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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.

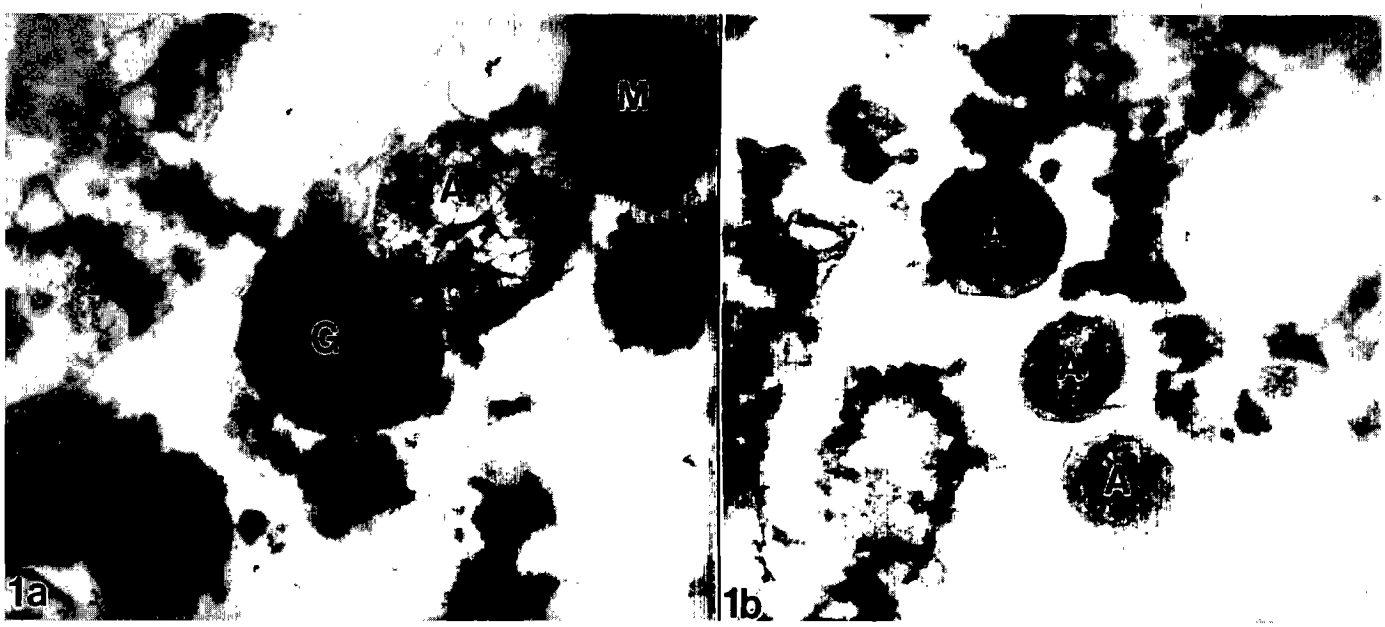


*Photo 1 : Electron micrographs of an inoculum prepared from salivary glands of* Dermacentor andersoni *infected with* Anaplasma marginale. *a) An individual* Anaplasma *organism (A) in a host cell mitochondria (M) and a granule (G). (x 36,720). b) Three individual* Anaplasma *organisms (A) free from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

