Onderstepoort Journal of Veterinary Research, 62:277–280 (1995)

Immunohistochemical identification of *Cowdria ruminantium* in formalin-fixed tissue sections from mice, sheep, cattle and goats

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ABSTRACT

JARDINE, J.E., VOGEL, S.W., VAN KLEEF, M. & VAN DER LUGT, J.J. 1995. Immunohistochemical identification of *Cowdria rumantium* in formalin-fixed tissue sections from mice, sheep, cattle and goats. *Onderstepoort Journal of Veterinary Research*, 62:277–280

An immunohistochemical staining technique in which a monospecific serum was used against the major antigenic protein-1 (MAP-1) of *Cowdria ruminantium*, was evaluated for the detection of *C. ruminantium* in formalin-fixed tissues of experimentally infected mice and field cases of heartwater in sheep, cattle and goats. Mice were infected with the mouse-pathogenic stocks: Mara, Kwanyanga, Welgevonden, Nonile, Vosloo, Kümm, Mali and Omatjenne. In all these cases and in the naturally infected cattle, sheep and goats, *Cowdria* colonies were identified as clearly-defined, brown-staining rickettsial colonies within the cytoplasm of endothelial cells. No positive staining was observed in the control group. This technique was shown to be reliable for detecting infection with *C. ruminantium* in the formalin-fixed tissues of mice and domestic ruminants.

Keywords: Cattle, *Cowdria ruminantium*, formalin-fixed tissue sections, goats, heartwater, identification, immunohistochemical, mice, sheep, staining technique

INTRODUCTION

Heartwater is an economically important tick-transmitted disease of cattle, sheep and goats and some wild ruminants in Africa and the Caribbean, and is caused by the rickettsial parasite *Cowdria ruminantium* (Bezuidenhout, Prozesky, Du Plessis & Van Amstel 1994; Brown & Skowronek 1990). Several isolates (stocks) of the organism have been identified which vary in their antigenicity and pathogenicity. Some stocks have been used to establish experimental infections in mice, a frequently used laboratory model for the study of the disease (Bezuidenhout, Prozesky, Du Plessis & Van Amstel 1994; Brown & Skowronek 1990; Rossouw, Neitz, De Waal, Du Plessis, Van Gas & Brett 1990).

At present, post-mortal confirmation of heartwater in ruminants is based on the microscopic demonstration of parasites in endothelial cells in Giemsa- or Romanowsky-stained brain smears (Bezuidenhout *et al.* 1994; Prozesky 1987). Infection in mice is diagnosed by examination of squash smears of the lungs or, more reliably, by sub-inoculation of infective material into ruminants (Prozesky 1987). *Cowdria ruminantium* can be detected in haematoxylin- and eosin-stained (H&E) histological sections; however, this is tedious and possibly equivocal in cases where only a small number of parasites are present or when animals have succumbed following treatment (Bezuidenhout

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Accepted for publication 27 September 1995—Editor

et al. 1994; Prozesky 1987). Should no parasites be detected while the gross lesions and clinical signs are highly suggestive of the disease, no reliable back-up or second-tier diagnostic method would be available. Furthermore, in cases where only formalin-fixed tissues are submitted for diagnostic purposes, diagnosis of heartwater is often time-consuming, occasionally inadequate and, the results considered, unreliable (Bezuidenhout *et al.* 1994).

Immunohistochemical staining for specific parasite antigens allows for precise identification of the parasites in the tissues. Immunohistochemistry on formalin-fixed tissues allows submission of samples in a readily available preservative without deterioration of the diagnostic accuracy, and this would enable a diagnostic service to be delivered even to those areas where only the most rudimentary laboratory support is available (Haines & Chelak 1991). Rickettsial organisms such as *Rickettsia rickettsii* and *C. ruminantium* have previously been demonstrated by means of immunohistochemical techniques (Brown & Skowronek 1990; Dumler, Gage, Pettis, Azad & Kuhadja 1990). An immunoperoxidase method for the identification of *C. ruminantium* in formalin-fixed, paraffin-wax-embedded sections has been published, but was successful only in demonstrating the parasite in the mouse model; staining of *C. ruminantium* in experimentally infected goats was largely unsuccessful (Brown & Skowronek 1990).

