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## The immunodominant 32-kilodalton protein of *Cowdria ruminantium* is conserved within the genus *Ehrlichia*

JONGEJAN (F.), DE VRIES (N.), NIEUWENHUIJS (J.), VAN VLIET (A.H.M.), WASSINK (L.A.). La protéine immunodominante de *Cowdria ruminantium* Cr32 conservée dans le genre *Ehrlichia*. *Revue Elev. Méd. vét. Pays trop.*, 1993, 46 (1-2) : 145-152

Les tests sérologiques pour la cowdriose sont perturbés par des réactions croisées dues à des anticorps présents chez des animaux soupçonnés d'être infectés par *Ehrlichia* spp. On a contrôlé des infections expérimentales par *Ehrlichia bovis*, *E. ovina*, *E. canis* et *E. phagocytophila*, par l'ELISA de compétition, le western blotting et l'immunofluorescence, utilisant des antigènes de cultures de cellules endothéliales infectées de *Cowdria*. Les réactions croisées par des anticorps contre *Ehrlichia* sont attribuables à leur reconnaissance d'épitopes sur la protéine immunodominante de *Cowdria*, Cr32. Ceci est surtout vrai pour *E. canis* et *E. ovina*, beaucoup moins pour *E. bovis*, mais pas du tout pour *E. phagocytophila*. De plus, une réaction croisée forte fut démontrée entre *Cowdria* et des anticorps contre *E. chaffeensis*. Ces résultats concordent avec les relations phylogénétiques trouvées récemment entre *Cowdria* et d'autres membres de la tribu des Ehrlichieae par VAN VLIET *et al.* en 1992, qui montrent que *Cowdria* est étroitement apparentée à *E. canis* et également à *E. chaffeensis*. Bien que les tests utilisés au cours de cette étude restent des outils de valeur dans les conditions du laboratoire, leur spécificité doit être améliorée. Il est proposé d'étudier des antigènes recombinants de *Cowdria* pour le développement de tests sérologiques de deuxième génération pour la maladie.

**Mots clés :** Protéine - *Cowdria ruminantium* - *Ehrlichia* - Technique immunologique - Test ELISA - Western blotting - Immunofluorescence - Culture de cellule - Anticorps - Antigène.

### INTRODUCTION

Cowdriosis or heartwater, caused by the tick-borne rickettsia *Cowdria ruminantium*, occurs in domestic and wild ruminants and is considered one of the most important tickborne diseases in sub-Saharan Africa (17). The presence of the tick *A. variegatum*, one of the main vectors of cowdriosis, on most of the islands in the Caribbean region and the existence of the disease on at least three of these islands, constitutes a major threat for livestock on the American mainland (1).

Serodiagnosis of *Cowdria* is a possible means to assess the prevalence and present distribution of heartwater in Africa and the Caribbean in domestic and wildlife hosts (10,11,16).

So far four types of antibody detection assays, based on endothelial cell culture antigens, have been developed : an indirect fluorescent antibody (IFA) test, (13), an indirect ELISA (14) and two tests based on recognition of the immunodominant Cr32 *Cowdria* antigen, i.e. a competitive enzyme-linked immunosorbent assay (cELISA) (7,9) and Western blots (16). A recent critical interlaboratory comparison has however indicated that cross-reactions occur in all tests currently available, probably due to *Ehrlichia* species (5).

In this study we tested antisera, obtained from experimental animals infected with different species of *Ehrlichia* (*E. bovis*, *E. ovina*, *E. canis* and *E. phagocytophila*), using immunofluorescence, competitive ELISA and Western blots to determine whether epitopes on the immunodominant Cr32 *Cowdria* antigen are also recognized by antibodies to *Ehrlichia*.

### MATERIAL AND METHODS

#### Cultivation

*Cowdria* antigens for cELISA, immunofluorescence and Western blotting were prepared from rickettsiae cultivated in Bovine Umbilical Endothelial cells (BUE) as described previously (9). Briefly, monolayers of BUE cells were grown in HEPES buffered RPMI 1640 medium supplemented with antibiotics and 10 % newborn calf serum. Infection of BUE cell cultures with *Cowdria* was initiated by inoculation of BUE culture supernatant, which had been stored at -80 °C in sucrose-phosphate-glutamate (SPG) buffer (2). Infected cultures were maintained in Glasgow Minimal Essential Medium (GMEM), supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (1.25 µg/ml) HEPES buffer (20 mM; pH 7.0 to 7.2), L-glutamine (2 mM), 10 % newborn calf serum and tryptose phosphate broth (2.9 g/l).

Incubation was carried out at 37°C on a slowly rocking platform. Samples of BUE cells were scraped from the bottom of the culture flask, smeared onto a glass slide, and examined for *Cowdria* inclusions after staining with Diff-Quik (Merz & Dada AG, Dudingon, Switzerland). Cultures with virtually all BUE cells infected with reticulate bodies of *Cowdria* and containing large numbers of extracellular elementary bodies in the supernatant were used as antigen for serology.

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## Competitive ELISA

Infected BUE cultures were centrifuged at 4 °C for 15 min at 15,000 g. Pellets were resuspended in PBS and sonicated on ice for four periods of 15 sec with 1 min intervals (Vibracell, Sonics materials Inc., USA). The protein concentration was determined according to Bradford and sonicates were stored at -20 °C.

