Experimental & Applied Acarology, 22 (1998) 111–122

Evidence to show that an agent that cross-reacts serologically with *Cowdria ruminantium* in Zimbabwe is transmitted by ticks

Deckster T. Savadye[a], Patrick J. Kelly[b] and Suman M. Mahan[a]*
[a]*University of Florida/USAID/SADC Heartwater Research Project, Veterinary Research Laboratory Diagnostic and Research Branch, PO Box CY 551, Causeway, Harare, Zimbabwe*
[b]*Department of Clinical Veterinary Science, Faculty of Veterinary Science, University of Zimbabwe, PO Box MP 167, Mount Pleasant, Harare, Zimbabwe*
(Received 5 July 1997; accepted 26 September 1997)

ABSTRACT

The serological diagnosis of heartwater based on reactions to the immunodominant *Cowdria ruminantium* major antigen protein-1 (MAP-1) is impaired by the detection of false-positive reactions. In this study, the prevalence of false-positive reactions on seven heartwater-free farms in Zimbabwe was determined to be 8–94% by immunoblotting against *C. ruminantium* antigens. The highest prevalence of false-positives on Spring Valley Farm correlated with the presence of *Rhipicephalus evertsi evertsi* ticks. The other tick species found on these seven farms were *Hyalomma truncatum* and *Hyalomma marginatum rufipes*. *Rhipicephalus evertsi evertsi* ticks collected from Spring Valley Farm and fed on seronegative sheep caused seroconversion in one of two sheep. This sheep developed a mild febrile reaction and *C. ruminantium* MAP-1 antigen reactive antibodies 3 weeks after the ticks started feeding. Polymerase chain reactions (PCRs), conducted using *C. ruminantium*-specific primers on ticks collected from the seven farms and on some of the *R. e. evertsi* ticks that had caused seroconversion in one sheep, were negative. However, some of these ticks gave positive PCRs with DNA primers which amplify a 350 bp DNA fragment of the 16s rRNA gene from all ehrlichial agents indicating the presence of infection with one or more *Ehrlichia* species. Although attempts to isolate the cross-reacting agent from the sheep were unsuccessful, this study demonstrates that false-positive reactions with the MAP-1 *C. ruminantium* antigen are associated with agents transmitted by ticks.
Exp Appl Acarol 22: 111–122 © 1998 Chapman & Hall Ltd

Keywords: *Cowdria ruminantium*, tick transmission, *R. e. evertsi*, *Ehrlichia*, serological cross-reaction.

INTRODUCTION

Heartwater is a disease of domestic ruminants that is of economic importance in Africa and the Caribbean where it is a major constraint to livestock improvement. The disease is caused by a rickettsia, *Cowdria ruminantium* (Uilenberg, 1983) and the only known vectors are ticks of the genus *Amblyomma* (Uilenberg, 1983), the

*To whom correspondence should be addressed at: Tel: 263–4–794980; Fax: 263–4–794980; e-mail: Sumanmah@sumara.co.zw

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most important species being *Amblyomma hebraeum* and *Amblyomma variegatum*. The diagnosis of heartwater by serological assays is hindered by the serological cross-reactions between *C. ruminantium* and members of the genus *Ehrlichia* (Logan *et al*., 1986; du Plessis *et al*., 1987). These have been detected by the immunofluorescent antibody test (IFAT) (Logan *et al*., 1986; du Plessis and Malan, 1987; Holland *et al*., 1987; Jongejan *et al*., 1989b), MAP-1-specific competitive enzyme-linked immunosorbent assay (cELISA) (Jongejan and Thielemans, 1989a; Jongejan *et al*., 1991; de Vries *et al*. 1992) and immunoblotting (Mahan *et al*., 1993). The immunodominant MAP-1 antigen of *C. ruminantium* has been proposed for the diagnosis of heartwater (Jongejan *et al*., 1991; Mahan *et al*., 1993; Barbet *et al*., 1994). This antigen was previously known as the *C. ruminantium* 32 kDa protein (Cr 32) and thought to be antigenically specific to *C. ruminantium* (Jongejan and Thielemans, 1989), but has since been shown to also be antigenically conserved in the genus *Ehrlichia* (Jongejan *et al*., 1993; Mahan *et al*., 1993; Kelly *et al*., 1994). Studies based on the analysis of 16s ribosomal RNA gene sequences demonstrate that *C. ruminantium* is phylogenetically closely related to *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia phagocytophila* and *Ehrlichia equi* and this relationship explains the presence of common antigenic determinants between the two genera (van Vliet *et al*., 1992).

