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

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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

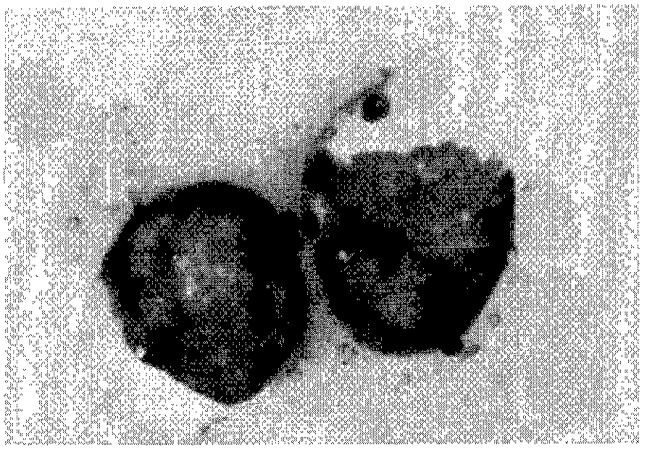

Photo 3 : Ring-like colony of ehrlichial organisms in the monocyte of Lamb 3 (x 5,000).

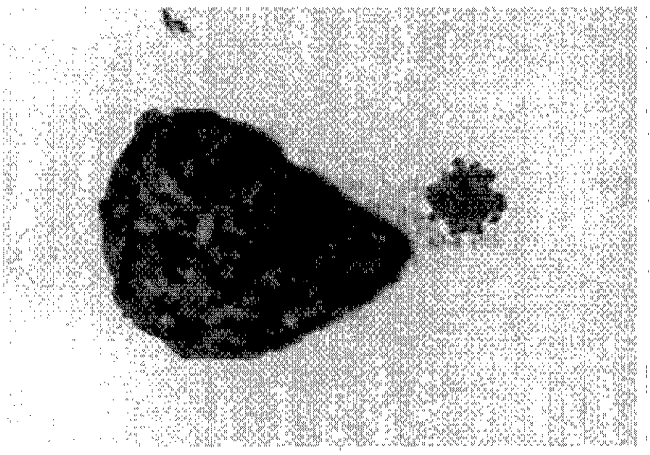

Photo 4 : Ehrlichial morula in the monocyte of Lamb 3 (x 5,000).

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.

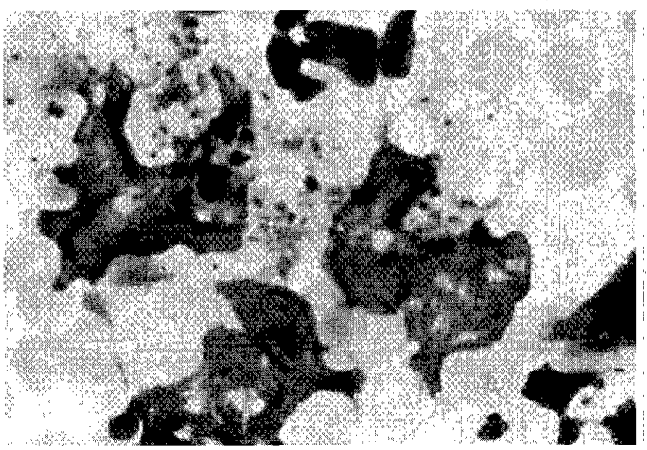

Photo 5 : Large monocytes with bizarre-shaped nuclei and organisms in their cytoplasm in the blood smear of Sheep 35 (x 5,000).

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).

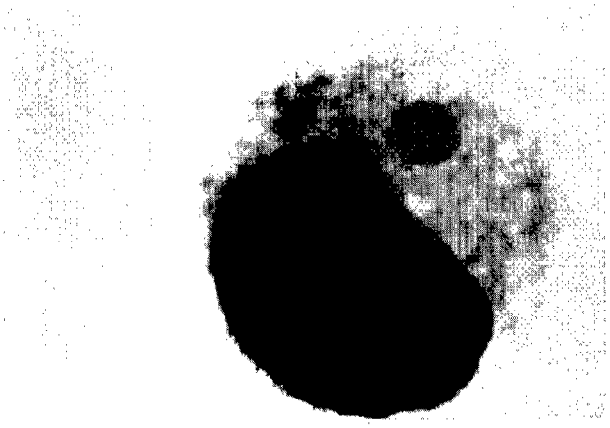

Photo 8 : Ring-like colony in the cytoplasm of a mouse peritoneal macrophage, with the organisms distributed on the periphery of the inclusion (x 5,000).

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.