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INCREASED PATHOGENICITY OF AN *EHRLICHIA*-LIKE AGENT AFTER PASSAGE THROUGH *AMBLYOMMA HEBRAEUM:* A PRELIMINARY REPORT

J. L. DU PLESSIS, Veterinary Research Institute, Onderstepoort 0110

ABSTRACT

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After being passaged through 3 generations of *Amblyomma hebraeum,* an *Ehrlichia*-like agent isolated from an adult *Hyalomma truncatum* female became more pathogenic and elicited a disease in sheep indistinguishable from heartwater. Cross-immunity between this agent and several stocks of *Cowdria ruminantium* and high levels of antibody elicited by the agent against 2 stocks of *C. ruminantium* in the indirect fluorescent antibody test, confirmed its close relationship to *Cowdria.*

INTRODUCTION

The serological cross-reactions between *Cowdria ruminantium* antigen used in the indirect fluorescent antibody (IFA) test and the sera of animals infected with different species of *Ehrlichia* (Du Plessis, Camus, Oberem & Malan, 1987; Holland, Logan, Mebus & Ristic, 1987), complicate the interpretation of the IFA test results, aimed at studying the epidemiology of heartwater. Apart from the fact that these cross-reactions suggest that *C. ruminantium* and *Ehrlichia* share common antigens and that their developmental forms in the macrophages of mammalian hosts are morphologically indistinguishable (Unpublished data, 1989), very little is known about the relationship between these 2 agents.

When the occasion arose to study ticks collected from cattle in an *Amblyomma*-free region of southern Africa, a high percentage of which reacted positively in the IFA test, it was decided to characterize the agent presumably responsible for these cross-reactions.

MATERIALS AND METHODS

Origin of sera and ticks

Forty-three sera, collected from cattle on the farm Omatjenne in the Otjiwarongo district of Namibia, were subjected to the IFA test for *C. ruminantium* (Du Plessis & Malan, 1987b). When 81 % of these sera was found to be positive, ticks were collected from cattle on the same farm, identified and injected individually into mice along the intravenous (i.v.) route as previously described (Du Plessis, 1985). Two *Rhipicephalus oculatus,* 11 *Hyalomma marginatum rufipes,* 10 *H. truncatum* and 5 *Rhipicephalus evertsi mimeticus* adult females were inoculated into mice. Four weeks later the sera of the mice were subjected to the IFA test.

Infection of experimental animals

The agent, isolated from a female *H. truncatum,* hereafter referred to as the Omatjenne agent, was passaged in mice, calves and sheep, either by inoculating them i.v. with the tissues from animals infected during earlier passages or through *Amblyomma hebraeum* ticks. Homogenates were prepared in buffered lactose peptone (BLP) from the liver and spleens of infected mice on a 10 % mass/volume basis and stored in liquid nitrogen. Blood collected in heparin was added to equal volumes of BLP and stored in liquid nitrogen.

Tick feeding

Larvae and nymphae of the Spesbona strain of *A. hebraeum,* reared free from infection by *C. ruminantium* (Heyne, Elliot & Bezuidenhout, 1987), were used to infest mice and sheep under tick-free conditions. When unfed, uninfected nymphae were required, larvae were fed on rabbits and left to moult. The Velcro corset was used in the case of mice and calico bags were glued to the backs of sheep as described elsewhere (Heyne *et al.,* 1987). Engorged, infected ticks were collected and allowed to moult at 80 % relative humidity and 27 °C.

Serology

Pre- and post-exposure sera of mice, sheep and calves were subjected to the IFA test, in which 2 different stocks of *C. ruminantium* were used as antigen. The peritoneal macrophages of mice infected with the Kümm stock (Du Plessis, 1982) were used as previously described (Du Plessis & Malan, 1987b). In addition, the Kwanyanga stock (MacKenzie & Van Rooyen, 1981), grown in tissue culture on E-5 endothelial cells (Bezuidenhout, 1987), was also used. The cells of a 2-day-old culture were removed with activated trypsin versene, pelleted by centrifugation at 300 *g* for 5 min and washed in IFA test buffer. The cells were centrifuged again and the pellet taken up in a small volume of buffer, so that a platinum loop of cell suspension placed on a 15-well antigen slide left a monolayer of cells. The subsequent handling of the antigen slides, the execution of the IFA test and the evaluation of the fluorescence of intracellular colonies of *C. ruminantium,* were identical to that described for the Kümm stock-infected mouse peritoneal macrophage test (Du Plessis & Malan, 1987b). Unless otherwise stated, mice sera were tested at a dilution of 1:10 and sheep and calf sera at a dilution of 1:20.