In Zimbabwe, serological cross-reactions with *C. ruminantium* have been detected in sera collected from heartwater-free areas and this limits the application of the currently available assays for the diagnosis of heartwater. Even the most specific MAP-1B indirect ELISA for *C. ruminantium* detects approximately 8% of the false-positive sheep sera from Zimbabwe (van Vliet *et al*., 1995). The identity of the agents responsible for serological cross-reactions in field sera is unknown, although *Ehrlichia* species are suspected to be responsible for these cross-reactions based on laboratory evidence. Evidence from a previous study in cattle (Mahan *et al*., 1993) suggested that the serological cross-reactions were due to agents transmitted by ticks other than *Amblyomma* since a high frequency of false-positive reactors in heartwater-free areas correlated with relaxed tick control. The isolation of the agents responsible for serological cross-reactions with *C. ruminantium* would allow an antigenic comparison so that unique *C. ruminantium* antigens may be identified for the serodiagnosis of heartwater. The studies presented here attempted to determine whether the agent responsible for the serological cross-reactions was transmitted by ticks, by attempted isolation of the cross-reacting agent from sheep fed on by ticks that were polymerase chain reaction (PCR) positive for ehrlichial 16s RNA.

MATERIALS AND METHODS

Determination of the prevalence of false-positive reactors by immunoblotting

Serum samples were collected from cattle, sheep and goats on seven farms in heartwater-free areas of Zimbabwe based on the absence of *Amblyomma* ticks and heartwater. These farms are Munenga Farm (Arcturus), Kent Estates (Norton),

Chikwaka Communal Lands (Murewa), Henderson Research Station (Mazowe), Chitara Estate (Bromley), Spring Valley Farm (Ruwa) and Sandringham Farm (Darwendale). All these sites are located in the highveld of Zimbabwe. The sera were diluted to 1:100 and then tested against *C. ruminantium* antigen immunoblots (Mahan *et al*., 1993) to determine the prevalence of false-positive reactors on each farm and to compare the reaction patterns with *C. ruminantium*-specific sera. The antigen used for immunoblotting was derived from *C. ruminantium*, Crystal Springs strain, cultured in bovine endothelial cells (Byrom and Yunker, 1990).

Tick collection and tick transmission tests

Ticks were collected off livestock from the seven farms at the same time as the sera. This was during the last half of the rainy season of 1994 (from February to April), when abundant numbers of ticks were available in the field. Information on tick control programmes on the seven farms was obtained to determine a relationship between the ticks and the prevalence of false-positive reactors. The ticks that were collected from the various farms were identified by the Tick Section of the Veterinary Research Laboratory in Harare, Zimbabwe. Ticks that were still alive after collection were used for transmission tests in the laboratory to determine their ability to transmit *C. ruminantium* cross-reacting agent(s) to seronegative sheep. The remaining ticks were tested for infection with *C. ruminantium* and *Ehrlichia* species by PCR assays (Mahan *et al*., 1992; Peter *et al*., 1995; L.A. Matthewman, N. Lally, K. Sumption, P.J. Kelly and D. Raoult, unpublished).

At the time of the experiment, live adult ticks were available from Spring Valley Farm and Chitara Estate. Seronegative sheep (Wiltiper breed) were obtained from the heartwater-free Sandringham Farm in Darwendale, Zimbabwe. The sheep were prepared for tick feeding by fixing body bags onto their dorsum. Forty *Rhipicephalus evertsi evertsi* ticks from Spring Valley Farm were placed on each of sheep 2525 and 2404. Three *Hyalomma marginatum rufipes* ticks from Chitara Estate (the only live ticks available in the sample at the time of the experiment) were placed on sheep number 2420. The sheep were kept in concrete-floored pens under tick-free conditions and were monitored daily by recording their morning rectal temperatures to determine the onset of febrile responses. The sheep were bled once a week and the sera were tested for the presence of cross-reactive antibodies to *C. ruminantium* antigen immunoblots (Mahan *et al*., 1993). Following seroconversion in sheep 2525, buffy coat cells were collected from its blood and seeded onto DH82 cells (Kelly *et al*., 1994) or autologous monocyte cultures were started to isolate the agent from the blood. These sheep were subsequently challenged with 5 ml of culture-derived *C. ruminantium* Crystal Springs strain to determine their susceptibility to heartwater. Brain biopsies were conducted on these sheep on day 3 of the febrile reaction to confirm the presence of *C. ruminantium* infection in brain endothelial cells (Synge, 1978). Death of the sheep was prevented by treating them with 10 mg kg^{-1} of oxytetracycline intravenously on days 2 and 3 of the febrile reaction.

