JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1993, p. 2729–2737
0095-1137/93/102729-09$02.00/0

Vol. 31, No. 10

An Immunoblotting Diagnostic Assay for Heartwater Based on the Immunodominant 32-Kilodalton Protein of *Cowdria ruminantium* Detects False Positives in Field Sera

S. M. MAHAN,[1]* N. TEBELE,[1]† D. MUKWEDEYA,[1] S. SEMU,[1] C. B. NYATHI,[2] L. A. WASSINK,[1] P. J. KELLY,[2] T. PETER,[1] AND A. F. BARBET[3]

Veterinary Research Laboratory, University of Florida/United States Agency of International Development/SADC Heartwater Research Project, Box 8101, Causeway,[1] and Faculty of Veterinary Medicine, University of Zimbabwe, Mount Pleasant, Harare,[2] Zimbabwe, and Department of Infectious Diseases, University of Florida, Gainesville, Florida 32611-0880[3]

Received 21 January 1993/Returned for modification 11 February 1993/Accepted 21 June 1993

Heartwater, a major constraint to improved livestock production in Zimbabwe, threatens to invade areas which have been previously unaffected. To monitor its spread in Zimbabwe, an immunoblotting diagnostic assay based on the responses of animals to the immunodominant, conserved 32-kDa protein of *Cowdria ruminantium* was evaluated. In this assay, no false reactions were detected with sera known to be positive and negative, but sera from some cattle, sheep, and goats from heartwater-free areas of Zimbabwe reacted strongly with the 32-kDa protein, suggesting that either these animals had previous exposure to heartwater or they were false positives. To investigate the possibility of previous exposure to heartwater, 11 immunoblot-positive and 6 immunoblot-negative sheep from heartwater-free areas of Zimbabwe were compared regarding their susceptibilities to challenge with *C. ruminantium*. Prior to challenge, *C. ruminantium* could not be detected in any sheep by transmission to *Amblyomma hebraeum* ticks or by the polymerase chain reaction (PCR) conducted with plasma samples. All sheep were equally susceptible to the challenge, and infection was confirmed by brain biopsy, necropsy, PCR, and transmission of *C. ruminantium* to ticks. Our data suggest that the immunoblot-positive reactions of sera from heartwater-free areas were due not to previous *C. ruminantium* infection but rather to antigenic cross-reactivity between *C. ruminantium* and another agent(s), such as *Ehrlichia* species. In conclusion, the immunodominant 32-kDa protein is not antigenically specific to *C. ruminantium* and its use in serological diagnosis of heartwater requires reevaluation.

Heartwater is an economically important tick-transmitted disease of livestock which affects food production in subsaharan Africa (33) and on certain Caribbean islands (3, 28). The disease, caused by the rickettsia *Cowdria ruminantium* and transmitted by ticks of the genus *Amblyomma* (33), is responsible for mortality ranging between 20 and 90% in susceptible herds of animals (33). The presence of heartwater on the Caribbean islands has the potential to spread to North, Central, and South America (2). Effective detection of heartwater in areas in which it is endemic and control of its spread to new areas cannot be accomplished as long as reliable diagnostic tests are not available for use in epidemiological studies and field surveys.

Several serological tests have been developed to diagnose heartwater. These may be differentiated into those methods that detect antibodies against the whole organism, such as the indirect fluorescent-antibody test (IFAT) (11, 16, 19, 32), and those detecting antibodies produced against defined proteins, such as immunoblotting (14, 30) and the 32-kDa-protein-specific competitive enzyme-linked immunosorbent assay (cELISA) (15). Although the IFAT and cELISA have been used in serological surveys, they have neither the sensitivity nor the specificity required of a reliable diagnostic test. For example, the IFATs based on the infected neutrophil (16, 20) or mouse macrophage (11) have poor specificity, as they detect cross-reactions with *Ehrlichia phagocytophila*, *Ehrlichia bovis*, *Ehrlichia ovina* (10, 16), *Ehrlichia equi*, and *Ehrlichia canis* (19). The immunodominant 32-kDa-protein-specific cELISA does not have adequate sensitivity; in one study, it detected only 79% of the known positive samples from animals that had recovered from and were immune to heartwater (15). The remaining 21% of the samples were immunoblotted against the *Cowdria* antigen, and their reaction against the 32-kDa protein was determined to be positive. On the basis of these criteria, immunoblotting is considered to be the most sensitive serological assay for heartwater (15). Hence, because of the recognized specificity and sensitivity limitations of the IFAT and cELISA, these tests were used concurrently with the immunoblotting assay to determine the probable extended distribution of heartwater in the Caribbean region (18).

The 32-kDa protein of *C. ruminantium* has been proposed by various investigators as a diagnostic antigen for heartwater because it is immunodominant and antigenically conserved between *C. ruminantium* isolates (14, 30). We evaluated and investigated the 32-kDa-protein-based immunoblotting assay as a diagnostic test for heartwater in Zimbabwe, and we discuss here the specificity problems that were encountered.

MATERIALS AND METHODS

Animals and antisera. Antisera from cattle and sheep at the University of Florida, Gainesville, were used as known

* Corresponding author.
† Present address: International Laboratory for Research on Animal Diseases, Nairobi, Kenya.

negative samples, as heartwater does not exist on the North American continent (2). Known positive sera were prepared from blood of cattle and sheep which were intravenously (i.v.) infected with tick (5 ml) or blood stabilate (5 to 20 ml) or infected-cell culture supernatant (2 to 3 ml) of the Palm River, Crystal Springs (4), Ball-3 (13), or Welgevonden (8) *C. ruminantium* strain. Serum samples were prepared from blood collected between 3 and 12 weeks after a primary infection or 1 and 11 weeks after a secondary infection of these animals. For the time course study, cattle were i.v. inoculated with the Palm River and Ball-3 strains by using 2 to 3 ml of cell culture supernatant or 5 ml of tick or 10 to 20 ml of blood stabilate. After infection, sera were collected weekly for 24 weeks. Also tested were sera from cattle (Friesian, Mashona, Hereford-Tuli crosses), rams, ewes, lambs, and goats from Headlands, Darwendale, Ruwa, Norton, Banket, and Mazowe, which are areas in the high veld, close to Harare, the capital of Zimbabwe, and are known to be free of heartwater and *Amblyomma* ticks (26). Sera were also obtained from cattle from a heartwater-endemic area at the Heartwater Research Project, Veterinary Quarantine Station at Mbizi, in southern Zimbabwe.

A monospecific rabbit serum was prepared against the gel-purified 32-kDa protein of *C. ruminantium*. Following electrophoresis of *C. ruminantium* proteins on sodium dodecyl sulfate (SDS) gels, the 32-kDa protein was sliced out and electroeluted for 3.5 h at 50 mA into a buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 50 mM NaCl, 1% Nonidet P-40, 0.1% SDS). Rabbits were inoculated three times, at 3-week intervals, with 1.16 mg of the protein, emulsified initially with Freund's complete adjuvant and subsequently with incomplete adjuvant. The immunized rabbits were bled 2 weeks after the last booster injection.

E. canis antiserum was obtained from a dog that was inoculated i.v. with 3×10^3 *E. canis*-infected DH82 cells (6). Serum was collected on day 42 postinfection and had an IFAT titer of 1/2,560. Serum samples from cattle infected with *E. bovis* were obtained from blood collected on days 9 and 51 post-i.v. inoculation with tissue culture-derived *E. bovis* organisms. The titers of these sera were unknown.