Cross-immunity tests

Five heartwater susceptible Dorper sheep were infected with the Omatjenne agent either by allowing adult *A. hebraeum* ticks that had engorged as nymphae on Sheep 7 during its febrile reaction to feed on them (Sheep 8 & 9, Table 2), or by inoculating them i.v. with 5 mℓ of heparinized blood collected from Sheep 8 at the height of the febrile reaction (Sheep 10 & 11). Sheep 12 was infected with 5 mℓ of heparinized, pooled blood collected from Sheep 10 & 11. This inoculum was also used to subject Sheep 8 & 9 to an homologous challenge. Sheep 13 was immunized with the Kümm and Sheep 14 & 15 with the Ball 3 stock.

Sheep 8–12 were cross-challenged a month later with 5 mℓ sheep blood infected with the Kümm, Germishuys, Ball 3, Kwanyanga and Breed stocks of *C. ruminantium* (Du Plessis, Van Gas, Olivier & Bezuidenhout, 1989), respectively. Four control animals (Sheep 16–19) were inoculated at the same time. Sheep 13, immune to the Kümm stock, was chal-

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TABLE 1 Reactions and IFA test results of mice, calves and sheep infected with the Omatjenne agent

Experimental animal No.	Inoculum	Reaction	IFA test titre Antigen	
			Kümm	Kwanyanga
Mouse 1	*H. truncatum* homogenate	No clinical signs	1:10	ND
Mice 2	Mouse 1 tissue homogenate	1/7[1]	−ive	−ive
Calf 1	Mouse 1 tissue homogenate	16/3/40,9[2]	1:20	1:20
Calf 2	Blood, Calf 1	8/2/39,8	1:20	1:20
Sheep 1	Mouse 1 tissue homogenate	11/7/40,6	−ive	−ive
Sheep 2	Blood, Sheep 1	No reaction	−ive	−ive

[1] 1/7 = 1 out of 7 mice with ruffled hair coat and huddled in corner of cage

[2] 16/3/40,9 = febrile reaction commenced 16 days after inoculation, lasted for 3 days and attained a maximum temperature of 40,9 °C.

lenged with ticks from the same batch used to infect Sheep 8 & 9. Sheep 14 & 15 were challenged with the pooled blood from Sheep 10 & 11. Early morning rectal temperatures were recorded. Animals showing no reaction or a reaction index (Du Plessis *et al.*, 1989) below 10 when they were challenged, were considered immune and those above 10 as susceptible.

RESULTS

Serology

Twenty-four, 8 and 3 out of 43 sera of the Namibian cattle subjected to the IFA test, in which Kümm stock antigen was used, were positive to titres of 1:20, 1:80 and 1:320, respectively, and the other 8 were negative at a dilution of 1:20. The same 35 sera, tested at a dilution of 1:20 only, were also positive with the Kwanyanga antigen. All the pre-exposure mice-, sheep- and calf-sera were negative with both antigens.

Tissue sub-inoculation in mice, calves and sheep

The serum of Mouse 1, inoculated with the homogenate of a *H. truncatum* adult female, reacted positively in the IFA test (Table 1), but all the other mice injected with tick homogenates were negative. Mouse 1 showed no clinical signs and, when autopsied, a marked splenomegaly was the only salient feature.

Ten mice (Mice 2, Table 1), injected i.p. with 0,3 mℓ of a homogenate prepared from the spleen and liver of Mouse 1, with the exception of a single mouse, also failed to show any clinical signs and were serologically negative a month later. Giemsa-stained smears, prepared from the peritoneal macrophages of 3 of these mice killed 8–11 days after being infected and those of the single mouse with clinical signs 14 days p.i. (huddled in a corner of the cage with a ruffled hair coat), revealed small numbers of cells with intra-cytoplasmic inclusions. These were basophilic, dense bodies that were either homogeneous or consisted of several poorly outlined fragments of varying sizes and shapes. Some of the inclusions consisted of basophilic granules of consistent size and shape. These inclusions were indistinguishable from the developmental stages of *C. ruminantium* in the peritoneal macrophages of mice infected with the Kümm stock (Du Plessis, 1975).