An immunodominant 31–33 kDa protein, designated the major antigenic protein-1 (MAP-1), which is antigenically conserved and common among 14 stocks of *C. ruminantium* of differing virulence, pathogenicity and origin, has been identified (Barbet, Semu, Chigagure, Kelly, Jongejan & Mahan 1994; Jongejan & Thielemans 1989; Rossouw *et al.* 1990). This protein was found to be present in the electrophoretic profiles of Ball 3, Kwanyanga, Welgevonden and Senegal stocks, and is recognized by sheep antiserum to the Welgevonden, Ball 3, Kwanyanga, Mali, Comoro, Breed, Germishuys, Kümm and Mara stocks; bovine and goat antiserum to the Welgevonden stock, and goat antisera to the Guadeloupe, Ball 3, Kümm and Kwanyanga stocks, as well as stocks from Senegal, Sudan, Kenya and Nigeria, (Jongejan & Thielemans 1989; Rossouw *et al.* 1990)

TABLE 1 Inoculation protocol and immunoblot titres obtained from mono-specific anti-MAP-1 serum produced in a goat

Day	Inoculum/bled	Reciprocal of immunoblot titre
0	Antigen/FCA (1:1)	
14	Bled	125 00
28	Antigen/FIA (1:1)	
42	Bled	125 00
56	Antigen	
70	Bled	125 00
84	Antigen	
98	Bled	125 00

FCA = Freund's complete adjuvant
FIA = Freund's incomplete adjuvant

The purpose of this study was to assess the use of mono-specific anti-MAP-1 *C. ruminantium* serum for immunohistochemical identification of *C. ruminantium* in formalin-fixed tissues of experimentally infected mice and field cases of heartwater in goats, sheep and cattle, with the aim of producing a simple, but reliable test for the post-mortal confirmation of heartwater.

MATERIALS AND METHODS

Antiserum production

Mono-specific anti-MAP-1 serum was prepared in a goat as described previously (Van Kleef, Neitz, & De Waal 1993). Briefly, this was accomplished as follows: preparative SDS-PAGE was performed with crude Welgevonden-stock-infected culture extracts and the gel was stained with 0,3 M $CuCl_2$. The MAP-1 protein band was excised, fragmented in PBS and the goat immunized subcutaneously and intramuscularly with 266 g of the MAP-1 protein according to the schedule in Table 1. The serum, collected at intervals indicated in Table 1, was evaluated and the titres determined by IFA and/or immunoblotting (Du Plessis & Malan 1987; Rossouw *et al.* 1990). The IFA titre of the goat anti-MAP-1 serum (day 98) was determined to be 1:5100. The mono-specific anti-MAP-1 goat serum appeared unspecific at low serum dilutions in the immunoblot, but the specificity increased as the dilution of the serum increased.

Model system

Disease-free Balb/c mice were experimentally infected with the mouse-pathogenic *C. ruminantium* stocks: Mara, Kwanyanga, Welgevonden, Nonile, Vosloo, Kümm, Mali and Omatjenne. Six mice were infected with each specific stock of *C. ruminantium* by intravenous injection of 0,2 mℓ of infective blood stabilate in BLP. Six uninfected mice were included as controls. All fatalities in the post-infection period were necropsied, and all the mice that survived to day 15 post infection were killed and necropsied. In each case, the brain, lungs, heart, liver, kidneys and spleen were fixed in 10% buffered formalin for at least 24 h. Tissue blocks were embedded in paraffin wax at a temperature not exceeding 60 °C and subsequently processed according to standard histological practices.

Cases of heartwater were selected in six sheep, six goats and six cattle that had died of naturally acquired infections, confirmed at post mortem by the microscopic detection of Giemsa-stained parasites

in the endothelial cells of the cerebral blood vessels. Portions of the cerebral cortex from each case were placed in 10% buffered formalin and routinely processed for histological examination.

Immunohistochemical staining procedure

Sections were stained according to an avidin-biotin immunohistochemical procedure, following standard procedures. Briefly, the procedure was performed as follows: sections were affixed to poly-L-lysine-coated glass slides (Sigma Chemical Company, P.O. Box 14508, St Louis, Missouri, USA) and dried overnight in an incubator at 60°C. The tissue sections were dewaxed in xylene for 5 min, rehydrated through graded alcohols and washed in distilled water for 3 min. Endogenous peroxidases were quenched by immersing sections in 3% hydrogen peroxide in methanol for 5 min, after which the sections were rinsed twice consecutively for 5 min in distilled water. Sections were subjected to an antigen-retrieval protocol to expose sequestered antigens where the sections were placed in distilled water and boiled in a microwave on full power for two consecutive 5-min periods. Sections were cooled for 15 min, rinsed twice in distilled water, and then rinsed in phosphate-buffered saline (PBS, pH 7,6) containing 0,1% bovine-serum albumin. Non-specific antibody-binding was blocked with 10% normal rabbit serum (Labotec, P.O. Box 5103, Durban 4000, South Africa) for 20 min. The sections were blotted and the slides covered in a 1:500 dilution of primary goat anti-*Cowdria ruminantium* antibody for 60 min at room temperature. Following two further consecutive 5-min washes in PBS, the sections were incubated for 30 min at room temperature, with a 1:500 dilution of biotinylated rabbit anti-goat secondary antibody (Dako A\S, Produktionsvej 42, DK-2600 Glostrup, Denmark). Two further washes in PBS were followed by covering the sections with avidin-biotin-complex (Dako A\S, Produktionsvej 42, DK-2600 Glostrup, Denmark) for 30 min according to the manufacturer's recommendations. The sections were again rinsed in PBS and treated for 8 min with the peroxidase substrate, diaminobenzamine tetrahydrochloride (DAB) (BDH Laboratory Supplies, Poole, Dorset, England). Slides were finally rinsed in distilled water, counterstained with Mayer's haematoxylin, dehydrated, mounted and examined with a standard light microscope.

