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

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

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

INTRODUCTION

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

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

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MATERIALS AND METHODS

Sera

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

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

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

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

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

Serological tests

IFA test

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

Competitive ELISA (cELISA)

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

Indirect ELISA

The antigen for the indirect ELISA (Guadeloupe) was prepared from *C. ruminantium*-infected bovine umbilical endothelial cell cultures (17). When approximately 80 % of the cell monolayer was destroyed, the remaining adherent cells were scraped off, mixed with the supernatant and certifuged at 2500 g for 15 min. The pellet was resuspended in sterile PBS and sonicated 5 times for 30 s in an ethanol-dry ice bath. Microplates were coated overnight at 37°C with 5 µg/ml of antigen in a carbonate-bicarbonate 0.1M buffer, pH 9.5 (100 µl per well). The plates were washed 3 times with phosphate buffered saline (0.1M, pH 7.2), supplemented with 0.1 % Tween-20. To each well was added 100 µl of test serum (diluted 1:800 for cattle, 1:100 for sheep and 1:400 for goats) in PBS-Tween, with 3 % skimmed cow milk as a blocking buffer. The plates were incubated for 1h at 37°C and washed 5 times in PBS-Tween. Horse radish peroxidase conjugated rabbit anti-goat, anti-bovine or anti-sheep IgG, optimally diluted in the blocking buffer, was added at 100 µl per well and the plates incubated for 1h at 37°C. After 5 washings, each well was filled with 100 µl of citrate buffer (0.1M, pH 5.5) containing 0.5 mg/ml 0-phenylene diamine and 3 µl/ml of 9 % H_20_2. The enzymatic reaction was stopped after 30 min of incubation at room temperature by adding 50 µl of 2N H_2S0_4 and the absorbance read at 495 nm.

Western blots

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

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

RESULTS

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

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

TABLE I Results of 5 serological tests on sheep sera.

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

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

TABLE II Results of 5 serological tests on bovine sera.

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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