Calf 1 (Table 1), injected i.v. with 2 mℓ of tissue homogenate from Mouse 1, had a mild febrile response for 3 days but no other clinical signs. Calf 2, inoculated i.v. with 10 mℓ of blood collected from Calf 1 during its febrile response, likewise showed only a mild febrile reaction. The sera of both calves at a low titre were positive to both IFA test antigens.

Sheep 1, inoculated i.v. with 2 mℓ of tissue homogenate from Mouse 1, showed a moderate febrile reaction but no other clinical signs. Sheep 2, inoculated with 10 mℓ of blood from Sheep 1, failed to show any febrile reaction and no antibody was detectable with the IFA test in the sera of either sheep, irrespective of the antigen used.

Tick passage of the Omatjenne agent

The mice (Mice 3, Fig. 1), infected i.v. with 0,1 mℓ of tissue homogenate from Mouse 1, showed no clinical signs but were seropositive to a low titre a month later. Kümm antigen only was used in the IFA test. Four unfed *A. hebraeum* nymphae, fed on a rabbit as larvae, were allowed to engorge on each of 3 of these mice 13 days after being infected. The engorged nymphae were allowed to moult and 2 months later 2 adult males were allowed to attach to Sheep 3, followed 2 days later by 8 females. Sheep 3 showed a mild, intermittent febrile response of short duration 13 days later, but no other clinical signs and remained seronegative with both antigens. The lar-