The cELISA was performed as described previously (9), with some modifications. Polystyrene 96-well flat-bottomed plates (Nunc) were coated overnight at 37 °C with 6 µg/ml sonicated *Cowdria* antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Plates were washed three times with tap water. Test serum (1:50 dilution) was applied, simultaneously with Mab 4F10B4 (9) (1:400), both diluted in phosphate-buffered saline (PBS) (pH 7.2), containing 3 % skimmed milk and 0.05 % Tween 20 and incubated at 37 °C for 1 h. After washing, peroxidase labeled rabbit anti-mouse immunoglobulin (Dakopatts, Denmark), at a 1:750 dilution, was added and incubated at 37 °C for 1 h. After washing, 100 µl of 0.1 M citrate buffer (pH 5.5), containing ABTS and hydrogen peroxide were added to each well. The optical density (OD) was read at 405 nm on an ELISA plate reader after 30 min incubation at room temperature in the dark.

*Cowdria* positive control sera and negative control sera were included on each plate in duplicate. Percentage inhibition was calculated based on the extinction obtained after incubation with monoclonal antibody without test serum.

## Western blots

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 modified after an earlier description (9). Electrophoretic transfer was carried out at 100 V for 1 h or at 20 V overnight. Blots were quenched for 1 h in PBS/5 % skimmed milk and incubated for 3 h with test serum, positive or negative control serum or monoclonal antibody 4F10B4, diluted 1:100 in PBS containing 0.02 % Tween 20 and 5 % skimmed milk.

Bound antibodies in test sera were visualized by incubation with either rabbit anti-bovine (1:2000), rabbit anti-ovine (1:2500), rabbit anti-goat (1:5000), rabbit anti-dog (1:5000) or rabbit anti-mouse (1:500) immunoglobulins conjugated with alkaline phosphatase (Sigma) diluted in PBS containing 5 % skimmed milk. After washing, binding of conjugate was visualized by the addition of 100 mM Tris-HCl buffer (pH 9.5), supplemented with 100 mM NaCl, 5 mM MgCl<sub>2</sub>, nitroblue tetrazolium (NBT) and 5-bromo-4-dichloro-3-indocyl phosphate (BCIP). The reaction was stopped by extensive washing with a 20 mM TrisHCl buffer of pH 8.0 containing 5 mM EDTA.

## Immunofluorescence

BUE cultures infected with *Cowdria* were centrifuged at 4 °C for 15 min at 15,000 g. Pellets were resuspended in PBS, spotted onto microscope slides, dried and fixed in acetone. The slides were incubated with two-fold titrations of antisera in PBS starting from 1:80 up to 1:20,480. Fluorescein isothiocyanate-labeled antibodies, *i.e.* rabbit anti-bovine, rabbit anti-sheep, rabbit anti-goat, or rabbit anti-dog immunoglobulins were used as second antibodies. Fluorescence was observed with an Olympus BH2-RFL microscope.

## Experimental infections

Clinical manifestations of the experimental infections with *Ehrlichia* spp. in calves (Friesian), sheep (Tesselaar), goats (Saanen) and dogs (Beagle) are shown in table I.

TABLE I. Clinical manifestations of experimental *Ehrlichia* infections.

| Animal number | Incubation period | Peak temp. (°C) | Rickettsaemia* | Treatment           |
|---------------|-------------------|-----------------|----------------|---------------------|
| dog 8765      | 12                | 41.0            | (+)            | oxytetracycline (1) |
| dog 8302      | 11                | 41.1            | nps            | oxytetracycline (1) |
| calf 456      | 14                | 40.0            | (+)            | —                   |
| calf 70       | 17                | 40.4            | nps            | —                   |
| sheep 8440    | 11                | 41.2            | (+)            | —                   |
| sheep 8513    | 9                 | 40.5            | (+)            | —                   |
| goat 8769     | 5                 | 41.3            | ++             | —                   |
| goat 8727     | 4                 | 41.0            | ++             | —                   |

\* rickettsaemia: (+): scanty *Ehrlichia* inclusion bodies in monocytes; ++: large number of *Ehrlichia* inclusion bodies in granulocytes; nps: no parasite seen.

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### *Ehrlichia bovis*

The strain of *E. bovis*, originating from Kenya (15), caused moderate but prolonged fever in Friesian calves. In this study clinical reactions were monitored in two experimental calves (nos. 70 and 456). (Calf 456 was infected with *Cowdria* before infection with *E. bovis*).

### *Ehrlichia canis*

A pathogenic isolate of *E. canis* was obtained from a clinical case submitted to Dr. R.J. Slappendel of the Small Animal Veterinary Clinic at the University of Utrecht, The Netherlands. Two experimental dogs were infected and monitored (nos. 8765 and 8302).

### *Ehrlichia ovina*

*E. ovina* was monitored in two sheep (nos. 8440 and 8513). The isolate of *E. ovina* originated from Turkey and caused prolonged fever periods in Dutch splenectomized sheep, which eventually recovered spontaneously (G. UILENBERG, unpublished data).

### *Ehrlichia phagocytophila*

*E. phagocytophila*, causal agent of tick-borne fever of ruminants, was isolated from cattle on the North Sea Island of Ameland, The Netherlands. The infection was studied in two goats (Nos. 8727 and 8769).

### *Cowdria ruminantium*

Two stocks of *Cowdria* were used : the Senegal isolate (6) and an isolate from Zambia (Lutale) (6). Antisera to the Senegal isolate were raised in two experimental sheep (nos. 50 and 8849) and in two goats (nos. 8737 and 8907) after intravenous inoculation of 2 ml aliquots of thawed blood stabilate. Antiserum to the Lutale isolate was raised in calf no. 57 after inoculation of 20 ml infected bovine blood. Calf 456 was infected with *Cowdria* (Lutale isolate) through feeding of infected *Amblyomma variegatum* ticks and six months later the animal was infected with *E. bovis*. Goat no. 8769 was infected with the Senegal isolate of *Cowdria* after being initially infected with *E. phagocytophila*.