Isolation and quantitation of *C. ruminantium*. *C. ruminantium* organisms were harvested from infected bovine endothelial cell cultures by centrifugation of the supernatants for 15 min at 1,000 × *g* to remove host cell debris, followed by two washes of the suspended organisms in phosphate-buffered saline (PBS) at 30,000 × *g*. Quantitation of *C. ruminan-*

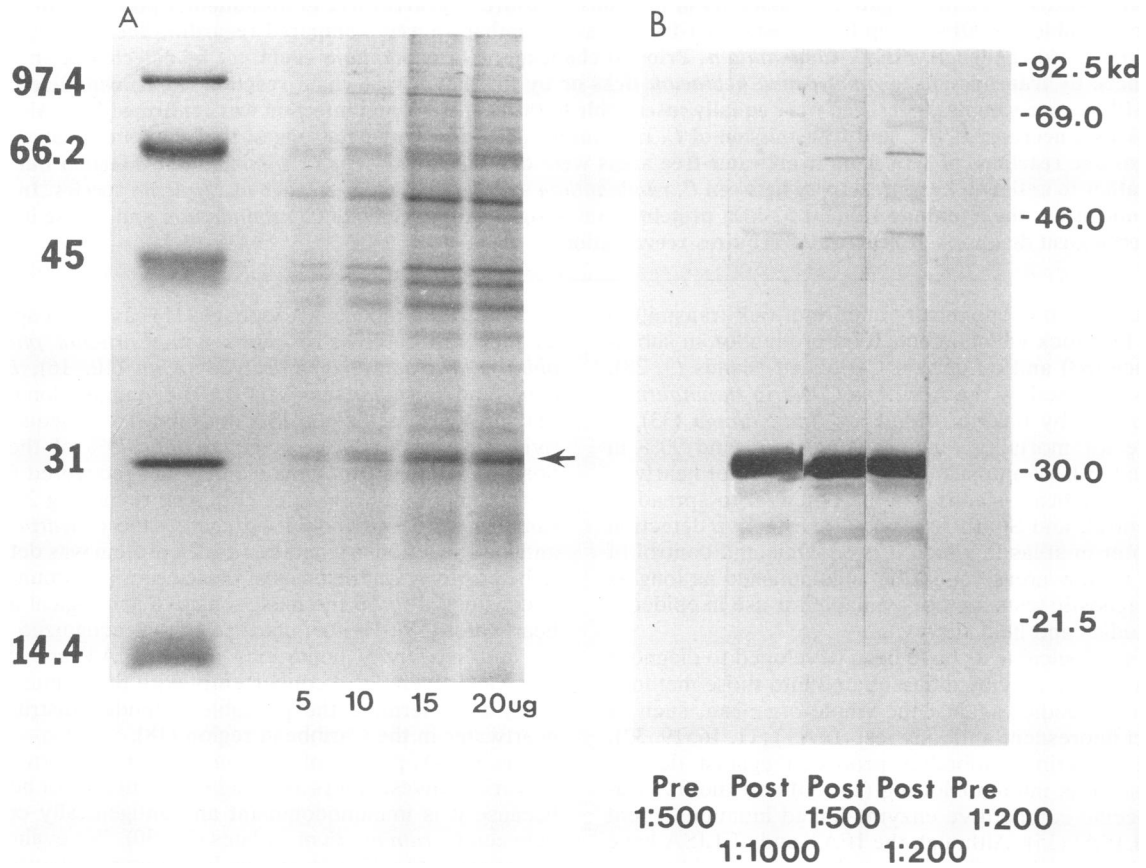

FIG. 1. (A) SDS-PAGE analyses of *C. ruminantium* proteins (Crystal Springs strain, purified elementary bodies [EBs]). *C. ruminantium* proteins (5, 10, 15, and 20 μg) were electrophoretically separated on an SDS-PAGE gel (12% polyacrylamide) and stained with Coomassie blue. These analyses show that the 32-kDa protein is a major structural protein of *C. ruminantium*. The positions of molecular size markers (in kilodaltons) are shown on the left. (B) Immunoblots show that the 32-kDa protein of *C. ruminantium* is immunodominant. The 32-kDa protein is immunodominant, as its reaction with the most dilute postinfection antiserum is stronger than that of other proteins. Reactions of preinfection 1/200- and 1/500-diluted antisera with *C. ruminantium* antigen are shown in lanes 1 and 5, lane 1 being closest to the molecular size markers (in kilodaltons) shown on the right. The reactions of postinfection antisera at dilutions of 1/200, 1/500, and 1/1,000 are shown in lanes 2, 3, and 4, respectively.

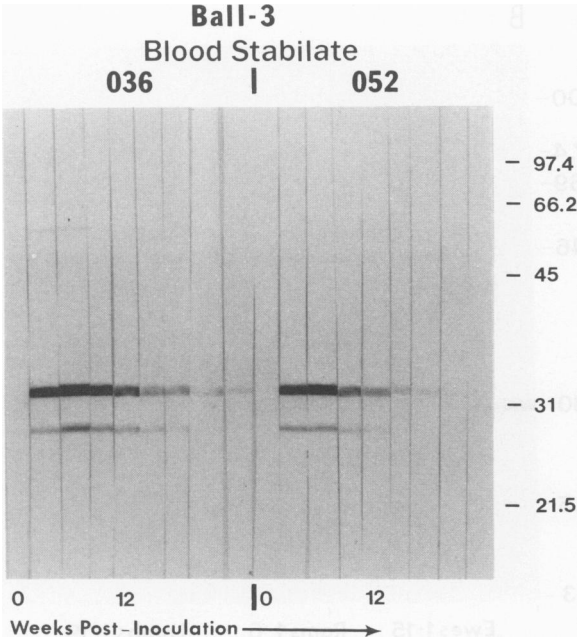

FIG. 2. Detection of anti-32-kDa-protein-specific immunoglobulin G antibody responses of cattle by immunoblotting. The blots show the reactions of cattle sera against homologous *C. ruminantium* antigen at three weekly intervals after infection with the Ball-3 strain of *C. ruminantium*. Responses of two animals (036 and 052) are shown, starting with week 0. The positions of molecular size markers (in kilodaltons) are shown on the right.

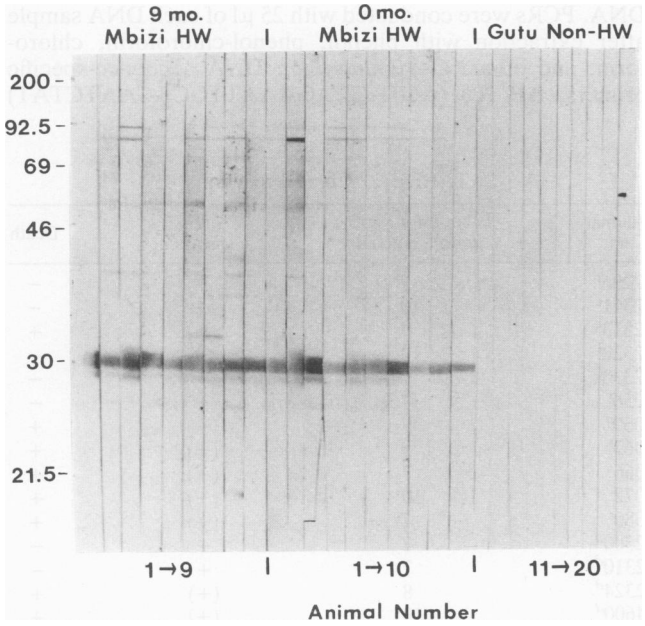

FIG. 3. Antibody responses of cattle sera from an area where heartwater (HW) is endemic against *C. ruminantium* proteins. Immunoblotting reactions of sera of cattle (Mbizi HW, 0 and 9 months) taken 9 months apart reveal a consistent recognition of the 32-kDa protein. The 9-month sample for animal number 10 was not tested. Negative control animal serum (Gutu Non-HW) reactions are shown for animals 11 to 20. The positions of molecular size markers (in kilodaltons) are shown to the left.