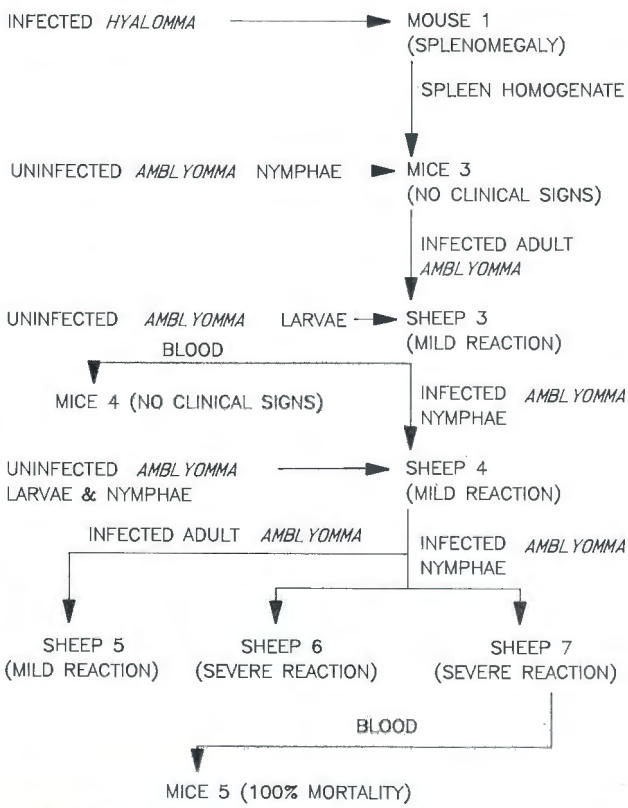

FIG. 1 Flow diagram of mice, sheep and ticks infected with Omatjene agent

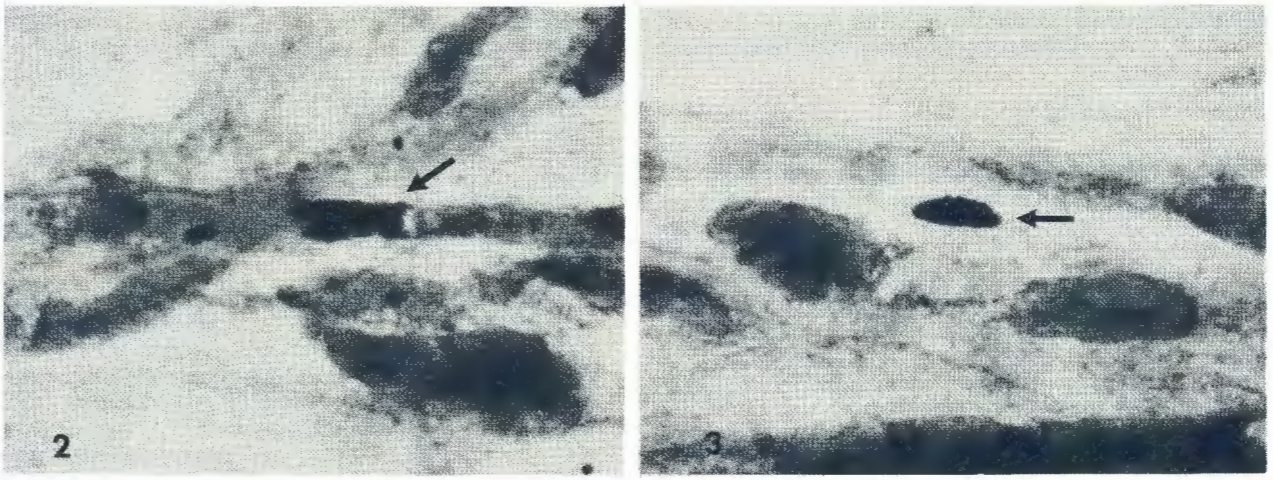

FIG. 2 & 3: Colonies of organisms (arrows) in capillary endothelial cells of a Giemsa-stained brain smear of Sheep 7

val progeny of a single *A. hebraeum* female, placed on this sheep on the 1st day of the febrile reaction, dropped 6–8 days later and were allowed to moult.

Five mice (Mice 4, Fig. 1), inoculated i.v. with 0,2 mℓ of blood collected from Sheep 3 on the first day of the febrile reaction, failed to show any clinical signs and remained serologically negative.

Sheep 4 (Fig. 1), on which 100–150 unfed nymphae that had engorged as larvae on Sheep 3, were allowed to feed 65 days after moulting, showed a moderate, intermittent febrile response, consisting of 4 peaks of short duration 12, 28, 45 and 50 days later. The larval progeny of a single female, placed on Sheep 4 on the 1st of these peaks, dropped 8 days later and were allowed to moult. Fifty unfed, uninfected nymphae, placed on Sheep 4 on the 3rd febrile peak, dropped 6–8 days later. Thirty-three days after moulting, 23 of these ticks were allowed to feed as adults on Sheep 5 (Fig. 1). The latter showed only a mild febrile response and remained seronegative with both antigens.

The 2 sheep on which approximately 300 unfed nymphae that had engorged as larvae on Sheep 4 were allowed to feed 3 and 4 months after moulting, developed severe reactions. The febrile reactions of Sheep 6 and 7 (Fig. 1) commenced 11 and 14 days after the attachment of the ticks, lasted for 8 and 6 days and attained a maximum temperature of 41,7 and 42 °C respectively. Both sheep showed depression, dyspnoea and loss of appetite before dying (Sheep 6) or before being killed *in extremis* (Sheep 7). The salient features at autopsy were oedema of the lungs, splenomegaly and degenerative swelling of the liver and kidneys. Capillary endothelial cells in the brain smears of both sheep revealed a small number of colonies of small size, consisting of basophilic granules which in size, shape and colour were indistinguishable from those of *C. ruminantium* in brain capillary endothelial cells (Fig. 2 & 3). The serum of Sheep 7, collected immediately prior to death, was positive to a titre of 1:1280 in the IFA test, in which both antigens were used. Sheep 6 died overnight and no serum was available for testing.

