWARDS THE ERADICATION PROGRAM

Four major resolutions developed from the workshop in 1987 with concurrent actions are presented below :

- The establishment of an *Amblyomma* Program Council. The council was established as the *Amblyomma* Steering Committee (ASC) at a special meeting of the Standing Committee of Ministers responsible for Agriculture (SCMA) of the Caribbean Community (CARICOM) held in FAO Headquarters in Rome, Italy, on 17 November 1987.

- The establishment of a pilot project for the control/eradication of the tropical bont tick on Antigua. The USDA and USAID were responsible for developed and implementation of a proposal to eradicate the tick from Antigua which included operational and research components.

The operational component was not completed because of a technical question which arose from an environmental assessment study where Bayticol® pour-on was identified as not registered by the U.S. Environmental Protection Agency and therefore could not be used in an eradication program sponsored by USAID. As a result of the environmental assessment study, all funds not used for the operational component were deobligated by USAID and returned to the U.S. treasury.

The research component was completed and included research on : a comparative study of commercially available pour-on acaricides which showed that the Bayticol® pour-on acaricide was the most effective in controlling populations of the tick and would be an excellent acaricide for use in an eradication program (4) ; the potential role of the cattle egret in the dissemination of the tick to other islands in the Caribbean (6) ; an economic assessment of an eradication program.

The convening of a donors' meeting to secure adequate resources for the successful eradication of the tick from the Caribbean.

The donors' meeting was held in Rome at the FAO Headquarters on 10 December 1992. Although results from the meeting are encouraging, no donor officially committed funding to support the regional eradication program. Additional effort will be needed to follow up with the interested donors identified prior to and during the donors' meeting.

That Caribbean countries already undertaking emergency tick control activities approach appropriate agencies for immediate emergency support.

FAO, through the Technical Cooperation Programme (TCP), has been approached by CARICOM and a number of individual Caribbean countries to assist in the development of control programs, preparation of documentation and organization of a donors' meeting for the

regional eradication program, and in the development of surveillance and prevention programs.

Results from the TCP program have been the establishment of the infrastructure for an effective control program against the tropical bont tick on St. Lucia which is still operational today. The TCP program has also organized and prepared the document entitled "Programme for the eradication of *Amblyomma variegatum* from the Caribbean" which was the document used to approach potential donors (see above). The establishment of surveillance and prevention programs, advice and assistance with drafting of legislation required to support a tick eradication program, and assistance with continuing control efforts of a number of member and associate member countries of CARICOM has been provided by FAO through the TCP programs of 1991 and 1992.

Additionally, related projects dealing with the eradication/control of *A. variegatum* have been funded by the British Development Division (BDD) of the Eastern Caribbean. The BDD has funded a small three year project, presently ongoing on Montserrat, that is designed to control the tick and prevent further disease outbreaks and spread. The BDD program on Montserrat has been assisted by the regional FAO TCP programs. Previously, the BDD had funded similar *A. variegatum* and dermatophilosis control programs on St. Kitts and Nevis.

CONCLUSIONS

Efforts by FAO and other organization/governments and interested parties over the last six years have resulted in :

1. the establishment of an infrastructure and cooperative relationships among all the principals involved in the regional eradication program ;

2. the completion of a program proposal for a Caribbean-wide eradication program including a co-operational element relating to the implementation of the eradication efforts on the French islands ;

3. assistance with review and drafting of legislation required to support a tick eradication campaign on each of the CARICOM islands presently infested with the *A. variegatum* tick ;

4. Identification of a number of potential donors who have expressed support for the regional eradication program but who have not officially committed themselves to the financial aspects of the program.

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G.I. Garris N. Barré E. Camus D.D. Wilson

GARRIS (G.I.), BARRÉ (N.), CAMUS (E.), WILSON (D.D.). Progress towards a program for the eradication of *Amblyomma variegatum* from the Caribbean. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 359-362

***Amblyomma variegatum* (Fabricius), the tropical bont tick, is now widely distributed in the Caribbean. Eighteen islands countries are now or were recently infested with the tick. To stop the spread of this tick to other non-infested islands and to the mainland areas of South, Central and North America, a regional eradication program has been proposed and endorsed by the respective governments on each of the *Amblyomma variegatum* infested islands, including the French government and CARICOM member and associate member countries. The Food and Agriculture Organization of the United Nations (FAO) was requested by CARICOM, on behalf of member and associate member governments to : develop eradication proposals ; assist CARICOM member countries to maintain existing *Amblyomma* tick control programs ; advise and assist with the drafting of legislation required for implementation of an eradication program ; assist in the identification of funds to implement the eradication program and, if funding was obtained, coordinate the eradication campaign against this tick on all infested islands. The current distribution of the tick and the status of the proposed eradication program in the Caribbean are discussed.**

Key words : Tick - *Amblyomma variegatum* - Tick control - Research projects - CARICOM - FAO - Caribbean.