tium organisms was conducted after purification of the organisms on discontinuous percoll density gradients (0 to 40%), made in either PBS or sucrose-phosphate-glucose buffer (0.22 M sucrose, 3 mM KH_2PO_4, 7.2 mM $K_2HPO_4 \cdot 3H_2O$, 4.9 mM potassium glutamate) and centrifuged at 400 × *g* for 30 min. The purified organisms taken from the 0% layer were washed once at 30,000 × *g*, diluted, and pelletted onto slides by cytospinning at 200 × *g* for 10 min. The slides were then stained with fluorescein diacetate, and a viable count (22) was determined by a quantitative method previously described for *Chlamydia trachomatis* (17).

SDS-PAGE and immunoblotting analyses. Twenty to thirty micrograms of *C. ruminantium* antigen per lane was electrophoretically separated on SDS–12% polyacrylamide gel electrophoresis (PAGE) and either stained with Coomassie blue or transferred to nitrocellulose membranes for immunoblotting. Immunoblotting was conducted as follows. After being blocked for 1 h with Tris-buffered saline (TBS) (0.1 M Tris HCl, 0.9% NaCl [pH 8.0])–0.25% gelatin, the blots were reacted overnight with serum samples diluted to 1/100 or as otherwise specified. The blots were washed three times in TBS containing 0.25% Tween 20, reacted with peroxidase-labeled protein G (Zymed) for 2 h, washed three times as described above, and developed by incubation with 4CN peroxidase substrate (Kirkegaard and Perry, Gaithersburg, Md.). Immunoblots against the *E. canis* antigen (Oklahoma strain) obtained from DH82-infected macrophage cultures (6) were prepared in a similar manner.

Challenge of immunoblot-positive and -negative sheep from heartwater-free areas of Zimbabwe with *C. ruminantium*. Eleven immunoblot-positive sheep (five Wiltiper [numbers 2296, 2311, 2313, 2322, and 2330] and six Merino [numbers 056, 060, 062, 066, 072, and 080]) and six immunoblot-negative sheep (three Wiltiper [numbers 2300, 2310, and 2324] and three Merino [numbers 4600, 4728, and 4780]) were purchased from heartwater-free areas of Zimbabwe (Norton and Ruwa). These sheep were challenged by i.v. inoculation with 4×10^6 *C. ruminantium* cell culture-derived organisms of the Crystal Springs strain from Zimbabwe.

TABLE 1. Application of immunoblotting assay in heartwater-free areas of Zimbabwe

Serum source	No. of sera tested	No. of immunoblot-negative sera[a]	No. of immunoblot-positive sera[a]
Cattle			
Friesian[b]	56	49	7
Mashona[c]	52	21	31
Hereford-Tuli[d]	30	28	2
Total no. of sera tested	138		
Sheep			
1[e]	109	43	66
2[f]	86	85	1
Total no. of sera tested	195		

[a] All reactions were judged against the 32-kDa immunodominant protein of *C. ruminantium*.
[b] Cattle from Norton.
[c] Cattle from Headlands.
[d] Cattle from Darwendale.
[e] Sera from sheep from heartwater-free areas (Mazowe and Ruwa), which were tested before being used in experimental studies.
[f] Sera from a farm in one heartwater-free area of Zimbabwe (Ruwa).

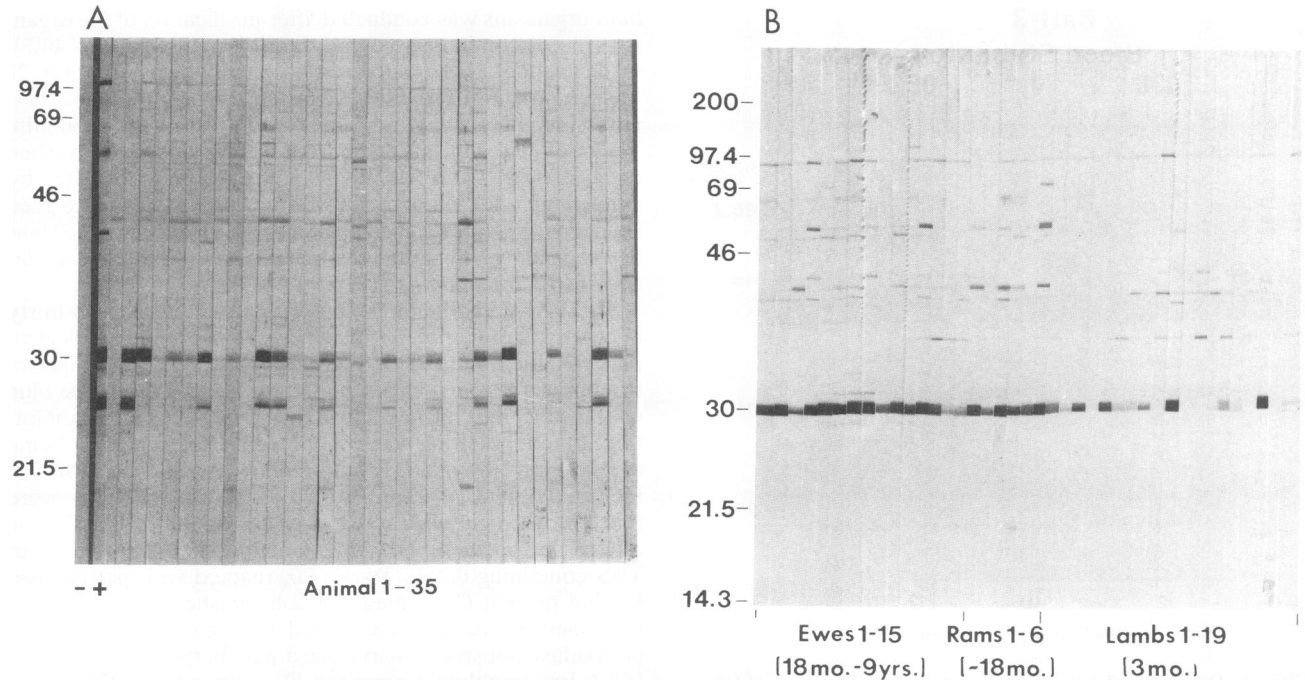

FIG. 4. Recognition of *C. ruminantium* proteins by sera of animals from heartwater-free areas of Zimbabwe by immunoblotting. (A) Immunoblotting reactions of Mashona cattle sera from heartwater-free areas (Headlands) against *C. ruminantium* proteins. The blots show positive reactions of some sera against the 32-kDa protein (and other proteins). Negative (−) and positive (+) control serum reactions are shown in the leftmost lanes. (B) Immunoblotting reactions of sera from Darwendale ewes (first 15 lanes), rams (next 6 lanes), and lambs (next 19 lanes) of different ages from heartwater-free areas against *C. ruminantium* proteins. The immunoblots show that the positive reaction of sera against the 32-kDa protein is not related to the age or sex of the animals. The positions of the molecular size markers (in kilodaltons) are shown to the left.