Five mice (Mice 5, Fig. 1) inoculated i.v. with 0,2 mℓ of blood collected from Sheep 7 on the first day of the febrile reaction, died 10–12 days later. At autopsy hydrothorax was a salient feature in all of

TABLE 2 Cross-challenges between Omatjenne agent and several stocks of *C. ruminantium*

Sheep No.	Infective agent	Reaction	Homologous challenge	Challenge inoculum	Reaction to challenge
8	Omatjenne-infected ticks fed	16/5/41,4;[1] T[2] (17,7)[3]	No reaction	Kümm sheep blood	8/5/40,1(4,7)
9	Omatjenne-infected ticks fed	14/6/40,6;T (16,5)	No reaction	Germishuys sheep blood	No reaction
10	5 mℓ blood Sheep 8	7/7/41,5;T (24,4)	ND[4]	Ball 3 sheep blood	13/6/41,9 (14,1)
11	5 mℓ blood Sheep 8	8/7/41,3;T (19,4)	ND	Kwayanga sheep blood	No reaction
12	5 mℓ pooled blood, Sheep 10 & 11	11/6/41,7;T (18,3)	ND	Breed sheep blood	No reaction
13	Kümm-infected ticks fed	14/14/42;T (27,3)	ND	Omatjenne-infected ticks fed	No reaction
14	Ball 3-infected sheep blood	10/8/42;T (24,2)	No reaction	5 mℓ pooled blood, Sheep 10 & 11	11/6/41,8 (21,2)
15	Ball 3-infected sheep blood	10/5/41,7;T (20,5)	No reaction	5 mℓ pooled blood, Sheep 10 & 11	11/5/41,7, died
16	Control to challenge Sheep 8			Kümm sheep blood	9/8/41,4, died
17	Control to challenge Sheep 9			Germishuys sheep blood	10/7/41,6, died
18	Control to challenge Sheep 11			Kwanyanga sheep blood	11/6/41,8 died
19	Control to challenge Sheep 12			Breed sheep blood	12/3/41,1 died

[1] 16/5/41,4 = The febrile reaction of Sheep 8 commenced on day 16, lasted for 5 days and attained a maximum temperature of 41,4 °C

[2] T = Sheep 8 was treated with oxytetracycline at a dosage rate of 10 mg/kg body mass on the 3rd day of the febrile reaction

[3] (17,7) = The reaction index of Sheep 8 was 17,7

[4] ND = Not done

them and inclusions similar to those seen in the case of Mice 2 were demonstrable in their peritoneal macrophages.

Cross-immunity tests

It can be seen from Table 2 that after 4 passages through *A. hebraeum*, the Omatjenne agent not only elicited severe febrile reactions in sheep, but also that these sheep were immune to challenge with the Kwanyanga, Germishuys, Breed and Kümm stocks of *C. ruminantium*. Sheep 8 showed a mild febrile reaction to challenge with the Kümm stock, but in the reverse challenge (Sheep 13), there was total cross-protection. Sheep 14 and 15, immune to the Ball 3 stock, however, were fully susceptible to challenge with the Omatjenne agent and so was Sheep 10 in the reverse challenge.

DISCUSSION

A. hebraeum has as yet not been reported in the Otjiwarongo district of Namibia where the sera and ticks were collected from cattle (Walker & Olwage, 1987). The *H. truncatum* female from which the Omatjenne agent was isolated originated from these cattle, 81 % of which reacted positively to both the Kümm and Kwanyanga stocks of *C. ruminantium* used as antigen in the IFA test. Thus far only members of the genera *Ehrlichia* and *Cytoecetes* have been shown to cross-react with *C. ruminantium* in the IFA test (Du Plessis *et al.*, 1987; Holland *et al.*, 1987). It is therefore assumed that the Omatjenne agent isolated in a mouse belonged to the *Ehrlichia* genus. The close resemblance between the intracytoplasmic inclusions in the macrophages of the 2nd generation mice, infected i.p. with the Omatjenne agent, and those described in the monocytes of dogs, infected with *E. canis* (Du Plessis, Fourie, Nel & Evezard, 1990), lends further support to this conclusion.

Passaging of the agent in mice, 2 calves and 2 sheep failed to change its pathogenicity in any way. Had it not been for the low level of antibody detected in the sera of the calves and the inclusions observed in the peritoneal macrophages of some of the mice, one might have suspected that the infection had been lost.

Passaging of the agent through 3 generations of *A. hebraeum*, however, enhanced its pathogenicity to the point where it caused a fatal infection in 2 susceptible sheep. The change was abrupt, as the 2 sheep on which the 1st and 2nd generation ticks had fed showed no clinical signs apart from a mild febrile reaction, and no antibody could be detected in their sera.