Clinical reactions were treated with Terramycin LA (Pfizer). Antisera were collected 4 to 6 weeks post infection (p.i.) and stored at -20°C until further use as positive control sera.

All *Ehrlichia* and *Cowdria* isolates were stored in liquid nitrogen as infected blood stabilates, cryopreserved with 10 % dimethylsulphoxide, before being used in this study. Aliquots of 2 ml of deepfrozen blood were rapidly thawed and inoculated intravenously. The animals were monitored by daily recording of their rectal temperature and by clinical inspection. Sera were collected from all experimental animals once a week. Preinfection sera were used as negative controls.

## RESULTS

Clinical manifestations of the different experimental *Ehrlichia* infections are given in table I. Only infections with *E. canis* required antibiotic treatment. Scanty ehrlichial inclusion bodies in monocytes were seen in dog 8765 and calf 456. Monocytes infected with morulae of *E. ovina* were detected on several occasions during the febrile reaction in sheep. Infections with *E. phagocytophila* in goats were characterized by large numbers of ehrlichial inclusion bodies in granulocytes during fever.

Figure 1 shows an SDS-PAGE of a *Cowdria* -infected BUE cell sonicate containing the Cr32 protein. This sonicate was used as antigen for cELISA and immunoblotting. Antibodies to *Ehrlichia canis* competed strongly with anti-Cr32 monoclonal antibody for binding sites on the Cr32 molecule in competitive ELISA. High antibody titres (5120) were also detected by immunofluorescence (fig. 2, table II). Immunodominant recognition of epitopes on Cr32 by *E. canis* antibodies is shown by immunoblotting

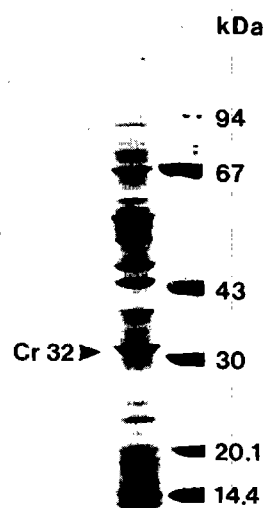


Figure 1 : SDS-PAGE of *Cowdria* (Senegal) infected BUE cell culture sonicate. The Cr32 antigen is indicated.

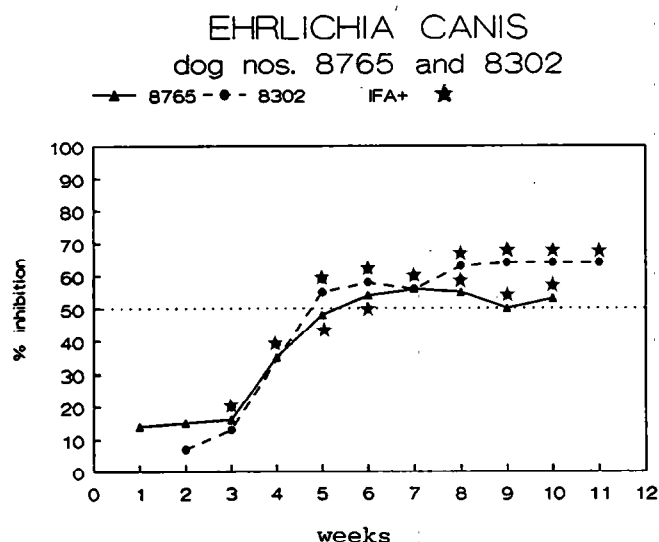


Figure 2 : Reactivity of sera from two dogs (nos. 8302 and 8765), infected with *Ehrlichia canis*, with epitopes on the Cr32 *Cowdria* antigen using competitive ELISA (cut-off 50% inhibition). Positive reactions determined by immunofluorescence are indicated by an asterisk (\*). Both animals were infected during the first week.

**TABLE II** Cross-reactions between Ehrlichia and Cowdria determined by IFAT

| Antigen                  | Cowdria ruminantium | Ehrlichia canis |
|--------------------------|---------------------|-----------------|
| antiserum                |                     |                 |
| Cowdria ruminantium      | 10240               | 5120            |
| Ehrlichia canis          | 5120                | 20480           |
| Ehrlichia bovis          | 320                 | nd              |
| Ehrlichia ovina          | 640                 | nd              |
| Ehrlichia phagocytophila | 80                  | nd              |
| Ehrlichia chaffeensis*   | 1280                | nd              |

nd : not determined.

\* antiserum from a patient with human ehrlichiosis obtained from the Centers for Disease Control, Atlanta, Georgia, USA.

(fig. 3, 4). Several other proteins of approximate molecular mass of 27 kDa (fig. 3, 4), 40 kDa (fig. 3) and 50 kDa (fig. 4) were also recognized.

An antibody profile similar to the one shown in fig. 2 was found in cELISA when antibodies against *E. ovina* were used (fig. 5). However, titres detected by immunofluorescence were much lower (640) with *E. ovina* antibodies than with antibodies to *E. canis* (table II). Also, in Western blots no other epitopes were recognized than those on the Cr32 protein (fig. 6, 7).