Processing ticks for PCR

Fresh and dried ticks from Spring Valley Farm, Kent Estate and Chitara Estate were analysed by PCR to detect the presence of *C. ruminantium* or agents of the *Ehrlichia* species. Fresh ticks were dissected to obtain the internal organs (salivary glands and guts), as described previously (Peter *et al*., 1995). Dry ticks were crushed individually in labelled Eppendorf tubes using a sterile 1 ml pipette tip. DNA was isolated from all these ticks as described previously (Peter *et al*., 1995). The number of ticks analysed varied upon their availability.

PCR assay

DNA from 15 *R. e. evertsi* ticks collected from Spring Valley Farm, 15 *R. e. evertsi* ticks (from the same farm) that were fed on sheep number 2525 and six *H. marginatum rufipes* ticks from Chitara Estate were analysed by PCR to detect the presence of *C. ruminantium*, using the *C. ruminantium*-specific primers for pCS20 DNA probe, AB128 (5' ACT AGT AGA AAT TGC ACA ATC TAT 3') and AB129 (5' TGA TAA CTT GGT GCG GGA AAT CCT T 3') (Mahan *et al*., 1992; Peter *et al*., 1995). These DNA primers amplify a 279 bp DNA fragment. The PCR assay was conducted as described (Peter *et al*., 1995) with modifications made after optimization on DNA obtained from laboratory-reared *Rhipicephalus appendiculatus* ticks (supplied from the Tick Section of the Veterinary Research Laboratory, Harare). These ticks were used as genus control for *R. e. evertsi*. At primer concentrations of 0.1 μM and 1.5 mM $MgCl_2$ non-specific priming was reduced to minimal (data not shown). The PCR products were visualized by agarose gel electrophoresis using 1.5% agarose gels, followed by Southern hybridization (Maniatis *et al*., 1989), with a ^{32}P-labelled, *C. ruminantium*-specific pCS20 DNA probe (Waghela *et al*., 1991), as described previously (Mahan *et al*., 1992; Peter *et al*., 1995).

The DNA from the individual ticks from the Spring Valley farm including those that were fed on sheep number 2525 and six *H. marginatum rufipes* ticks from Chitara Estate was also analysed for the presence of *Ehrlichia* sequences by PCR. The PCRs were performed using the general *Ehrlichia* primers, E_2 (5' GTG GCA GAC GGG TGA GTA ATG C 3') and E_3(s)(5' GGT AAC GTC AAT ATC TTC CC 3'), designed from the conserved region of the 16s rRNA gene of the members of the tribe Ehrlichieae (L.A. Matthewman N. Lally, K. Sumption, P.J. Kelly and D. Raoult, unpublished). These primers amplify a fragment of approximately 350 bp from *Ehrlichia* DNA. The PCRs were conducted as for the *C. ruminantium* PCR and the PCR products were analysed by agarose gel electrophoresis.

RESULTS

Identification of ticks from the seven sampled farms

The composition of tick species collected from the different farms is shown in Table 1. *Ambylomma hebraeum* or *A. variegatum* ticks, the major vector ticks of heartwater in Zimbabwe, were not present on any of the farms sampled. There were three major

TABLE 1

Species composition of ticks collected from seven different farms

Tick species Farm/area	*H. marginatum rufipes*	*H. truncatum*	*R. e. evertsi*	*Rhipicephalus compositus*	*R. appendiculatus*	*B. decoloratus*	*A. hebraeum* and *A. variegatum*
Munenga	–	0	–	–	–	–	–
Kent Estate	+ (25) (63%)	+ (10) (25%)	+ (4) (10%)	0	+ (1) (3%)	0	0
Chikwaka communal lands	+	+	0	0	+	+	0
Henderson Research Station	0	0	+ (5) (100%)	0	0	0	0
Chitara Estate	+ (57) (50%)	0	+ (57) (50%)	+ (1) (1%)	0	0	0
Spring Valley Farm	+ (1) (1%)	+ (10) (8%)	+ (117) (91%)	0	0	0	0
Sandringham Farm	–	–	–	–	–	–	–