The change in murinotropism of the agent was also abrupt. While the mouse, infected initially with the *Hyalomma* homogenate, and the mice, inoculated with the blood of Sheep 3 infected through the first generation *Amblyomma* ticks, failed to show any clinical signs, blood from Sheep 7, that had developed a fatal infection after having been infected with the 3rd generation ticks, caused 100 % mortality in mice. The question arises whether the great variation in murinotropism of *Cowdria* stocks (MacKenzie & McHardy, 1987; Du Plessis *et al.*, 1989) is perhaps related to the passage history in nature of a particular stock of *C. ruminantium*, not only in *Amblyomma*, but possibly also at some stage in other tick species.

It was noteworthy that the 2 sheep on which nymphae that had engorged on Sheep 4 were allowed to feed, developed severe, fatal reactions, whereas the adult ticks that had engorged on the same Sheep 4 failed to elicit a reaction in Sheep 5. This can possibly be due to the fact that *Amblyomma* larvae are better able than nymphae to influence the pathogenicity of *Ehrlichia*. A more likely reason, though, is that the larvae had a better chance to pick up the infection from Sheep 4 during the first rise in temperature than the nymphae much later during a subsequent febrile peak.

The question arises whether the Omatjenne agent had increased in virulence and pathogenicity as an *Ehrlichia* sp. *per se*, or whether it adopted the biological characteristics and pathogenicity of *C. ruminantium* after passage through *Amblyomma* ticks. The oedema of the lungs, splenomegaly and nephrosis, observed at autopsy, are consistent with what one sees in heartwater, and the absence of fluid in the pericardial sac and thoracic cavity are by no means rare in cases of heartwater. Colonies of organisms in brain capillary endothelial cells that resemble those seen in heartwater suggest that the Omatjenne agent elicited a disease which in several important respects was indistinguishable from heartwater. It is important to note that, also during the early passaging of the Kümm stock of *C. ruminantium* in sheep, very few colonies of small size were detectable in brain smears (Du Plessis, 1982), so that this phenomenon is not unprecedented in heartwater.

The close resemblance between the colonies in the brain smears of sheep infected with the Omatjenne agent and those seen in the case of Kümm stock *C. ruminantium*, does perhaps raise the possibility that the latter may accidentally have been introduced into the ticks or other infective material used in the present experiments. The solid immunity of sheep that had recovered from infection with the Omatjenne agent against challenge with the Kwanyanga, Breed and Germishuys stocks of *C. ruminantium*, however, proved beyond doubt that the pathogenic agent that had evolved from the Omatjenne agent, was distinct from the Kümm stock, because there is no cross-protection between the latter and the 3 other stocks (Du Plessis *et al.*, 1989; Logan, Birnie & Mebus, 1987).

The high level of antibody, directed against both the Kümm and the Kwanyanga stocks of *C. ruminantium*, detected in the serum of Sheep 7, is consistent with the severe reaction elicited by the Omatjenne agent. Since the level of antibody parallels the severity of the reaction in heartwater (Du Plessis & Malan, 1987a), the high antibody titre in association with the pathological features and positive brain smear points to the involvement of a *Cowdria*-like agent rather than an *Ehrlichia*.

Another possibility that should be considered is that *C. ruminantium* had at some stage been introduced into the Otjiwarongo area of Namibia, e.g. by cattle infested by *A. hebraeum*. Because the latter could not establish itself, *C. ruminantium* was picked up by *H. truncatum*, in which it survived and persisted, but was unable to transmit heartwater. This is unlikely, since experience with a method to determine the *C. ruminantium* infection rate of *A. hebraeum* (Du Plessis, 1985) has repeatedly shown that the inoculation into a susceptible sheep of homogenized spleen of a serologically positive but clinically healthy mouse that had been injected with a tick homogenate, consistently elicited clinical or fatal heartwater. In the case of the Omatjenne agent this did not happen.

If one were to accept that a mildly pathogenic *Ehrlichia*-like agent had been transformed into a highly pathogenic *Cowdria,* the deduction would be justified that *Ehrlichia* and *Cowdria* are one and the same parasite, manifesting biological differences, dependent on whether they parasitize *H. truncatum* and possibly other tick species on one hand or *Amblyomma* on the other.