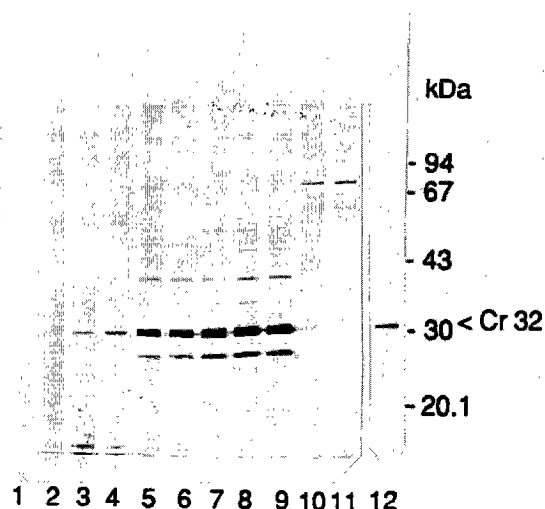


Figure 3 : Western blot analysis of sera from dog no. 8765 infected with Ehrlichia canis on Cowdria antigen.

Lane 1, pre-infection serum; lanes 2-9, sera collected respectively during week 2 and 4-10 post infection; lane 10, non-infected control serum from dog no.138; lane 11, non-infected control serum from dog no. 143; lane 12, monoclonal antibody 4F10B4 directed against Cr32. Molecular mass markers are indicated on the right.

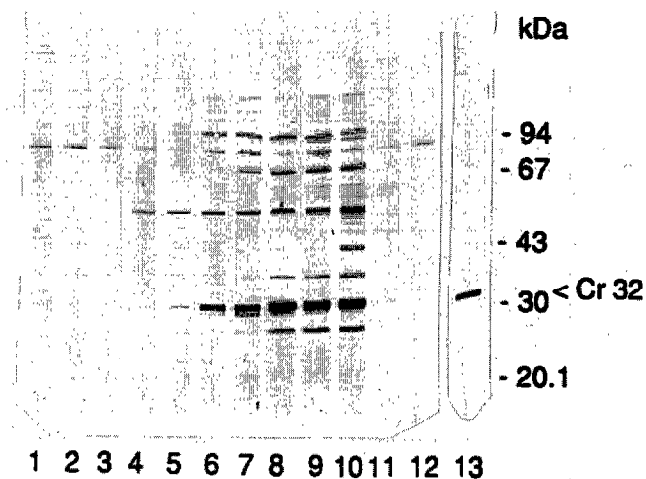


Figure 4 : Western blot analysis with sera from dog no. 8302 infected with Ehrlichia canis on Cowdria antigen.

Lane 1, pre-infection serum; lanes 2-9, sera collected respectively during week 2-10 post infection; lane 10, non-infected control serum from dog no. 138; lane 11, non-infected control serum from dog no. 143; lane 12, non-infected control serum from dog no. 143; lane 13, monoclonal antibody 4F10B4 against Cr32.

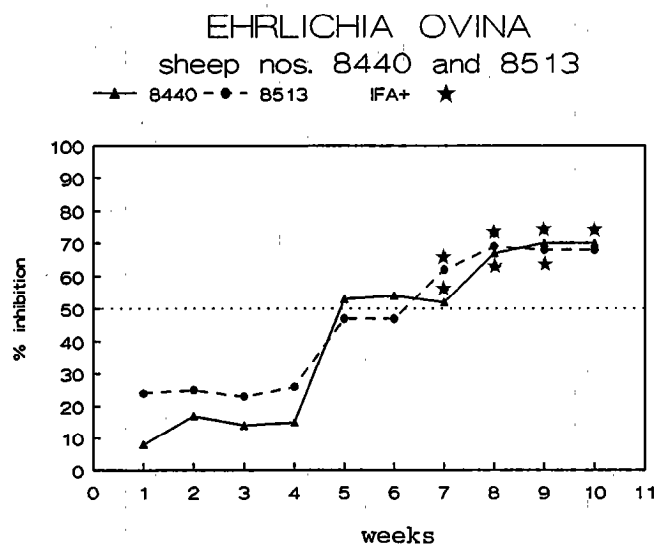


Figure 5 : Reactivity of sera from two sheep (nos. 8440 and 8513) infected with Ehrlichia ovina with epitopes on the Cr32 Cowdria antigen using competitive ELISA (cut-off 50 % inhibition). Positive reactions determined by immunofluorescence are indicated by an asterisk (\*). Both animals were infected at week 2.

Calf 456 was infected with Cowdria (Lutale) before infection with *E. bovis*. This explains the additional antibody peak in cELISA (fig. 8) and the reactivity with the Cr32 protein before the animal was infected with *E. bovis* (fig. 9). Interestingly, antibodies to *E. bovis* also recognized epitopes on an approximately 21 kDa Cowdria protein (fig. 9). Antibodies raised against *E. bovis* in calf no. 70

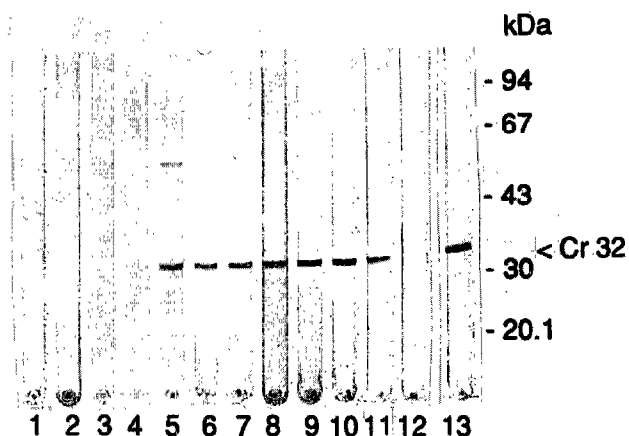


Figure 6 : Western blot analysis with sera from sheep no. 8440, infected with Ehrlichia ovina, on Cowdria antigen.