Rectal temperatures of all sheep were monitored daily, and on the third day of the febrile reaction, brain biopsies were prepared and examined for the presence of intracytoplasmic *C. ruminantium* colonies in brain capillary endothelium (29). Additionally, heartwater infection in sheep that died was confirmed by brain crush smears and necropsy.

Tick feeds and DNA extractions and hybridization. Tick feeds were conducted with *Amblyomma hebraeum* nymphs from a laboratory colony which had previously been shown to be free of infection by its failure to transmit *C. ruminantium* to susceptible sheep. Two hundred uninfected *A. hebraeum* nymphs from this laboratory colony were fed twice on individual sheep, prior to and 7 days after challenge with *C. ruminantium* organisms. After the replete ticks had moulted, DNA of midgut and salivary glands from 10 ticks (5 males and 5 females) from each feed and each individual sheep was extracted and blotted onto nylon membranes (GeneScreen Plus; Du Pont) by the method described previously (37). *C. ruminantium* DNA was detected in tick DNA samples by hybridization to the *C. ruminantium*-specific, ^{32}P-labeled pCS20 DNA probe, which can detect infections in *Amblyomma variegatum* and *A. hebraeum* ticks (21, 36, 37).

PCR. Ten milliliters of plasma from blood collected in EDTA tubes was centrifuged at 30,000 × *g* for 30 min at 4°C to pellet *C. ruminantium* organisms. The pellet was washed once in PBS, suspended in 100 μl of polymerase chain reaction (PCR) lysis buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% Nonidet P-40), and digested with lysozyme (1 mg/ml) for 30 min and with proteinase K (100 μg/ml) overnight at 37°C to access the DNA. PCRs were conducted with 25 μl of each DNA sample after extraction with phenol, phenol-chloroform, chloroform, and ether. *C. ruminantium* DNA sequence-specific primers, AB 128 (ACTAGTAGAAATTGCACAATCTAT)

TABLE 2. Clinical reactions

Animal no.	No. of days to febrile reaction[a]	Brain smears[b]	Death
2296[c]	7	+	−
2311[c]	10	+	−
2313[c]	7	(+)	+
2322[c]	10	+	−
2330[c]	7	+	−
056[c]	7	+	−
060[c]	7	(+)	+
062[c]	9	(+)	+
066[c]	7	(+)	+
072[c]	10	(+)	+
080[c]	7	(+)	+
2300[d]	7	+	−
2310[d]	7	+	−
2324[d]	8	(+)	+
4600[d]	10	(+)	+
4728[d]	10	(+)	+
4780[d]	7	+	−

[a] A rectal temperature of 40.5°C or higher was taken as a febrile reaction.
[b] +, sheep were positive for *C. ruminantium* by brain biopsy performed on day 3 of the febrile reaction; (+), at death, brain crush smears were positive for *C. ruminantium* colonies.
[c] "False-positive" sheep detected by immunoblotting.
[d] Negative sheep detected by immunoblotting.

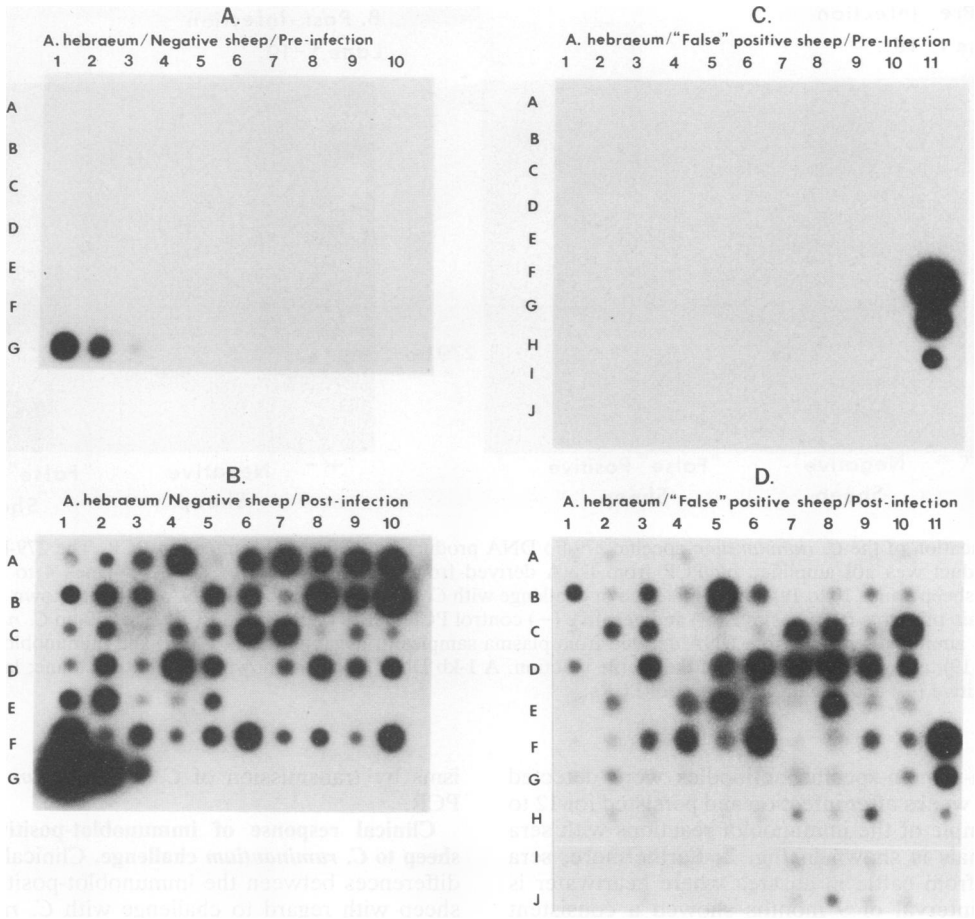

FIG. 5. Hybridization of the pCS20 DNA probe to tick DNA. (A) Hybridization of the pCS20 DNA probe to combined DNA from midgut and salivary glands of *A. hebraeum* ticks fed on individual immunoblot-negative sheep before infection with *C. ruminantium*. Different rows represent the DNA samples of five female (lanes 1 to 5) and five male (lanes 6 to 10) ticks fed on individual sheep. Rows A through F represent samples from ticks fed on sheep 2300, 2310, 2324, 4600, 4728, and 4780, respectively. Row G, lanes 1 to 3, represents 100, 10, and 1 ng of Crystal Springs DNA, respectively, blotted as positive control samples. (B) Hybridization of the pCS20 DNA probe to combined DNA from midgut and salivary glands of *A. hebraeum* ticks fed on individual immunoblot-negative sheep after infection with *C. ruminantium* as described for panel A. Sheep 4728 (Row E) died before the others, and only five ticks were collected; their DNA samples are in lanes 1 to 5. (Lane 1 contains the sample from one female tick, and lanes 2 to 5 contain samples from four male ticks.) Row G, lanes 1 to 3, represents 100, 10, and 1 ng of Crystal Springs DNA, respectively, blotted as positive control samples. (C) Hybridization of the pCS20 DNA probe to DNA from midgut and salivary glands of *A. hebraeum* ticks fed on individual immunoblot ("false")-positive sheep before infection or challenge with *C. ruminantium*. Different rows represent the DNA samples of five female (lanes 1 to 5) and five male (lanes 6 to 10) ticks fed on individual sheep. Rows A through J represent samples from ticks fed on sheep 056, 060, 062, 080, 072, 2296, 2311, 2313, 2330, and 2322, respectively. Crystal Springs positive control DNA samples of 100, 10, and 1 ng are in lane 11 of rows F, G, and H, respectively. (D) Hybridization of the pCS20 DNA probe to combined DNA from midgut and salivary glands of *A. hebraeum* ticks fed on individual immunoblot ("false")-positive sheep after infection or challenge. Different rows represent the DNA samples of five female (lanes 1 to 5) and five male (lanes 6 to 10) ticks fed on individual sheep as described in the legend to panel C.

and AB 129 (TGATAACTTGGTGCGGGAAATCCTT), were used to amplify a 279-bp DNA fragment (21), which was visualized on 1.5% agarose gels (31).