This would have important epidemiological implications. Firstly, if *Amblyomma* spp. free from *C. ruminantium* exist or were able to establish in an area where other ticks infected with *Ehrlichia* infest wild and domestic hosts, the transformation of the latter into *Cowdria* may lead to the appearance of heartwater. Secondly the interpretation of IFA test serology would be easier, in that seropositives in areas where *Amblyomma* does not occur, would be attributable to *Ehrlichia.* Seropositives in areas where *Amblyomma* does occur would, on the other hand, rather be attributable to *C. ruminantium,* since *Ehrlichia* would sooner or later be transformed into *Cowdria.*

This is merely a preliminary communication, and the author is well aware of the fact that the conclusions drawn are based on a single experiment that must be repeated.

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REFERENCES

BEZUIDENHOUT, J. D., 1987. The present state of *Cowdria ruminantium* cultivation in cell lines. *Onderstepoort Journal of Veterinary Research,* 54, 205–210.

DU PLESSIS, J. L., 1975. Histopathological studies on the pathogenesis of heartwater as manifested in mice infected with a strain of *Cowdria ruminantium.* M. Med. vet thesis, University of Pretoria.

DU PLESSIS, J. L., 1982. Mice infected with a *Cowdria ruminantium*-like agent as a model in the study of heartwater. D. V. Sc. thesis, University of Pretoria.

DU PLESSIS, J. L., 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum:* Effects in mice injected with tick homogenates. *Onderstepoort Journal of Veterinary Research,* 52, 55–61.

DU PLESSIS, J. L. & MALAN, LETITIA, 1987a. The block method of vaccination against heartwater. *Onderstepoort Journal of Veterinary Research,* 54, 493–495.

DU PLESSIS, J. L. & MALAN, LETITIA, 1987b. The application of the indirect fluorescent antibody test in research on heartwater. *Onderstepoort Journal of Veterinary Research,* 54, 319–325.

DU PLESSIS, J. L., CAMUS, E., OBEREM, P. T. & MALAN, LETITIA, 1987. Heartwater serology: Some problems with the interpretation of results. *Onderstepoort Journal of Veterinary Research,* 54, 327–329.

DU PLESSIS, J. L., VAN GAS, L., OLIVIER, J. A. & BEZUIDENHOUT, J. D., 1989. The heterogenicity of *Cowdria ruminantium* stocks: Cross-immunity and serology in sheep and pathogenicity to mice. *Onderstepoort Journal of Veterinary Research,* 56, 195–202.

DU PLESSIS, J. L., FOURIE, N., NEL, P. W. & EVEZARD, D. N., 1990. Concurrent babesiosis and ehrlichiosis in the dog: Serology and blood smear examination. *Onderstepoort Journal of Veterinary Research,* 57, 151–155.

HEYNE, HELOISE, ELLIOTT, E. G. & BEZUIDENHOUT, J. D., 1987. Rearing and infection techniques for *Amblyomma* species to be used in heartwater transmission experiments. *Onderstepoort Journal of Veterinary Research,* 54, 461–471.

HOLLAND, C. J., LOGAN, L. L., MEBUS, C. A. & RISTIC, M., 1987. The serological relationship between *Cowdria ruminantium* and certain members of the genus *Ehrlichia. Onderstepoort Journal of Veterinary Research,* 54, 331.

LOGAN, L. L., BIRNIE, E. F. & MEBUS, C. A., 1987. Cross-immunity between isolates of *Cowdria ruminantium. Onderstepoort Journal of Veterinary Research,* 54, 345.

MACKENZIE, P. K. I. & VAN ROOYEN, R. E., 1981. The isolation and culture of *Cowdria ruminantium* in albino mice. *Proceedings of International Congress on Tick Biology and Control, Rhodes University, Grahamstown,* 1981, 33–39.

MACKENZIE, P. K. I. & MCHARDY, N., 1987. *Cowdria ruminantium* infection in the mouse: A review. *Onderstepoort Journal of Veterinary Research,* 54, 267–269.

WALKER, JANE B. & OLWAGE, A., 1987. The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus *Amblyomma*) and their distribution. *Onderstepoort Journal of Veterinary Research,* 54, 353–379.