+ , tick species present in sample; 0, tick species not present in sample; –, no collection made; (), number of that tick species present in the sample; (%), percentage of sample made by a tick species

tick species identified from these farms and they were *R. e. evertsi*, *H. marginatum rufipes* and *Hyalomma truncatum*. Of these the *R. e. evertsi* ticks made up the largest proportion of the samples. Tick control on all of the farms except in the communal areas (areas mainly for subsistence agriculture), such as Chikwaka, was conducted by either dipping or spraying the cattle using acaricide at 1 or 2 week intervals. In the communal areas dipping was not regular due to a shortage of acaricide. The sheep and goats were dipped irregularly on all of the farms visited except at Sandringham Farm where the sheep were dipped once every month. Tick infestation was visually assessed. The tick infestation on livestock was high at Kent Estates, Chitara Estates, Spring Valley Farm and on sheep at Henderson Research Station (Table 1). The cattle at Munenga Farm and Henderson Research Station and the sheep at Sandringham Farm were almost uninfested at the time of sampling.

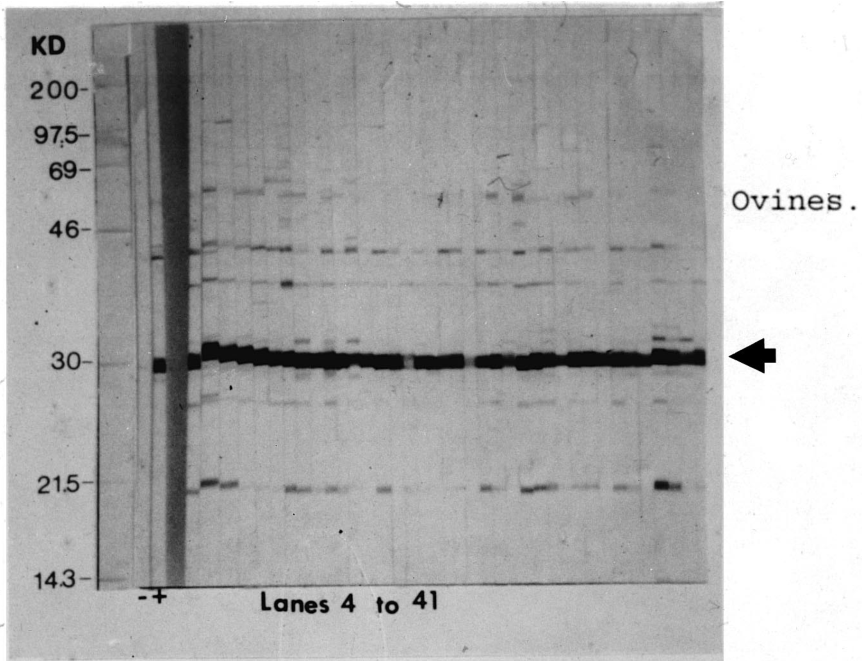
Prevalence of false-positive reactors on the seven heartwater-free farms

