J.A. Lawrence[1]

A.P. Whiteland[1]

J. Malika[1]

P. Kafuwa[1]

F. Jongejan[2]

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-

1. Project GCP/RAF/259/DEN, Production of Anaplasma, Babesia and Heartwater Vaccines for East and Central Africa, P.O. Box 30750, Lilongwe 3, Malawi.

2. Institute of Infectious Diseases and Immunology, Department of Tropical Veterinary Medicine and Protozoology, Faculty of Veterinary Medicine. Utrecht State University, P.O. Box 80 165, 3508 TD Utrecht, Pays-Bas.

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.