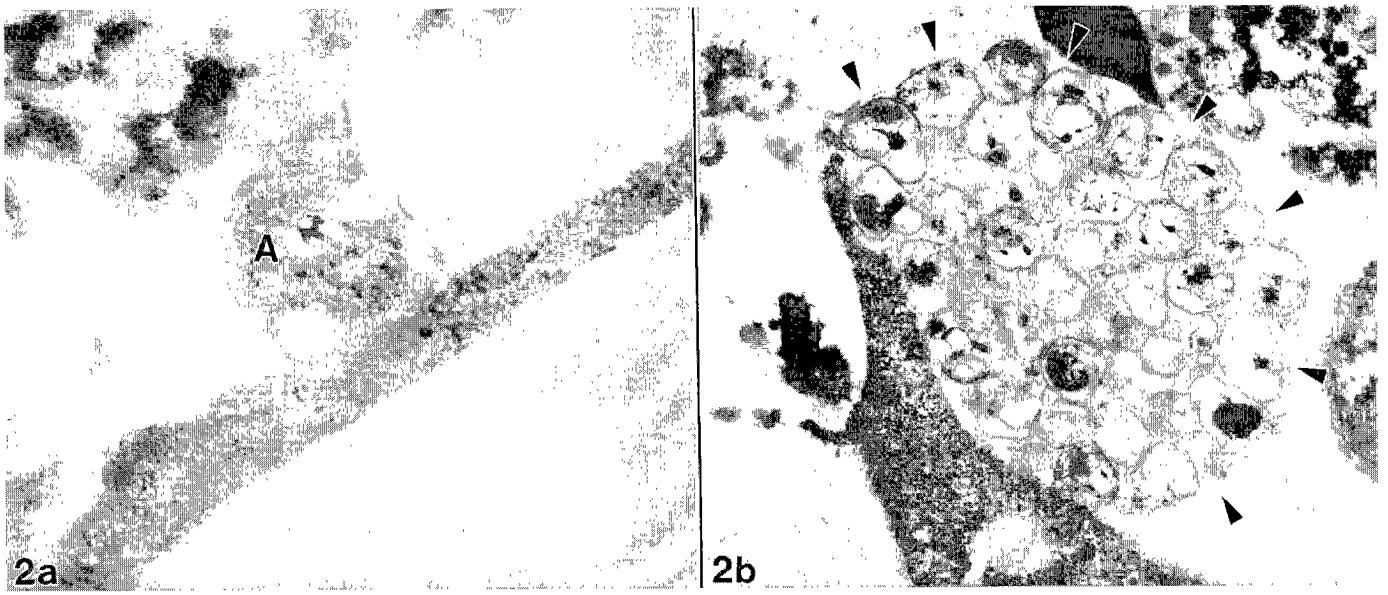


*Photo 2 : Electron micrographs of bovine turbinate cell cultures at 60 min PI with tick salivary gland material infected with* A. marginale. *a) An individual* Anaplasma *organism (A) in contact with a host cell membrane (x 60,780). b) A colony (arrows) of* A. marginale *in association with a bovine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

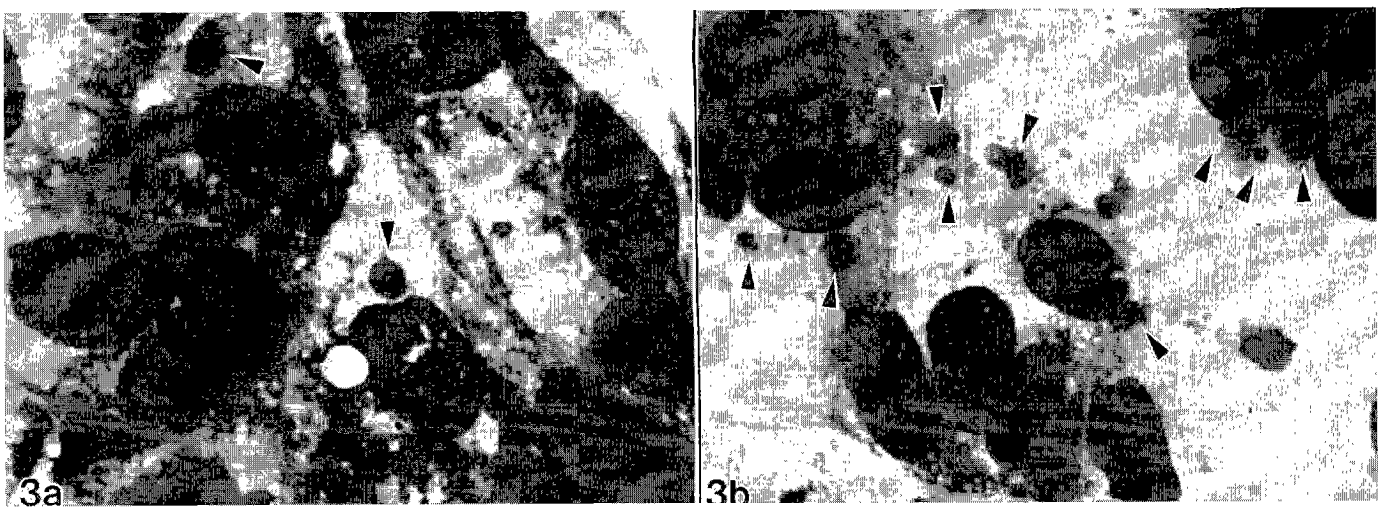


*Photo 3 : Bovine turbinate cell culture inoculated with the salivary gland stage of* A. marginale. *a) Intracellular inclusions (arrows) at 2 weeks PI. Note inclusions appear to contain multiple subunits. b) Intracellular inclusions (arrows) at 3 weeks PI (passage 1).*

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).