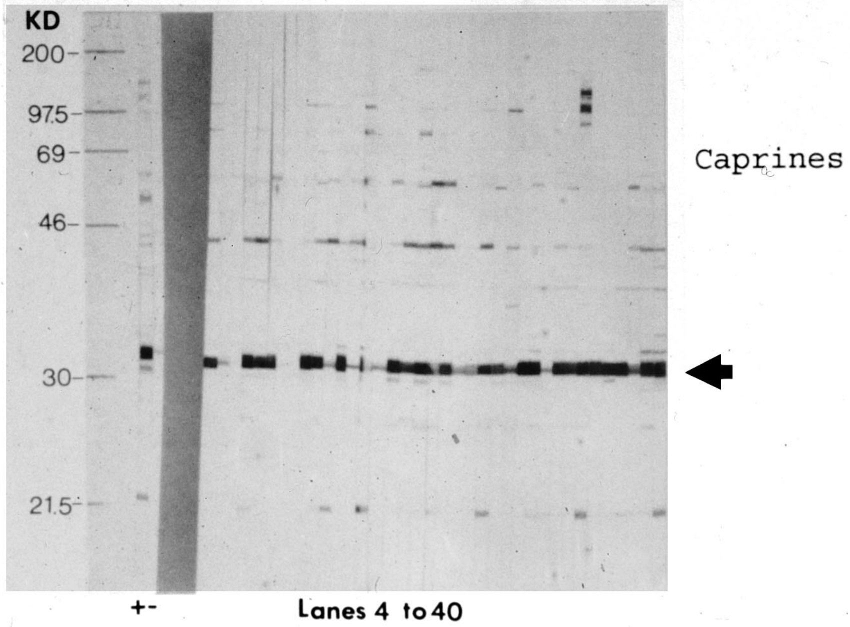
Two hundred and twenty-four serum samples collected from the seven heartwater-free farms were tested by immunoblotting for reaction with *C. ruminantium* antigen immunoblots. A breakdown of the samples collected is shown in Table 2. The prevalence of false-positive reactors on the seven farms varied from 8% in bovines from Henderson Research Station to 100% in ovines from Spring Valley Farm. In

TABLE 2
Prevalence of false-positive reactors from seven heartwater-free farms around Harare, Zimbabwe as determined by immunoblotting assay based on recognition of *C. ruminantium* MAP-1 and other antigens

Area	Animal species	Breed	Sample size	Prevalence of false-positives (%)
Munenga Farm	Cattle	Friesian	10	70
Kent Estate	Cattle	Brahman	14	46
Chikwaka communal lands	Cattle	Mashona	10	42
Henderson Research Station	Cattle	Hereford Brahman	25	8
	Goats	Unknown	25	28
	Sheep	Dorper	25	72
Chitara Estate	Cattle	Friesian	17	47
Spring Valley Farm	Sheep	Merino	37	100
	Goats	Angora	38	88
Sandringham Farm	Sheep	Wiltiper	23	65

Fig. 1. Immunoblot reactions of ovine and caprine sera from Spring Valley Farm with *C. ruminantium* Crystal Springs strain antigen blots. (A) Ovine sera are represented in lanes 4–41. Lanes 2: (−) and 3: (+) (sheep anti-*C. ruminantium* serum) are the negative and positive control serum reactions, respectively. (B) The reactions of caprine sera are represented in lanes 4–40. Lane 2: (+) (sheep anti-*C. ruminantium* serum) and lane 3: (−) are the positive serum and negative control serum reactions, respectively. Lane 1 in both (A) and (B) blots is the protein molecular weight markers (size in kDa). The arrows demonstrate the recognition of MAP-1 by the respective sera.


KD
200-
97.5-
69-
46-
30-
21.5-
14.3-
-+
Lanes 4 to 41
Ovines.


KD
200-
97.5-
69-
46-
30-
21.5-
+-
Lanes 4 to 40
Caprines

most cases the reaction of false-positive sera with *C. ruminantium* antigen was similar to that of antisera to *C. ruminantium*. A sample of the reaction of the sera from the sheep and goats from Spring Valley Farm is shown in Fig. 1 and highlights the dominant reaction of the sera with the MAP 1 antigen (32 kDa).

Transmission of the C. ruminantium cross-reactive agent by ticks

To prove that the varying degrees of seropositivity for the *C. ruminantium* antigen in the heartwater-free farms was due to a tick-transmitted cross-reacting agent, any live ticks that were collected from these farms were fed on seronegative sheep. Forty *R. e. evertsi* ticks from Spring Valley Farm were fed on each of sheep numbers 2525 and 2404. Seroconversion occurred in sheep number 2525 21 days after tick feeding. The reaction of the sera from sheep number 2525 with *C. ruminantium* MAP-1 became stronger with time and the sera recognized two other *C. ruminantium* proteins of 44 and 58 kDa molecular size (Fig. 2). Sheep number 2525 showed a