RESULTS

Properties of the 32-kDa protein of *C. ruminantium*. SDS-PAGE analysis of *C. ruminantium* proteins followed by Coomassie blue staining and immunoblotting revealed that the 32-kDa protein is a major structural protein (Fig. 1A) and that it is immunodominant (Fig. 1B). It has also been shown to be surface exposed and antigenically conserved between different strains (14, 15, 30). These data and those of others describing consistent reactions of heartwater infection sera against the 32-kDa protein (14, 30) in immunoblots provided rationale for the use of the immunoblotting assay for diagnosis of heartwater in Zimbabwe.

Evaluation of the 32-kDa-protein-specific immunoblotting assay. The *C. ruminantium*-specific immunoblotting assay was evaluated as a diagnostic test for heartwater by studying the anti-32-kDa-protein reactions of known negative sera from the University of Florida (57 cattle and 85 sheep) and known positive sera (22 cattle and 36 sheep) from experimentally infected animals. These sera did not yield any false-positive or -negative reactions with the 32-kDa protein in this assay.

A time course study in cattle experimentally infected with the Palm River or Ball-3 strain of *C. ruminantium* showed

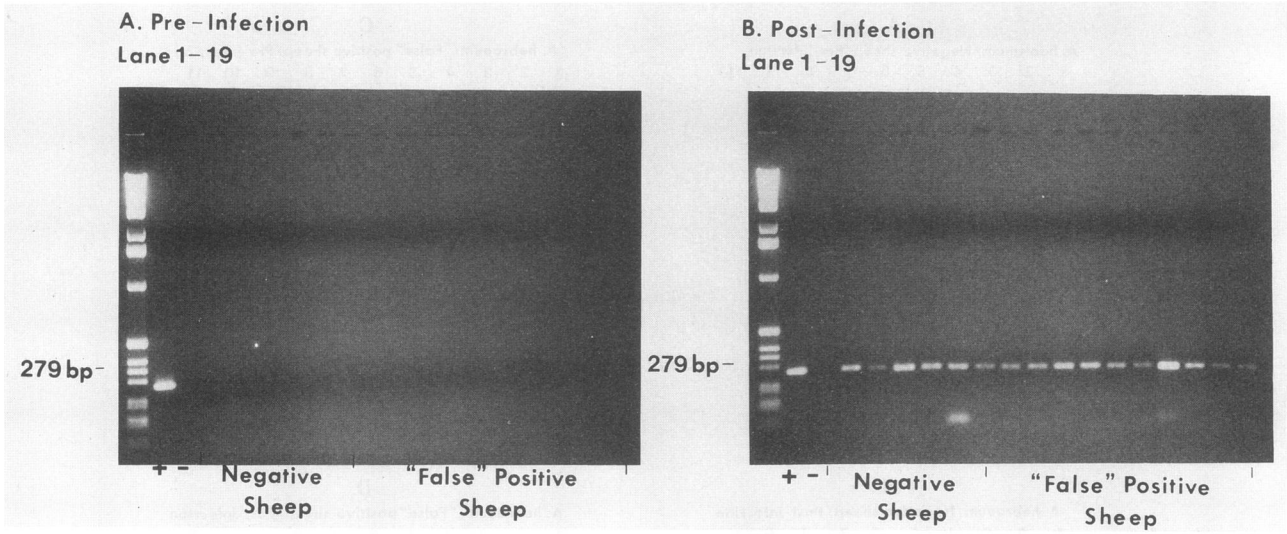

FIG. 6. Amplification of the *C. ruminantium*-specific 279-bp DNA product by PCR. (A) Preinfection PCR. The 279-bp *C. ruminantium*-specific DNA product was not amplified by PCR from DNA derived from plasma samples of negative (lanes 4 to 9) and immunoblot ("false")-positive sheep (lanes 10 to 19) before infection or challenge with *C. ruminantium*. A 1-kb DNA ladder is shown in the leftmost lane; lanes 2 and 3 contain products from positive (+) and negative (−) control PCRs. (B) Postinfection PCR. The 279-bp *C. ruminantium*-specific DNA product was amplified by PCR from DNA derived from plasma samples of negative (lanes 4 to 9) and immunoblot ("false")-positive sheep (lanes 10 to 19) taken on the third day of the febrile reaction. A 1-kb DNA ladder is shown in the leftmost lane; lanes 2 and 3 contain products from positive (+) and negative (−) control PCRs.

that the 32-kDa-protein-specific antibodies were detected consistently at 3 weeks after infection and persisted for 12 to 24 weeks. A sample of the immunoblot reactions with sera from these animals is shown in Fig. 2. Furthermore, sera collected twice from cattle in an area where heartwater is endemic at an interval of 9 months showed a consistent reaction with the 32-kDa protein of *C. ruminantium* (Fig. 3). Because of the high sensitivity and specificity of this assay with the above-mentioned sera, it was evaluated further for the diagnosis of heartwater in Zimbabwe.

Application of the immunoblotting assay in heartwater-free areas of Zimbabwe. The specificity of the immunoblotting assay was further tested by studying the reactions of cattle and sheep field sera from heartwater-free areas of Zimbabwe (see Materials and Methods). In these areas, neither clinical heartwater nor the *Amblyomma* tick vectors have ever been reported (26). In addition, the owners confirmed that their animals had never left the respective farms since birth. A total of 138 cattle and 195 sheep sera were tested, and many of these sera reacted strongly with the 32-kDa protein of *C. ruminantium* (Table 1, Fig. 4A). Positive immunoblot reactions were also detected with sera from goats from the same areas. There was no correlation between these immunoblot-positive reactions and age or sex, as sera from lambs, rams, and ewes of different ages reacted in a similar manner with the 32-kDa protein (Fig. 4B). Furthermore, some of these immunoblot-positive sera reacted with the 32-kDa protein at dilutions of 1/500 to 1/1,000, which are similar to endpoint titers in animals that have recovered from heartwater. Modifications introduced to optimize the specificity of the assay also did not alter the positive reactions.

Since the reaction of the 32-kDa *C. ruminantium* protein with sera from animals from heartwater-free areas did not appear artifactual, it became necessary to determine whether these animals were previously exposed to heartwater or were false positives. This was accomplished by examining their susceptibility to challenge with *C. ruminantium* organisms by transmission of *C. ruminantium* to ticks and by PCR.

Clinical response of immunoblot-positive and -negative sheep to *C. ruminantium* challenge. Clinically, there were no differences between the immunoblot-positive and -negative sheep with regard to challenge with *C. ruminantium*. The febrile reaction commenced 7 to 10 days after challenge in all sheep, and thereafter six immunoblot-positive and three immunoblot-negative sheep died (Table 2). Brain biopsies prepared on the third day of the febrile reaction from all sheep were positive for heartwater infection (Table 2) (29).