RESULTS

Positively stained colonies of *C. ruminantium* were identified as clearly defined, brown-staining, rickettsial colonies in the cytoplasm of endothelial cells (Fig. 1). The results of the survey are recorded in Table 2. In the mice, positively stained colonies were most frequently encountered in the lungs and myocardium, with fewer in the spleen and kidneys, and only sporadic organisms visible in the brain and liver. In ruminants, the positively stained colonies were observed in the capillaries and small blood vessels of the grey matter, particularly, but also in the white matter and meninges.

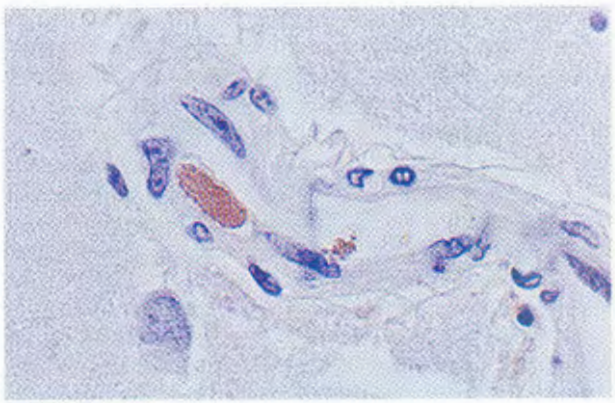

FIG. 1 A brown, positive-staining colony of *Cowdria ruminantium* in the cytoplasm of a cerebral vascular endothelial cell of a naturally infected bovine. Avidin-biotin-peroxidase complex technique, Mayer's haematoxylin counterstain (330X)

TABLE 2 Immunohistochemical staining of formalin-fixed, paraffin-wax-embedded tissues from experimentally infected and uninfected mice, and naturally infected sheep, cattle and goats

Animal	*C. ruminantium* stock	No. positive	No. negative
Balb/c mice	Mara	6	0
Balb/c mice	Kwanyanga	6	0
Balb/c mice	Welgevonden	6	0
Balb/c mice	Nonile	6	0
Balb/c mice	Vosloo	6	0
Balb/c mice	Kümm	6	0
Balb/c mice	Mali	6	0
Balb/c mice	Omatjenne	6	0
Balb/c mice	Uninfected controls	0	6
Sheep	Field infection	6	0
Cattle	Field infection	6	0
Goats	Field infection	6	0

DISCUSSION

The use of a mono-specific goat antibody against the immunodominant MAP-1 protein of *C. ruminantium* in an immunoperoxidase-staining procedure in which use was made of formalin-fixed, paraffin-wax-embedded tissues of mice experimentally infected with a wide range of stocks of *C. ruminantium*, and naturally infected sheep, cattle and goats, was highly successful in demonstrating the causative organisms. In all instances, all infected cases were positively identified and there was no indication of positive staining in the uninfected control mice.

The MAP-1 protein of *C. ruminantium* has been shown to be conserved within the closely related genus *Ehrlichia* (Jongejan, De Vries, Nieuwenhuijs, Van Vliet & Wassink 1993). This has hampered the use of the protein in serological tests due to the cross-reactions encountered with animals infected with *Ehrlichia* spp. Although this may be seen as a drawback in the immunohistochemical diagnosis of heartwater infection, *Ehrlichia* species parasitize neutrophils, platelets and monocytes, whereas *Cowdria* infects endothelial cells. The immunohistochemical techniques on histological sections allow visualization of the *Cowdria* colonies within the cytoplasm of the endothelial cells, thereby preventing any confusion and misdiagnoses caused by the cross-reactivity.

This technique allows for accurate and specific diagnosis of heartwater in formalin-fixed tissue sections and thereby offers a second-tier diagnostic test in cases where organisms were impossible to identify in routine brain smears. This technique may also find application in allowing submission of samples of suspected cases of heartwater in a preservative, making a diagnosis possible in areas where even the most rudimentary laboratory facilities are lacking. An added advantage is the accurate identification of rickettsial parasites in species not normally associated with heartwater infection. This would help clarify some of the unconfirmed reports of heartwater in wild ruminants and other game species. Furthermore, this technique can be applied to confirm the diagnosis in experimental infection in mice, alleviating the need for sub-inoculation into susceptible ruminants.

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