GARRIS (G.I.), BARRÉ (N.), CAMUS (E.), WILSON (D.D.). Progresos de un programa de erradicación de *Amblyomma variegatum* en el Caribe. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 359-362

Actualmente *Amblyomma variegatum* (Fabricius) se encuentra ampliamente distribuída en el Caribe. Dieciocho islas estan o estuvieron recientemente infestados con esta garrapata. Con el fin de parar el avance de la garrapata hacia otros países de Norte, Sur y Centroamérica, se propuso un programa de erradicación regional, patrocinado por los gobiernos de cada estado insular infestado con *Amblyomma variegatum*, así como por los miembros de CARICOM y Francia. En representación de los países miembros y de los países miembros asociados, CARICOM pidió a la Organización para la Alimentación y la Agricultura de las Naciones Unidas (FAO), lo siguiente : desarrollo de proposiciones de erradicación ; ayudar a los países miembros de CARICOM en el mantenimiento de los programas de control existentes contra *Amblyomma* ; aconsejar y asistir en la elaboración de un programa de legislación, necesario para la puesta en marcha del programa de erradicación ; ayudar en la recolección de fondos para implementar el programa de erradicación y, si se obtiene el financiamiento adecuado, coordinar la campaña de erradicación contra esta garrapata en las islas infestadas. Se discute la distribución actual de la garrapata, así como el lugar que ocupa en el Caribe el programa de erradicación propuesto.

Palabras claves : Garrapata - *Amblyomma variegatum* - Control de garrapatas - Proyecto de investigación - CARICOM - FAO - Caribe.

Poster

Flumethrin distribution on cattle haircoat after pour-on application *

H.D. Hamel [1]

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HAMEL (H.D.), STENDEL (W.), SIEVEKING (H.U.). La distribution de la fluméthrine sur le pelage de bovins après application topicale. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 363

La distribution de la fluméthrine dans le pelage de bovins après application topicale ("pour-on") a été déterminée par analyse chimique. La fluméthrine a été appliquée à 1 mg de substance active (s.a.) par kg de poids corporel, le long de la ligne dorsale. Il a été démontré que le produit pouvait être retrouvé dans tous les échantillons de poils prélevés de régions dorsales, latérales, ventrales et distales 1 jour après l'application, dans des concentrations allant de 670 à 1 µg de s.a. par g de poils, selon la distance du site d'application. Trois, 5 et 10 jours après l'application ces concentrations variaient respectivement de 125-1,5, 23-1 et 44-0,9 µg de s.a. Une corrélation de ces valeurs avec la surface corporelle de bovins montre qu'il y avait plus de 0,01 µg de s.a./cm^2 de surface corporelle sur toutes les régions du corps et tous les jours des prélèvements. Cette quantité est suffisante pour une activité acaricide efficace, comme l'ont montré des données de laboratoire et de terrain.

HAMEL (H.D.), STENDEL (W.), SIEVEKING (H.U.). Flumethrin distribution on cattle haircoat after pour-on application. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 363

By means of chemical analysis, the distribution behaviour of flumethrin was determined in the haircoat of cattle following topical pour-on application. Flumethrin was applied at 1 mg active ingredient kg^{-1} body weight along the backline of cattle. It was demonstrated that this compound could be recovered from all hair samples taken on Day 1 following application from dorsal, lateral, ventral and distal body regions in concentrations ranging from 670 to 1 µg a.i. g^{-1} hair, depending on the distance from the site of application. On days 3, 5 and 10 after treatment, the corresponding concentrations were 125.0-1.5, 23.0-1.0 and 44.0-0.9 µg a.i. g^{-1} hair, respectively. When correlating these values to the body surface of cattle, it is evident that on all sample days and body regions, a concentration of more than 0.01 µg a.i. cm^{-2} body surface was present. This amount of active substance is sufficient for effective acaricidal action, as shown by laboratory and field data.