Lanes 1 and 2, pre-infection sera; lanes 3-10, sera collected during weeks 1-8 post infection, respectively; lane 11, positive control serum from sheep no. 8849; lane 12, negative control serum; lane 13, monoclonal antibody 4F10B4 to Cr32.

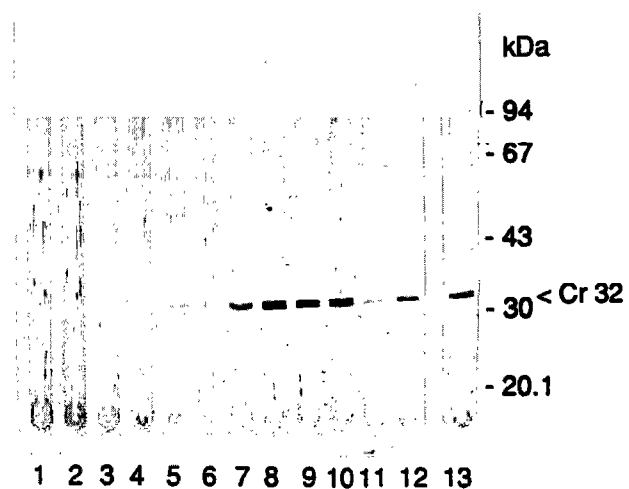


Figure 7 : Western blot analysis with sera from sheep no. 8513 infected with Ehrlichia ovina on Cowdria antigen.

Lanes 1 and 2, pre-infection sera; lanes 3-10, sera collected during weeks 1-8 post infection; lane 11, positive control serum from sheep no. 50; lane 12, positive control serum from sheep no. 8849; lane 13, monoclonal antibody 4F10B4 against Cr32.

did not recognize the Cr32 protein and antibodies of a maximum titre of only 320 were detected by immunofluorescence (fig. 10, table II).

Antibodies against *E. phagocytophila* were not detected in cELISA and Western blots (fig. 11, 12). Goat 8769 was challenged with *Cowdria* (Senegal) after the *E. phagocytophila* infection. Therefore, positive reactions in cELISA

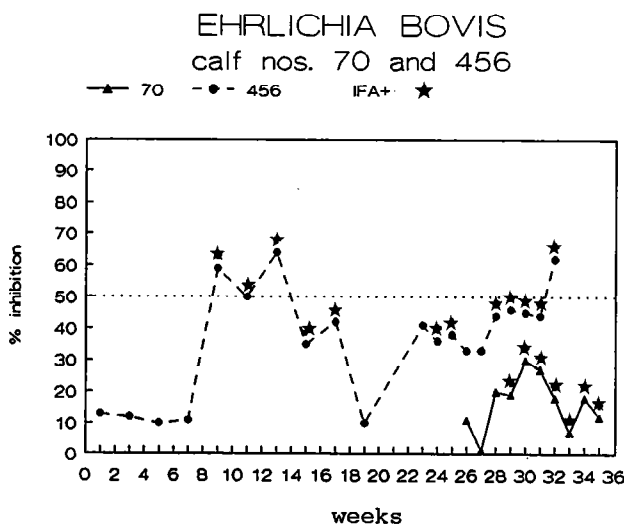


Figure 8 : Reactivity of sera from two calves (nos. 70 and 456), infected with Ehrlichia bovis, with epitopes on the Cr32 Cowdria antigen using competitive ELISA (cut-off 50 % inhibition). Positive reactions determined by immunofluorescence are indicated by an asterisk (\*). Calf 456 was infected with Cowdria (Lutale isolate) in week 3 and with E. bovis in week 26. Calf 70 was infected at week 26.

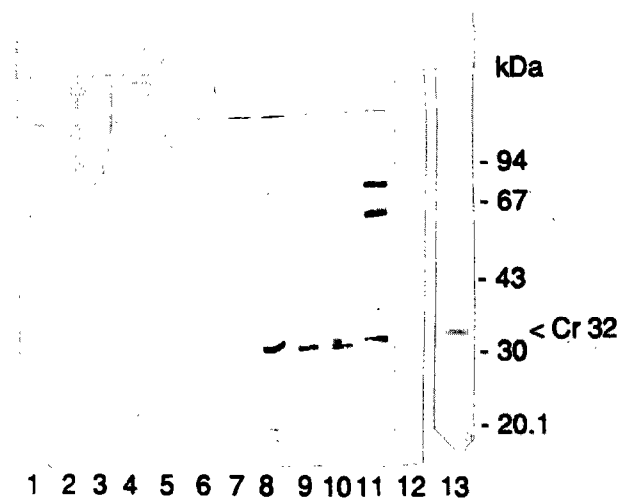


Figure 9 : Western blot analysis with sera from calf no. 456, infected with Ehrlichia bovis on Cowdria antigen.

Lanes 1-3, sera collected during weeks 23-25 post-Cowdria infection, lanes 4-10 (infection with E. bovis) sera collected between weeks 26 and 32 (fig. 8); lane 11, positive control serum from calf no. 57; lane 12, negative control from calf no 63; lane 13, monoclonal antibody 4F10B4 against Cr32. Calf 456 was infected with Cowdria (Lutale isolate) at week 3 and with E. bovis at week 26.

at week 4, see fig. 11, corresponding to lane 10 in fig. 12, are due to infection with *Cowdria*. Low levels of cross-reacting antibodies (80 or less) were detected by immunofluorescence (table II).