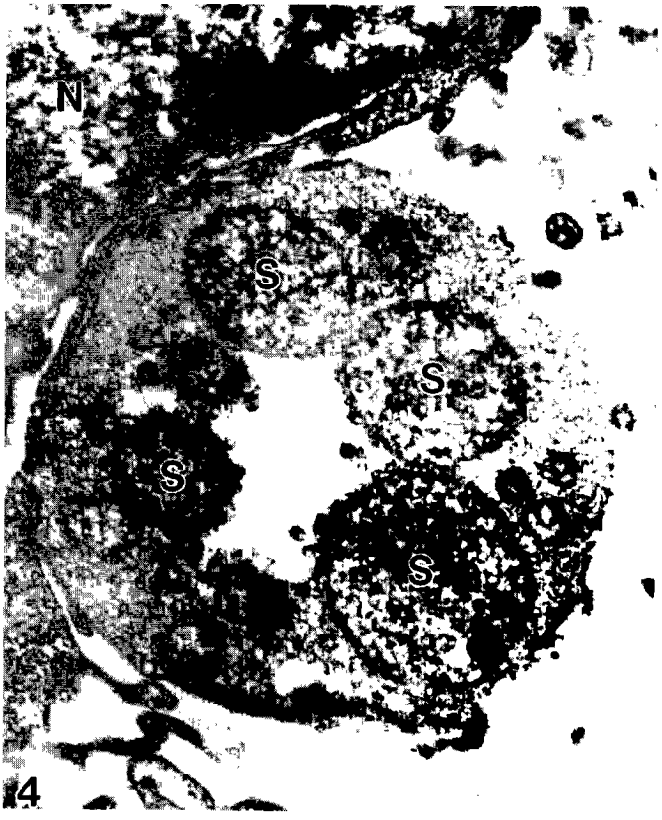


*Photo 4 : Electron micrograph of an inclusion body in association with a bovine turbinate cell nucleus (N) at 2 weeks PI. Note dense subunits (S). (x 18,465)*

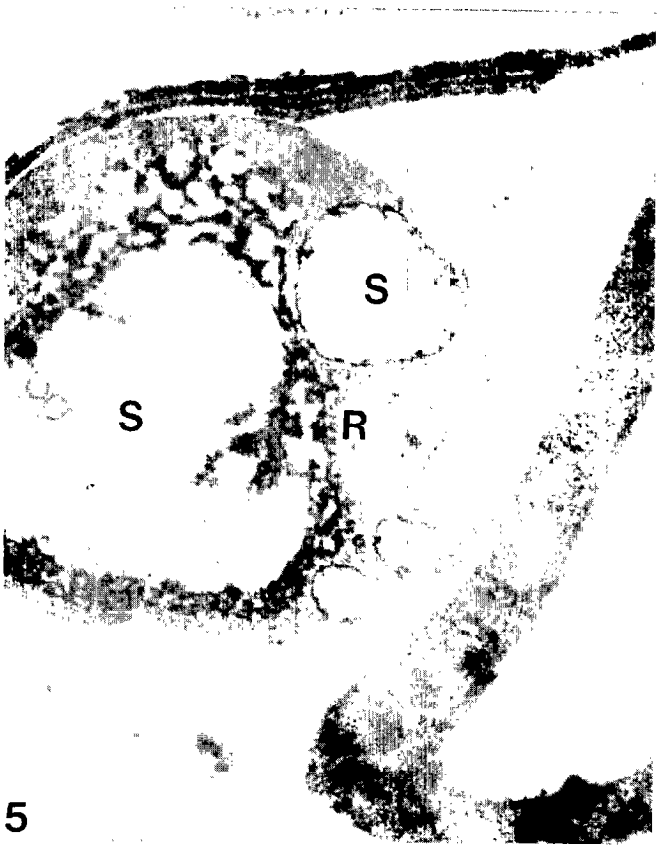


*Photo 5 : Electron micrographs of an inclusion body from turbinate cell culture at 41 days PI. Subunits (S) appear to be devoid of substance but 1 distinct rickettsial organism (R) is present. (x 18,900).*