Detection of *C. ruminantium* DNA in ticks by the pCS20 DNA probe. To detect *C. ruminantium* in these immunoblot-positive sheep, *A. hebraeum* ticks were fed on these sheep and on immunoblot-negative sheep before and after sheep were challenged with *C. ruminantium* organisms. Tick DNA samples were prepared and hybridized to the ^{32}P-labeled pCS20 DNA probe, which detected *C. ruminantium* DNA in DNA of ticks that had been fed on the sheep after challenge with *C. ruminantium* (Fig. 5B and D) but not before challenge (Fig. 5A and C). There were no obvious differences in the abilities of the immunoblot-positive and -negative sheep to transmit *C. ruminantium* to the ticks or in the levels of infection that developed subsequently in the ticks.

Detection of *C. ruminantium* in sheep by PCR. By PCR, before challenge, *C. ruminantium* infection was not detected in the DNA samples derived from plasma of immunoblot-positive and -negative sheep (Fig. 6A). However, after challenge, on the third day of the febrile reaction, *C. ruminantium* organisms were detected in the plasma of all sheep by PCR, as demonstrated by the positive amplification of the *C. ruminantium*-specific 279-bp DNA fragment (Fig. 6B) (21). This result, supported by results of clinical reactions of the sheep, brain biopsies, and DNA probe hybridizations, further proves that the sheep were not infected with *C. ruminantium* before challenge.

Relationship of immunoblot-positive reactions to tick control

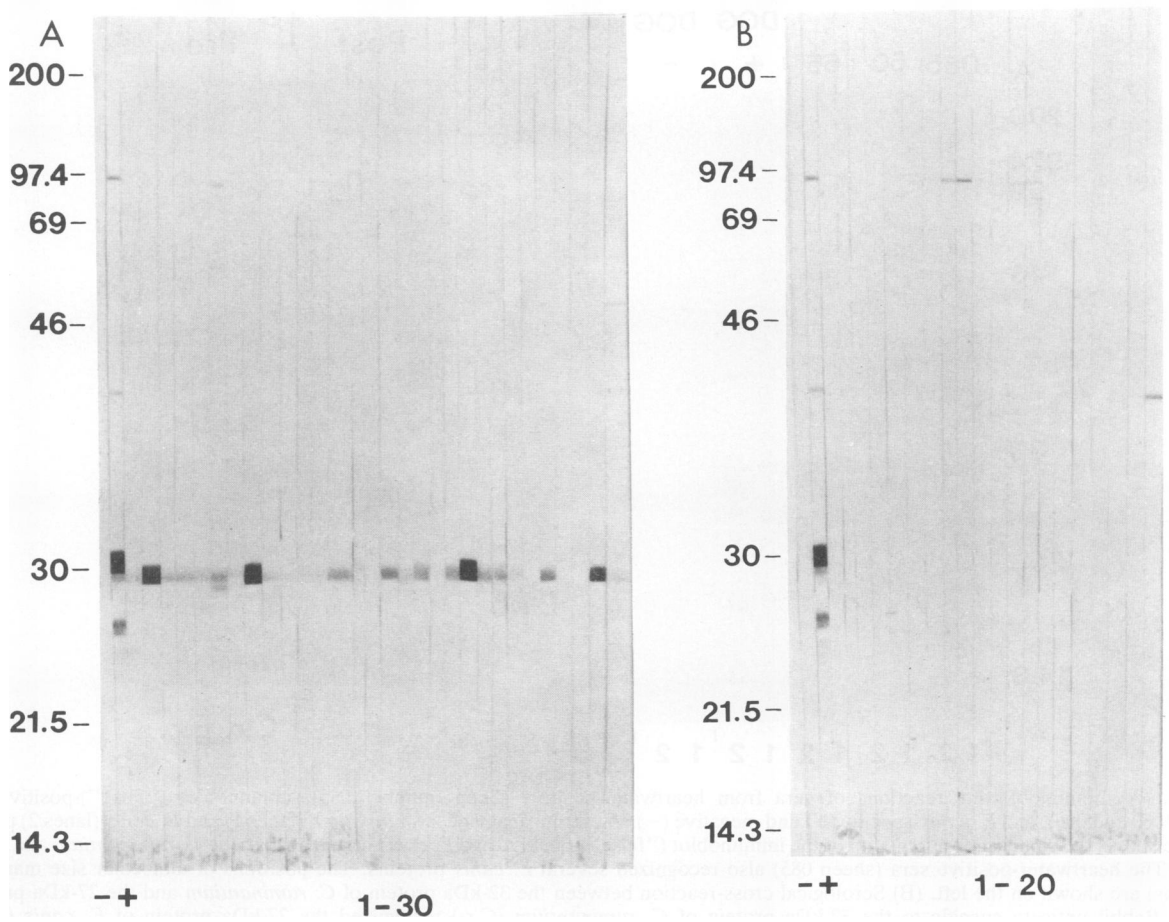

FIG. 7. (A) Immunoblots of 30 cattle sera (Mashona) from a heartwater-free area of Zimbabwe (Banket) where minimal tick control is practiced show frequent reaction with the 32-kDa protein of *C. ruminantium*. Positive (+) and negative (−) control serum reactions and the positions of the molecular size markers (in kilodaltons) are shown to the left. (B) Immunoblots of 20 cattle sera from a heartwater-free area of Zimbabwe (Banket) where strict tick control is practiced did not react against the 32-kDa *C. ruminantium* protein. Positive (+) and negative (−) control serum reactions and the positions of the molecular size markers are shown to the left.

and to serological cross-reactions. A higher frequency of immunoblot-positive reactions was associated in one heartwater-free area (Banket) with reduced tick control (Fig. 7A and B), suggesting that the agent(s) responsible for the serological cross-reactions with *C. ruminantium* is transmitted by ticks. In order to identify possible cross-reacting organisms, antisera to *C. ruminantium* and *E. canis* were reacted with *C. ruminantium* and *E. canis* antigens and antiserum to *E. bovis* was reacted with *C. ruminantium* antigen. The *E. canis* dog serum recognized the 32-kDa protein and several other proteins of *C. ruminantium* (Fig. 8A). Conversely, the *C. ruminantium* infection serum also recognized several *E. canis* proteins. A monospecific rabbit antiserum to the 32-kDa protein of *C. ruminantium* recognized a 27-kDa protein of *E. canis*, demonstrating antigenic cross-reactivity between these two proteins (Fig. 8B). The *E. bovis* infection sera also recognized the 32-kDa protein of *C. ruminantium*.

The prechallenge sera of two immunoblot-positive sheep (sheep 060 and 066) recognized both *C. ruminantium* and *E. canis* antigens in a pattern similar to that of homologous sera (Fig. 8A), demonstrating that the cross-reactivity properties of *C. ruminantium* antigens may not be restricted to the 32-kDa protein.

DISCUSSION

Although the 32-kDa-protein-specific immunoblotting assay has been used to confirm results of serological surveys initially conducted by using the IFAT and cELISA (14, 18), its true sensitivity and specificity as a diagnostic test for heartwater have not been determined. This study was conducted to determine these parameters.