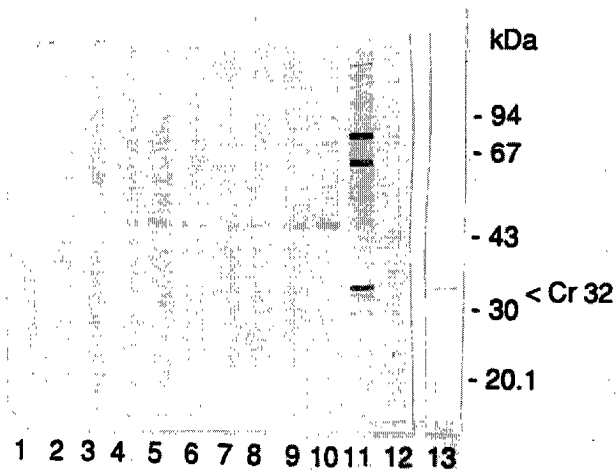


Figure 10 : Western blot analysis with sera from calf no. 70, infected with *Ehrlichia bovis*, on *Cowdria* antigen.

Lane 1, pre-infection serum ; lanes 2-10, sera collected during weeks 2 to 10 post-infection ; lane 11, positive control serum from calf no. 57 ; lane 12, negative control serum from calf no. 63 ; lane 13, monoclonal antibody 4F10B4 against Cr32. Molecular mass markers are indicated on the right.

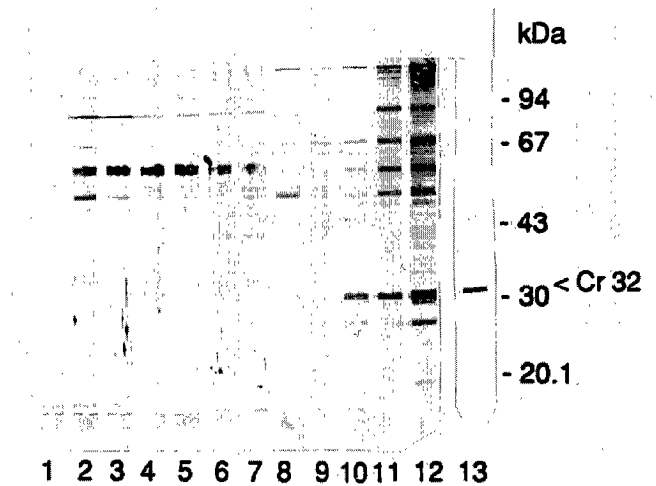


Figure 12 : Western blot analysis with sera from goats (nos. 8727 and 8769), infected with *Ehrlichia phagocytophila*, on *Cowdria* antigen.

Lanes 1-7, sera from goat no. 8727. Lane 1, pre-infection serum ; lanes 2-7, post-infection sera ; lanes 8-10, sera from goat no. 8769 ; lane 8, one week post-Ehrlichia infection ; lane 9, three weeks post-Ehrlichia infection ; lane 10, four weeks post-Ehrlichia infection and two weeks post-Cowdria infection ; lane 11, positive control serum from goat no. 8737 ; lane 12, positive control serum from goat no. 8907 ; lane 13, monoclonal antibody 4F10B4 against Cr32. Molecular mass markers are indicated on the right.

### EHRlichia PHAGOCYTOPHILA goat nos. 8727 and 8769

—▲— 8727 —●— 8769 IFA+ ★

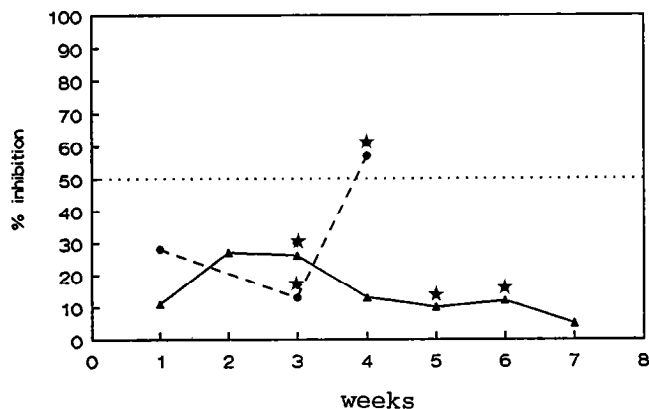


Figure 11 : Reactivity of sera from two goats (nos. 8727 and 8769), infected with *Ehrlichia phagocytophila*, with epitopes on the Cr32 *Cowdria* antigen using competitive ELISA (cut-off 50 % inhibition). Positive reactions determined by immunofluorescence are indicated by an asterisk (\*). Goat no. 8769 was infected with *Cowdria* (Senegal) after infection with *phagocytophila*.

Finally, antibodies against the human pathogen *E. chaffeensis* strongly cross-reacted with *Cowdria* antigens in the IFA test (table II).

### DISCUSSION

Already the IFA tests based on infected neutrophils or macrophages were known to have limited specificity due to cross-reactions with *Ehrlichia* species (3, 4, 8, 12). Here we demonstrate that serological tests based on endothelial cell culture antigens are also hampered by cross-reactive epitopes shared between *Cowdria* and *Ehrlichia*. False positive serological results in competitive ELISA and Western blots due to cross-reacting antibodies against *Ehrlichia* can be attributed to the recognition of epitopes on the immunodominant Cr32 molecule. This is especially true for *E. canis* and *E. ovina*, much less so for *E. bovis* and not at all for *E. phagocytophila*.