HAMEL (H.D.), STENDEL (W.), SIEVEKING (H.U.). Distribución de la flumetrina en la piel del ganado después de una aplicación por depósito ("pour-on"). *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 363

El patrón de distribución de la flumetrina, después de una aplicación tópica en el pelaje del ganado, se determinó mediante análisis químicos. Se aplicó 1 mg de flumetrina activa por kg de peso vivo, a lo largo del dorso del animal. Se demostró que este compuesto se puede recuperar al día 1 después de la aplicación, de todas las muestras de pelo tomadas en las zonas dorsal, lateral, ventral y distal del cuerpo, en concentraciones comprendidas entre 670 y 1 µg a.i. g^{-1} de pelo, dependiendo de la distancia al sitio de aplicación. A los días 3, 5 y 10 después del tratamiento, se encontraron concentraciones de 125,0-1,5, 23,0-1,0 y 44,0-0,9 µg a.i. g^{-1} de pelo, respectivamente. Se llevó a cabo una correlación entre estos valores y la superficie corporal del ganado, la cual mostró que en todas las muestras, independientemente del día o de la región corporal, se encuentra una concentración de más de 0.01 µg a.i. cm^{-2} de superficie corporal. Esta cantidad de substancia activa proporciona una acción acaricida eficaz, tal y como ha sido demostrado tanto en trabajos de laboratorio como de campo.

1. AG Bayer, Leverkusen, Allemagne.

* *Ce texte, dont seuls les résumés sont publiés dans ce volume, a fait l'objet d'un poster.*

SYMPOSIUM EPILOGUE

Looking back on this first international symposium on cowdriosis, dermatophilosis and Amblyomma *ticks, it is apparent how far we have come in the last decade or so. I will certainly not attempt to summarize all that the participants have shown us, but try to highlight a few points of particular significance. It certainly does not mean that what is left unsaid is unimportant!*

Because of the close relationship between Cowdria *and* Ehrlichia, *we now have no specific serological tests for heartwater. Evaluating the specificity of the* Cowdria *DNA probes and of the polymerase chain reaction, in particular on those species of* Ehrlichia *which cross-react with* Cowdria *in serological tests, is therefore a priority. A promising start on this point was made by Tony BARBET* et al., *who reported in this meeting that sheep from heartwater-free regions but serologically positive (immunoblotting using the 32 kDa* Cowdria *protein), were negative for* C. ruminantium *by PCR, did not transmit heartwater to ticks, and were fully susceptible to the infection. Let us also hope that the 21 kDa immunodominant protein, on which BARBET* et al. *have also reported, will be more specific than the 32 kDa antigen.*

According to the work of Jean du PLESSIS, the distinction between the genera Cowdria *and* Ehrlichia *may become blurred. It should be remembered that certain ehrlichial organisms have been reported to occur in endothelial cells as well as in white blood cells, bridging as it were the gap between the two genera, for example the* Ehrlichia *described in pigs in Algeria as E. suis by Donatien and Gayot 50 years ago. Many questions are piling up and are waiting to be addressed. One priority for the Caribbean is to find out what causes the false positive reactions in serological tests for heartwater on islands where the disease appears to be absent, both on islands with* A. variegatum *and without this tick. So far no* Ehrlichia *has been demonstrated to occur in ruminants in the Antilles. It would also be interesting if it could be shown in southern Africa that the Omatjenne and Vosloo agents, after changing through* Amblyomma *into something indistinguishable from* Cowdria, *could be picked up again by their first tick vector and changed back into* Ehrlichia.

On the other hand, we should not become too obsessed with diagnostic methods for cowdriosis. As was pointed out by Dürr BEZUIDENHOUT in his review at this meeting, they will certainly not be used for the diagnosis of clinical heartwater in the field, but are essentially research tools for improving epidemiological studies and disease surveillance, which if necessary can be carried out, imperfectly, by the methods we have had to use until now. These tools should not become an end in themselves and we should already apply the knowledge on the epidemiology of the disease that has been gained so far to help in the control of the disease.

Much information has been acquired lately on the immunology and immunopathology of cowdriosis, and on the antigenic composition of the rickettsia. The role of cytokines, interferons and the major histocompatibility complex is being investiga-

ted, and there is again a case for thinking that neutralizing antibodies may play some role after all. Dominique MARTINEZ et al. *have shown at this meeting that inactivated elementary bodies can induce a protective immune response, which allows hope for the possibility of developing a molecular or recombinant vaccine that will protect, at least against homologous challenge. A vaccine that is effective in the field may well be another story, because of cross-protection differences between various strains.*

The use of attenuated culture material as a vaccine is presently being evaluated in Senegal, as indicated by Frans JONGEJAN et al. *at this meeting. We already know that it will not solve the problem of cross-immunity differences, but the practical importance of these differences has not yet been fully evaluated; it may not be equally important in all ruminant species and may also depend on the innate susceptibility of the given ruminant population.*

Until something better comes along, we will have to make do with existing methods of immunization, details of which might be improved by using lyophilized and titrated cell culture instead of blood or tick-derived material as the infective inoculum, and by such practical improvements as the doxycycline implant (Doximplant®).