The data described here demonstrate that the 32-kDa-protein-based immunoblotting assay has poor specificity, as it detects false-positives in field sera from heartwater-free areas of Zimbabwe. This is supported by the fact that in these areas there is no history of clinical heartwater disease or of *Amblyomma* tick vectors (26). The distribution of heartwater closely follows the distribution of the *Amblyomma* ticks (33). Additionally, the farms from which these sera originated have records to prove that the animals have never been to an area in which heartwater is endemic. The absence of previous exposure to heartwater in such animals was confirmed when false-positive sheep were shown to be as susceptible to challenge with *C. ruminantium* as the negative sheep. In addition, *C. ruminantium* infection was demonstrated in all sheep only after challenge, by brain

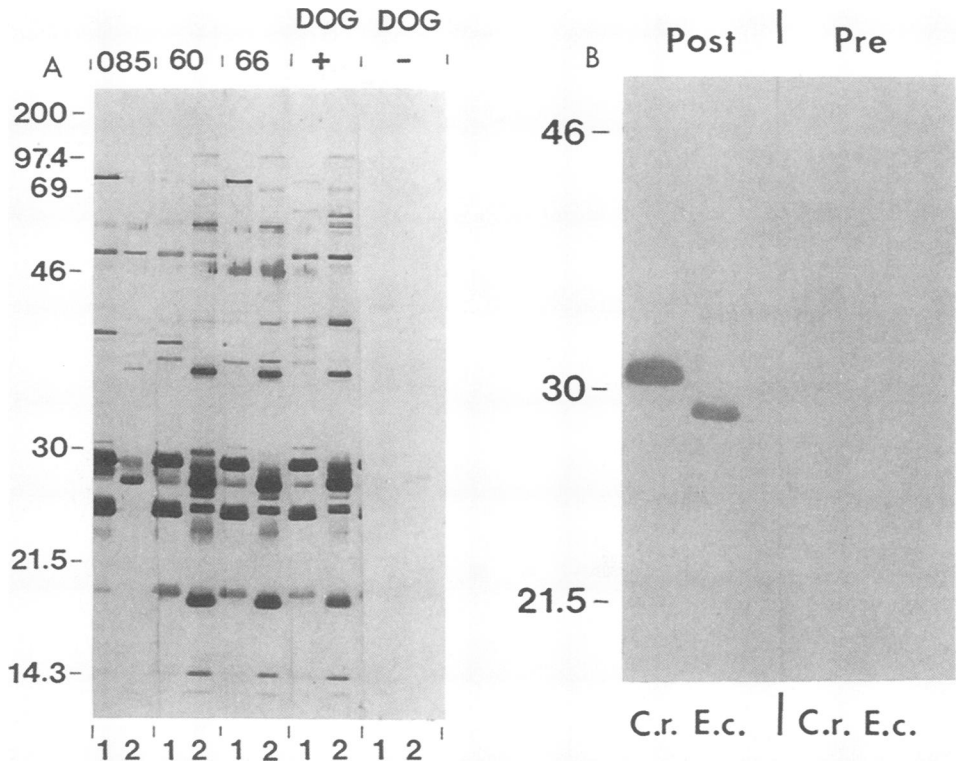

FIG. 8. (A) Immunoblotting reactions of sera from heartwater-positive sheep (number 085), immunoblot ("false")-positive sheep (numbers 060 and 066), and *E. canis*-specific (+) and -negative (−) dog serum against *C. ruminantium* (lanes 1) and *E. canis* (lanes 2) proteins. The figure shows the similarities in reaction of the immunoblot ("false")-positive and *E. canis* serum against the *C. ruminantium* and *E. canis* proteins. The heartwater-positive sera (sheep 085) also recognized several *E. canis* proteins. The positions of molecular size markers (in kilodaltons) are shown on the left. (B) Serological cross-reaction between the 32-kDa protein of *C. ruminantium* and the 27-kDa protein of *E. canis*. Rabbit antisera specific to the 32-kDa protein of *C. ruminantium* (C.r.) recognized the 27-kDa protein of *E. canis* (E.c.) in immunoblots. The preinoculation antiserum reaction (Pre) is shown on the right, and the postinoculation antiserum reaction (Post) is next to the molecular size markers (in kilodaltons), which are shown on the left.

biopsies, by transmission of *C. ruminantium* to ticks, and by PCR.

On the basis of these data we suggest that the immunoblot-positive reactions obtained with sera from heartwater-free areas were due to serological cross-reactions of *C. ruminantium* with an antigenically related organism(s). Serological cross-reactions between *C. ruminantium* and *E. canis* (19), *E. equi* (19), *E. phagocytophila* (16), *E. bovis* (10), and *E. ovina* (10) have been reported by using the IFAT. The cross-reaction of *E. bovis* and *E. canis* with the 32-kDa protein of *C. ruminantium* has been demonstrated here. These cross-reactions are not unexpected since, on the basis of 16S rRNA sequence analysis, *C. ruminantium* is very closely related to the *Ehrlichia* species (5) and in particular to *E. canis* (34).

Our observations also indicate that a higher frequency of immunoblot-positive reactions in heartwater-free areas of Zimbabwe was associated with minimal tick control. Tick species occurring in these areas are of the genera *Rhipicephalus* (23, 24, 27) and *Hyalomma* (25); they are known to feed on cattle, sheep, and goats and to transmit *E. bovis* (23), *E. canis* (12), and *E. ovina* (24). Although we demonstrated cross-reactions of *C. ruminantium* with *E. bovis* and *E. canis*, no attempt to isolate the actual cross-reacting agent was made, as this was beyond the scope of the study.

The occurrence of false-positive reactions has also been observed in heartwater-free areas of Zimbabwe by using the 32-kDa-protein-specific cELISA (7) and in South Africa by using the cELISA and the immunoblotting assay (9). In light of this information and the data presented here, the recent report of the possible extended distribution of heartwater in the Caribbean (18), based only on the serological reactions of livestock against the 32-kDa protein of *C. ruminantium*, needs reexamination. These serological results need to be confirmed by isolation of *C. ruminantium* organisms, since so far, heartwater has been confirmed to exist on the Caribbean islands of Guadeloupe, Marie Galant, and Antigua on the basis of isolation of *C. ruminantium* organisms (2, 3, 28).

In conclusion, this report shows that the immunodominant 32-kDa *C. ruminantium* protein is not antigenically unique to these organisms and that its use as a diagnostic antigen has limitations. In the future, development of reliable and specific serological tests for heartwater diagnosis would require identification of antigens or epitopes which are unique to *C. ruminantium*.

ACKNOWLEDGMENTS

This work received support from the United States Agency of International Development Cooperative Agreement no. AFR-0435-A-00-9084-00.

We thank the Centers for Disease Control and Prevention, Atlanta, Ga., for providing the DH82 cells and *E. canis* (Oklahoma strain). Bovine antisera to *E. bovis* were kindly provided by S. P.

Morzaria of the International Laboratory for Research on Animal Diseases, Nairobi, Kenya.