In addition, strong cross-reactivity between *Cowdria* antigens and antibodies to *E. chaffeensis* was demonstrated for the first time (table II). Cross-reactivity between *Cowdria* and *E. canis* goes beyond the recognition of Cr32 epitopes only, since several other proteins preliminarily characterized as 27 kDa, 40 kDa and 50 kDa were shown on blots (fig. 3, 4). This was not the case for anti-*E. ovina* antibodies, which recognized Cr32 epitopes only (fig. 6, 7).

Our findings are in agreement with the recently reported phylogenetic relationships between *Cowdria* and other members of the tribe Ehrlichieae (18), wherein *Cowdria*, *E. canis* and *E. chaffeensis* proved to be closely related.

Antibodies to the more distantly related *E. phagocytophila* did not compete in the cELISA and only a low level of cross-reactive antibodies were detected by immunofluorescence. Although the phylogenetic position of *E. ovina* and *E. bovis* has not been studied, one could predict, based on the results obtained in this study, that both rickettsiae may have an intermediate position between the *Cowdria/E. canis/E. chaffeensis* cluster and the *E. phagocytophila/E. equi* cluster.

In conclusion, it is clear that there is a serious specificity problem in all serological tests developed for cowdriosis so far. However, the different serological tests remain valuable tools for laboratory controlled conditions and can provide useful information when used in epidemiological surveys.

Production of *Cowdria* antigens in endothelial cell cultures is relatively laborious and expensive. Moreover, the quality varies between antigen batches and this requires checkerboard titrations for every antigen batch to determine the correct test conditions. Recombinant antigens can be produced in large batches of high quality and at a low cost. It is therefore particularly relevant that we have recently cloned the gene encoding the Cr32 protein of *C. ruminantium* (19). Cloning of this gene makes it feasible to investigate whether recombinant *Cowdria* antigen can be used for second generation serological tests for cowdriosis. Expression of parts of the Cr32 protein could be used to differentiate *Cowdria* and *Ehrlichia*-specific domains by screening with polyclonal species-specific antisera. This approach is currently under investigation.

## ACKNOWLEDGEMENTS

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## REFERENCES

- BARRÉ (N.), UILENBERG (G.), MOREL (P.C.), CAMUS (C.). Danger of introducing heartwater onto the American mainland: Potential role of indigenous and exotic *Amblyomma* ticks. *Onderstepoort J. Vet. Res.*, 1987, **54** : 405-417.
- BOVARNICK (M.R.), MILLER (J.C.), SNYDER (J.C.). The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. *J. Bacteriol.*, 1950, **59** : 509-522.
- CAMUS (E.). Contribution à l'étude épidémiologique de la cowdriose (*Cowdria ruminantium*) en Guadeloupe. Thèse Doct. ès-Sci., Centre d'Orsay, Université de Paris-Sud, 1987. 202 p.
- DU PLESSIS (J.L.), CAMUS (E.), OBEREM (P.T.), MALAN (L.). Heartwater serology : Some problems with the interpretation of results. *Onderstepoort J. vet. Res.*, 1987, **54** : 327-329.
- DU PLESSIS (J.L.), BEZUIDENHOUT (J.D.), BRETT (M.S.), CAMUS (E.), JONGEJAN (F.), MAHAN (S.M.), MARTINEZ (D.). The sero-diagnosis of heartwater : A comparison of 5 tests. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 123-129.
- JONGEJAN (F.), UILENBERG (G.), FRANSSSEN (F.F.J.), GUEYE (A.), NIEUWENHUIJS (J.). Antigenic differences between stocks of *Cowdria ruminantium*. *Res. Vet. Sci.*, 1988, **44** : 186-189.
- JONGEJAN (F.), THIELEMANS (M.J.C.). Identification of an immunodominant antigenically conserved 32-kilodalton protein from *Cowdria ruminantium*. *Infect. Immun.*, 1989, **57** : 3243-3246.
- JONGEJAN (F.), WASSINK (L.A.), THIELEMANS (M.J.C.), PERIÉ (N.M.), UILENBERG (G.). Serotypes in *Cowdria ruminantium* and their relationship with *Ehrlichia phagocytophila* determined by immunofluorescence. *Vet. Microbiol.*, 1989, **21** : 31-40.
- JONGEJAN (F.), THIELEMANS (M.J.C.), DE GROOT (M.), VAN KOOTEN (P.J.S.), VAN DER ZEIJST (B.A.M.). Competitive enzyme-linked immunosorbent assay for heartwater using monoclonal antibodies to a *Cowdria ruminantium*-specific 32 kilodalton protein. *Vet. Microbiol.*, 1991, **28** : 199-211.
- JONGEJAN (F.). Serodiagnosis of *Cowdria ruminantium* : Current status. In : DOLAN (T.T.), Ed. Recent Developments in the Control of Anaplasmosis, Babesiosis and Cowdriosis : Proceedings of a Workshop held at ILRAD, Nairobi, Kenya, 13-15 May 1991. Nairobi, ILRAD, 1992.
- KOCK (N.D.), JONGEJAN (F.), KOCK (M.D.), KOCK (R.A.), MOR-KEL (P.). Serological evidence for *Cowdria ruminantium* infection in free-ranging black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses in Zimbabwe. *J. Zoo Wildl. Med.*, 1992, **23** (4) : 409-413.
- LOGAN (L.L.), HOLLAND (C.J.), MEBUS (C.A.), RISTIC (M.). Serological relationship between *Cowdria ruminantium* and certain *Ehrlichia* species. *Vet. Rec.*, 1986, **119** : 458-459.
- MARTINEZ (D.), SWINKELS (J.), CAMUS (E.), JONGEJAN (F.). Comparaison de trois antigènes pour le sérodiagnostic de la cowdriose par immunofluorescence indirecte. *Revue Elev. Méd. vét. Pays trop.*, 1990, **43** : 159-166.
- MARTINEZ (D.) *et al.* (in press).
- MORZARIA (S.P.), IRVIN (A.D.), KOCAN (R.M.), VOIGT (W.P.). Isolation and characterization of an *Ehrlichia* sp. from cattle in Kenya. Workshop on diseases caused by leucocytic rickettsiae of man and animals, 1985, University of Illinois, Urbana-Champaign : Abstract 30.
- MULLER KOBOLD (A.C.), MARTINEZ (D.), CAMUS (E.), JONGEJAN (F.). Distribution of heartwater in the Caribbean determined on the basis of detection of antibodies to the conserved 32-kilodalton protein of *Cowdria ruminantium*. *J. Clin. Microbiol.*, 1992, **30** : 1870-1873.
- UILENBERG (G.). Heartwater (*Cowdria ruminantium* infection) : Current status. *Adv. Vet. Sci. Comp. Med.*, 1983, **27** : 427-480.
- VAN VLIET (A.H.M.), JONGEJAN (F.), VAN DER ZEIJST (B.A.M.). Phylogenetic position of *Cowdria ruminantium* (Rickettsiales) determined by analysis of amplified 16S ribosomal DNA sequences. *Int. J. Syst. Bacteriol.*, 1992 : 494-498.
- VAN VLIET (A.H.M.), JONGEJAN (F.), VAN KLEEF (M.), VAN DER ZEIJST (B.A.M.). Cloning and partial characterization of the Cr32 gene of *Cowdria ruminantium*. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 167-170.