Innate resistance to heartwater, or perhaps more correctly the genetically determined ability of developing a good immune response to first infection, well known to occur in local ruminant breeds in endemic areas, is being studied in Creole goats here on Guadeloupe. Should it depend on just one or very few genes, markers might perhaps be found to allow a short-cut in selection for resistance and in transferring the genes for resistance into more productive breeds by cross-breeding, or as some like to think perhaps in the more distant future by artificial gene transfer.

Our Belgian friends Philippe TOTTÉ et al. *have reported at this meeting that* C. ruminantium *will grow in human endothelial cells, and we cannot exclude the possibility that heartwater (or at least certain strains) may be a zoonosis. (However, in Zimbabwe, KELLY* et al. *have reported (in 1992) that sera of persons having apparently been bitten by* Amblyomma *ticks because they had antibodies to a rickettsia of the spotted fever group known to be transmitted by* A. hebraeum, *were nevertheless negative for* Cowdria *antibodies.)*

Much of the impetus for the progress in cowdriosis research has of course come about because of a real breakthrough, the first successful in vitro *culture of* C. ruminantium *(BEZUIDENHOUT* et al., *1985).*

Although there has been no breakthrough in research on dermatophilosis which is comparable to what has been accomplished in cowdriosis, considerable progress is being made. The cells that play a role in the immune response to the infection are being defined. Much of this work is still limited to mice and small ruminants, but as far as the association of severe dermatophilosis with the tropical bont tick is concerned, the real target host is the bovine.

There is still much to be discovered concerning the association between the tick and severe dermatophilosis. What has A. variegatum *got, and in particular the adult, what other ticks, including* A. hebraeum, *do not have? The key to the*

control of (severe) bovine dermatophilosis could lie there, if we can get a grip on the pathogenesis of the disease.

Severe dermatophilosis in cattle is especially rife when three main factors are associated:

** Prolonged humid and warm conditions. There is nothing one can do about that, except for protecting the animals indoors;*

** Susceptible livestock. Populations in regions where the disease is a problem have been subjected to long term natural selection and have an innate resistance. This leads to the possibility of selection;*

** Presence of large African* Amblyomma *ticks, in particular* A. variegatum. *Although the tick does not transmit the infection, it induces the development of severe dermatophilosis, if the other factors are present. It has been shown that only the adult ticks are responsible for this selective immunodepression, which appears to be caused by the tick saliva. Intensive tick control can control dermatophilosis.*

So far, immunization has not been very promising and has not been studied under controlled circumstances using susceptible cattle in the presence of the tick, which is the ultimate test. Antigenic strain diversity exists and might also be a stumbling block for successful immunization in the field.

There are innate, genetically determined differences in susceptibility to the disease between breeds and within breeds, just as for cowdriosis. Attempts to identify genetic markers for susceptibility or resistance have started and might provide a short-cut in selection procedures. The fact that none have as yet been identified should not stop anyone from attempting selection using classical methods in the meantime.

It has been shown that the nutritional status has a great influence on the immune response, which has important implications for eventual immunization campaigns in the dry season, when the nutritional status is low.

It is clear that the importation of susceptible cattle in areas with A. variegatum *is dangerous, as was shown once more by NAVES* et al. *(this meeting). At present only intensive tick control can prevent the disease in such circumstances, but acaricide resistance is certain to follow sooner or later.*

As far as the tick itself is concerned, different strategies are to be applied in different circumstances. In Africa, with limited financial resources, increasing acaricide resistance, and no possibility of eradicating the ticks, the focus in future may have to be on further research on acquired immunity to infestation, either following natural infestations (although this approach is not promising for A. variegatum, *at least not in ruminants), or through artificial immunization with internal antigens, where research is still in an early stage as far as this tick is concerned. Consistent individual differences in attractivity for this tick, if hereditary, might be useful as part of an integrated approach to control, as reported at this meeting by Frédéric STACHURSKI. Use of pheromones is also being further explored.*

But in the Caribbean the only sensible approach is the eradication of the Senegalese tick from the western hemisphere, before it reaches the mainland as it otherwise undoubtedly will, sooner rather than later. Fairly detailed plans exist and it is a matter of getting the donors to agree to a coordinated effort, which will be highly cost-effective even in the Caribbean itself, but especially in avoiding the huge potential losses on the mainland. Unfortunately, attempts since many years by a number of dedicated persons from various countries and organizations to obtain sufficient and assured funding have so far not been successful, and the danger is certainly not fully realized by the countries of the American continent. Let us be optimistic and hope that by the time the next symposium is held the eradication campaign in the Caribbean will be well underway.

We are all looking forward to the progress that will undoubtedly been achieved by the time the next meeting of the STVM will take place, in 1995 in Berg-en-Dal in the Kruger Park in South Africa. I hope you will be numerous.

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