REFERENCES

1. **Andrew, H. R., and R. A. I. Norval.** 1989. The carrier status of sheep, cattle and African buffalo recovered from heartwater. Vet. Parasitol. **34**:261–266.
2. **Barré, N., G. Uilenberg, P. C. Morel, and E. Camus.** 1987. Danger of introducing heartwater onto the American mainland: potential role of indigenous and exotic *Amblyomma* ticks. Onderstepoort J. Vet. Res. **54**:405–417.
3. **Burridge, M. J.** 1985. Heartwater invades the Caribbean. Parasitol. Today **1**:1–3.
4. **Byrom, B., and C. E. Yunker.** 1990. Improved culture conditions for *Cowdria ruminantium* (Rickettsiales), the agent of heartwater disease of domestic ruminants. Cytotechnology **4**:285–290.
5. **Dame, J. B., S. M. Mahan, and C. A. Yowell.** 1992. Phylogenetic relationship of *Cowdria ruminantium*, agent of heartwater, to *Anaplasma marginale* and other members of the order *Rickettsiales* determined on the basis of 16S rRNA sequence. Int. J. Syst. Bacteriol. **42**:270–274.
6. **Dawson, J. E., Y. Rikihisa, S. Ewing, and D. B. Fishbein.** 1991. Serologic diagnosis of human ehrlichiosis using two *Ehrlichia canis* isolates. J. Infect. Dis. **163**:564–567.
7. **De Vries, N., S. M. Mahan, U. Ushwokunze-Obatolu, R. A. I. Norval, and F. Jongejan.** Correlation between antibodies to *Cowdria ruminantium* (Rickettsiales) in cattle and the distribution of *Amblyomma* tick vectors in Zimbabwe. Submitted for publication.
8. **Du Plessis, J. L.** 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum*: effects in mice injected with tick homogenates. Onderstepoort J. Vet. Res. **52**:55–61.
9. **Du Plessis, J. L., J. D. Bezuidenhout, M. S. Brett, E. Camus, F. Jongejan, S. M. Mahan, and D. Martinez.** The sero-diagnosis of heartwater: a comparison of 5 tests. Rev. Elev. Med. Vet. Pays Trop., in press.
10. **Du Plessis, J. L., E. Camus, P. T. Oberem, and L. Malan.** 1987. Heartwater serology: some problems with the interpretation of results. Onderstepoort J. Vet. Res. **54**:327–329.
11. **Du Plessis, J. L., and L. Malan.** 1987. The application of the indirect fluorescent antibody test in research on heartwater. Onderstepoort J. Vet. Res. **54**:319–325.
12. **Greene, C. E., and J. W. Harvey.** 1984. Canine ehrlichiosis, p. 545–561. *In* C. E. Greene (ed.), Clinical microbiology and infectious diseases of the dog and the cat. W. B. Saunders Co., Philadelphia.
13. **Haig, D. A.** 1952. Note on the use of the white mouse for transport of strains of heartwater. J. S. Afr. Vet. Med. Assoc. **23**:167–170.
14. **Jongejan, F., and M. J. C. Thielemans.** 1989. Identification of an immunodominant antigenically conserved 32-kilodalton protein from *Cowdria ruminantium*. Infect. Immun. **57**:3243–3246.
15. **Jongejan, F., M. J. C. Thielemans, M. De Groot, P. J. S. Van Kooten, and B. A. M. Van der Zeijst.** 1991. Competitive enzyme-linked immunosorbent assay for heartwater using monoclonal antibodies to a *Cowdria ruminantium*-specific 32 kilodalton protein. Vet. Microbiol. **28**:199–211.
16. **Jongejan, F., L. A. Wassink, M. J. C. Thielemans, N. M. Perie, and G. Uilenberg.** 1989. Serotypes in *Cowdria ruminantium* and their relationship with *Ehrlichia phagocytophila* determined by immunofluorescence. Vet. Microbiol. **21**:31–40.
17. **Knight, S. T., V. R. Neece, and D. J. Witt.** 1989. Rapid culture-independent techniques for quantitation of *Chlamydia trachomatis* elementary bodies. J. Microbiol. Methods **10**:255–263.
18. **Kobold, A. M., D. Martinez, E. Camus, and F. Jongejan.** 1992. 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. **30**:1870–1873.
19. **Logan, L. L., C. J. Holland, C. A. Mebus, and M. Ristic.** 1986. Serological relationship between *Cowdria ruminantium* and certain *Ehrlichia* spp. Vet. Rec. **119**:458–489.
20. **Logan, L. L., C. J. Holland, C. A. Mebus, and M. Ristic.** 1987. The development of *Cowdria ruminantium* in neutrophils. Onderstepoort J. Vet. Res. **54**:197–204.
21. **Mahan, S. M., S. D. Waghela, T. C. McGuire, F. R. Rurangirwa, L. A. Wassink, and A. F. Barbet.** 1992. A cloned DNA probe for *Cowdria ruminantium* hybridizes with eight heartwater strains and detects infected sheep. J. Clin. Microbiol. **30**:981–986.
22. **Mishell, B. B., and S. M. Shiigi.** 1980. Selected methods in cellular immunology. W.H. Freeman and Company, New York.
23. **Norval, R. A. I.** 1979. Tick infestation and tick-borne diseases in Zimbabwe Rhodesia. J. S. Afr. Vet. Assoc. **50**:289–292.
24. **Norval, R. A. I.** 1981. The ticks of Zimbabwe. III. *Rhipicephalus evertsi evertsi*. Zimb. Vet. J. **12**:31–35.
25. **Norval, R. A. I.** 1982. The ticks of Zimbabwe. IV. The genus *Hyalomma*. Zimb. Vet. J. **13**:2–10.
26. **Norval, R. A. I.** 1983. The ticks of Zimbabwe. VII. The genus *Amblyomma*. Zimb. Vet. J. **14**:3–18.
27. **Norval, R. A. I., T. Daillecourt, and R. G. Pegram.** 1983. The ticks of Zimbabwe. VI. The *Rhipicephalus sanguineus* group. Zimb. Vet. J. **13**:38–46.
28. **Perreau, P., P. C. Morel, N. Barre, and P. Durrand.** 1980. Existence de la cowdriose (heartwater) a *Cowdria ruminantium* chez les ruminants des Antilles Françaises (La Guadeloupe) et des Mascareignes (La Reunion et Ile Maurice). Rev. Elev. Med. Vet. Pays Trop. **33**:21–22.
29. **Purchase, H. S.** 1945. A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry 1925) in heartwater brains. Vet. Rec. **57**:413–414.
30. **Rossouw, M., A. W. H. Neitz, D. T. De Waal, J. L. Du Plessis, L. Van Gas, and S. Brett.** 1990. Identification of the antigenic proteins of *Cowdria ruminantium*. Onderstepoort J. Vet. Res. **57**:215–221.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Semu, S., S. M. Mahan, C. E. Yunker, and M. J. Burridge.** 1992. Development and persistence of *Cowdria ruminantium* specific antibodies following experimental infection of cattle, as detected by the indirect fluorescent antibody test. Vet. Immunol. Immunopathol. **33**:339–352.
33. **Uilenberg, G.** 1983. Heartwater (*Cowdria ruminantium* infection): current status. Adv. Vet. Sci. Comp. Med. **27**:427–480.
34. **Van Vliet, A. H. M., F. Jongejan, and B. A. M. Van der Zeijst.** 1992. Phylogenetic position of *Cowdria ruminantium* (Rickettsiales) determined by analysis of amplified 16S ribosomal DNA sequences. Int. J. Syst. Bacteriol. **42**:494–498.
35. **Van Winkelhoff, A. J., and G. Uilenberg.** 1981. Heartwater: cross-immunity studies with strains of *Cowdria ruminantium* isolated in West and South Africa. Trop. Anim. Health Prod. **13**:160–164.
36. **Waghela, S. D., F. R. Rurangirwa, S. M. Mahan, C. E. Yunker, T. B. Crawford, A. F. Barbet, M. J. Burridge, and T. C. McGuire.** 1991. A cloned DNA probe identifies *Cowdria ruminantium* in *Amblyomma variegatum* ticks. J. Clin. Microbiol. **26**:2571–2577.
37. **Yunker, C. E., S. M. Mahan, S. D. Waghela, T. C. McGuire, F. R. Rurangirwa, A. F. Barbet, and L. A. Wassink.** 1993. Detection of *Cowdria ruminantium* by means of a DNA probe pCS20 in infected Bont ticks, *Amblyomma hebraeum*, the major vector of heartwater in Southern Africa. Epidemiol. Infect. **110**:95–104.