## 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.

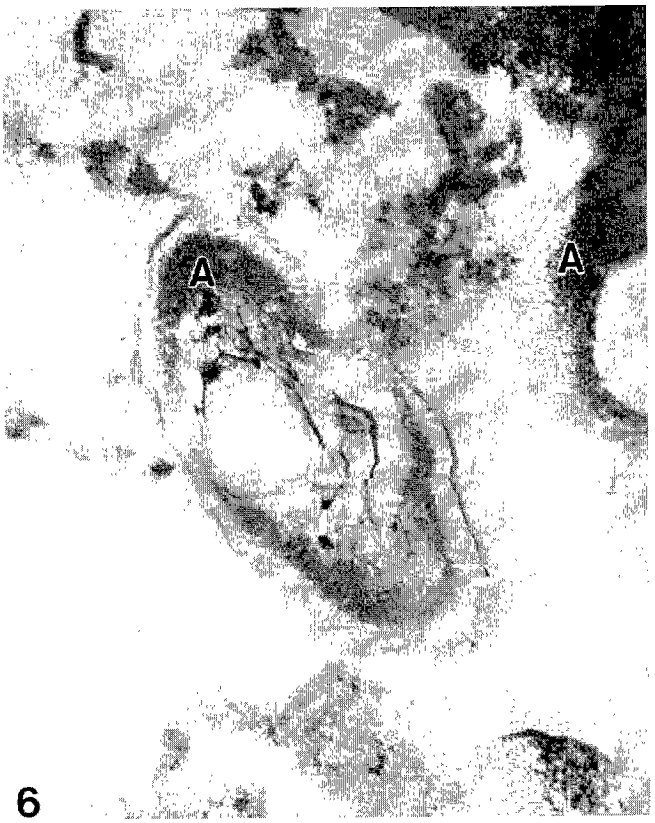


*Photo 6 : Electron micrograph of an individual* Anaplasma *organism (A) from bovine turbinate cell culture at day 43 PI. (x 64,800)*

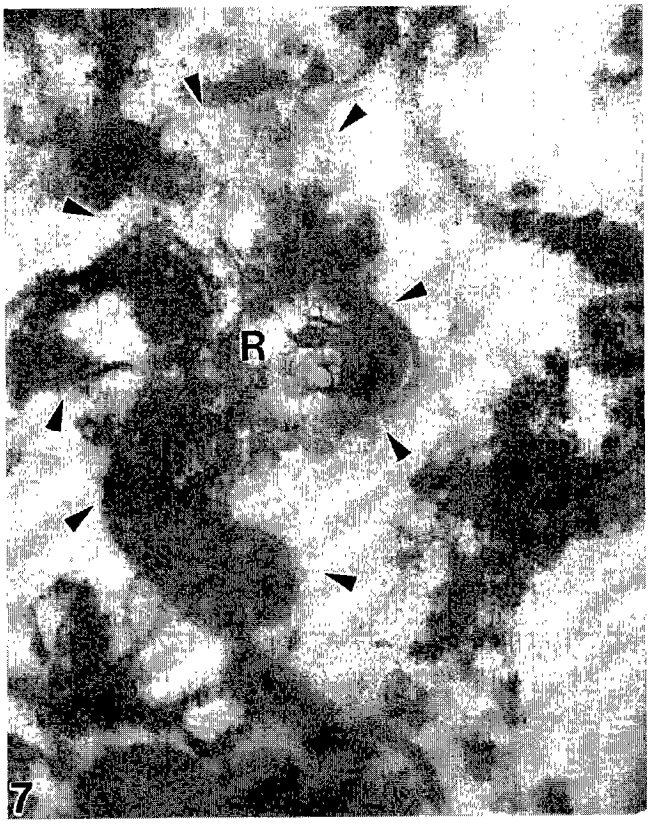


*Photo 7 : Electron micrograph of an amorphous inclusion (arrows) from bovine turbinate cell culture at day 43 PI. Note the central area of reticulation (R). (x 64,800).*

## 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-

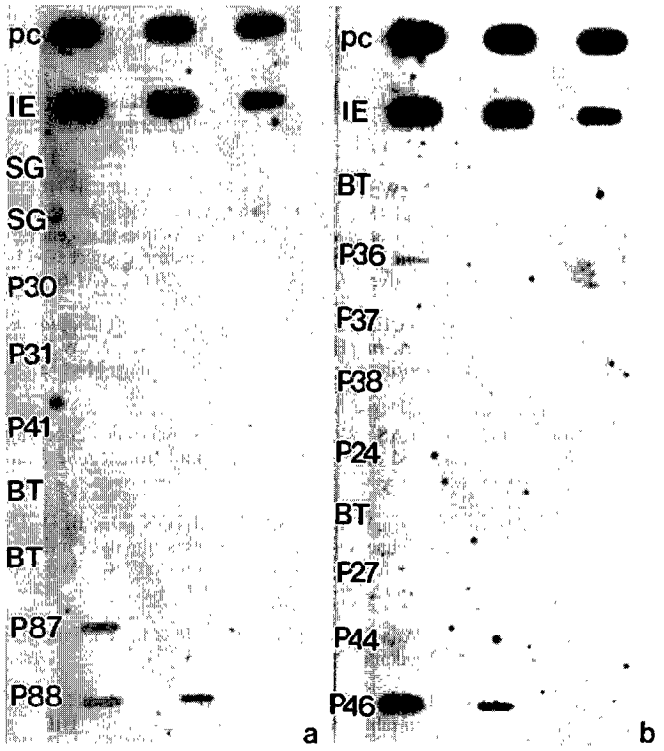


*Photo 8 : Hybridization of a $^{32}$P-labeled* A. marginale msp1ß *gene to cell culture samples. pc = plasmid control, IE =* A. marginale*-infected rbcs, SG = uninfected salivary gland control, BT = bovine turbinate cell inoculated with uninfected salivary gland inoculum. a) Two positive culture samples : 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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***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.