JONGEJAN (F.), DE VRIES (N.), NIEUWENHUIJS (J.), VAN VLIET (A.H.M.), WASSINK (L.A.). The immunodominant 32-kilodalton protein of *Cowdria ruminantium* is conserved within the genus *Ehrlichia*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 145-152

Serological tests for cowdriosis are hampered by cross-reacting antibodies from animals suspected to be infected with *Ehrlichia* species. We have monitored infections with *Ehrlichia bovis*, *E. ovina*, *E. canis* and *E. phagocytophila* in experimental animals by competitive ELISA, Western blotting and immunofluorescence using *Cowdria*-infected endothelial cell culture antigens. Cross-reactions due to *Ehrlichia* antibodies could be attributed to the recognition of epitopes on the immunodominant Cr32 *Cowdria* protein. This was especially true for *E. canis* and *E. ovina*, much less for *E. bovis*, but not at all for *E. phagocytophila*. In addition, strong cross-reactivity between *Cowdria* and antibodies to *E. chaffeensis* were demonstrated. These findings are in agreement with the phylogenetic relationships, recently reported by VAN VLIET *et al.* in 1992, between *Cowdria* and other members of the tribe Ehrlichieae, which showed *Cowdria* to be closely related to *E. canis* and also to *E. chaffeensis*. Although the tests used in this study remain valuable tools under laboratory conditions, their specificity requires improvement. It is suggested to study recombinant *Cowdria* antigens for the development of second generation serological tests for cowdriosis.

*Key words* : Protein - *Cowdria ruminantium* - *Ehrlichia* - Immunological technique - ELISA test - Western blotting - Immunofluorescence test - Cell growth - Antibody - Antigen.

JONGEJAN (F.), DE VRIES (N.), NIEUWENHUIJS (J.), VAN VLIET (A.H.M.), WASSINK (L.A.). La proteína inmunodominante 32 kilodalton de *Cowdria ruminantium* se conserva dentro del género *Ehrlichia*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 145-152

Las reacciones cruzadas con anticuerpos provenientes de animales sospechosos de *Ehrlichia* sp., perturban los tests serológicos para *Cowdria*. Se detectaron infecciones con *E. bovis*, *E. ovina*, *E. canis* y *E. phagocytophila* en animales experimentales mediante el ELISA, el "western blotting" y la inmunofluorescencia, gracias al uso de antígenos de cultivos celulares infectados con *Cowdria*. Las reacciones cruzadas con los anticuerpos de *Ehrlichia* podrían ser ocasionadas por el reconocimiento de epítomos en la inmunoproteína Cr32 de *Cowdria*. Esto se verificó principalmente para *E. canis* y *E. ovina*, en menor medida para *E. bovis*, pero no para *E. phagocytophila*. Se demostró también una fuerte reacción cruzada entre *Cowdria* y anticuerpos de *E. chaffeensis*. Estos hallazgos coinciden con los recientes reportes por VAN VLIET *et al.* en 1992 sobre las relaciones filogenéticas entre *Cowdria* y otros miembros de la familia Ehrlichieae, según los cuáles existe una relación estrecha entre *Cowdria* y *E. canis* y *E. chaffeensis*. A pesar de que los tests utilizados en este estudio representan un instrumento importante a nivel de laboratorio, es necesario mejorar la especificidad de los mismos. Se recomienda estudiar los antígenos recombinantes de *Cowdria*, con el fin de desarrollar una segunda generación de tests serológicos para la cowdriosis.

*Palabras claves* : Protéina - *Cowdria ruminantium* - *Ehrlichia* - Técnica inmunológica - Test ELISA - Western blotting - Inmunofluorescencia - Cultivo de célula - Anticuerpo - Antígeno.