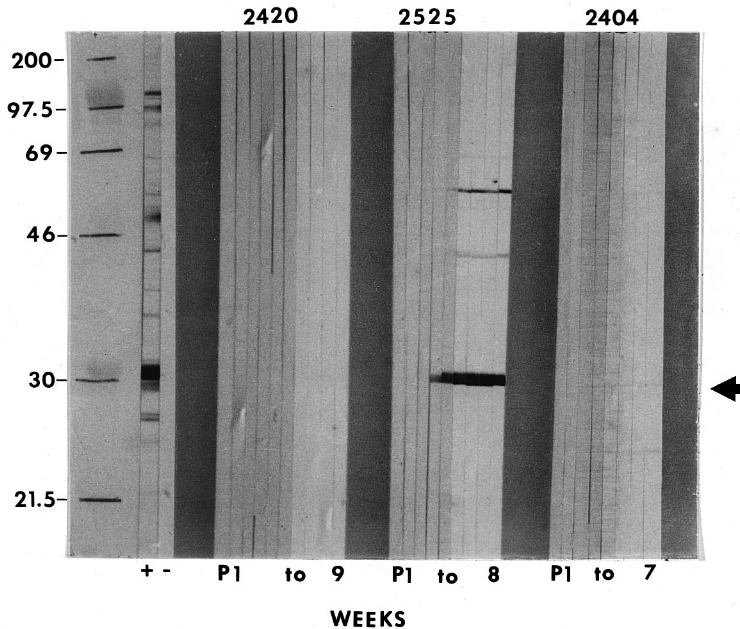


Fig. 2. Reaction of the pre- and post-tick feeding sera from sheep 2525, 2404 and 2420 with *C. ruminantium* Crystal Springs strain antigen blots. Forty *R. e. evertsi* ticks from Spring Valley Farm were fed on sheep 2525 and 2404 and three *H. marginatum rufipes* from Chitara Estate were fed on sheep 2420. Lane 1 represents the protein molecular weight markers (size in kDa). Lane 2: (+) is the reaction of goat anti-*C. ruminantium* serum (positive control). Lane 3: (−) is the negative goat serum reaction. The sheep numbers are on top of the blot. P represents the reaction of pre-tick feed serum with *C. ruminantium* blots and 1–9, 1–8 and 1–7 represent the reactions of the weekly serum samples for each sheep, respectively. The arrow demonstrates recognition of MAP-1 by sheep 2525 sera from week 3 after tick feeding.

mild febrile reaction (temperature above 40.5°C) 16–19 days after tick feeding, which was just before seroconversion was detected. Sheep numbers 2404 and 2420 remained seronegative and did not develop febrile reactions. Attempts to culture the agent from blood from sheep number 2525 in a DH82 cell culture or autologous monocyte culture were not successful (data not shown). To prove that the agent that caused seroconversion in sheep number 2525 was not *C. ruminantium*, a cell culture-derived *C. ruminantium* inoculum (Crystal Springs strain) was administered intravenously into sheep 2525, 2404 and 2420. All of the sheep were susceptible to the infection and were positive for *C. ruminantium* by brain biopsy on day 3 of the febrile reaction. Death in the sheep was prevented by treatment with oxytetracycline.

PCR identification of a potential C. ruminantium cross-reacting agent in ticks
The ticks collected from Spring Valley farm, Chitara Estate and Kent Estate were analysed by PCR assay to characterize the *C. ruminantium* cross-reacting agent. All of the ticks tested were negative for *C. ruminantium* infection by PCR and by pCS20 DNA probe hybridization and a sample of these analyses is presented in Fig. 3A (Lanes 4–36). The 15 *R. e. evertsi* ticks from this farm that caused seroconversion in sheep 2525 were also negative for *C. ruminantium* by PCR (Fig. 3A, lanes 22–36). However, PCR amplification conducted on the same ticks with *Ehrlichia* primers resulted in the detection of several positive ticks both from the unfed and the 15 ticks which caused seroconversion in sheep number 2525 (Fig. 3B, lanes 4–36). Two out of six *H. marginatum rufipes* unfed ticks from Chitara Estate were also positive for *Ehrlichia* sequences (data not shown).

DISCUSSION

The findings of this study confirmed the suggestion that the agent(s) that cross-react with *C. ruminantium* in heartwater-free areas of Zimbabwe were tick transmitted (Mahan *et al*., 1993). Mahan *et al*. (1993) observed that false-positive reactors are more frequent in areas that have minimal tick control. In the present study it was also observed that there was a relationship between the prevalence of false-positive reactors and tick infestation. On farms where the tick infestation was high, a greater prevalence of false-positive reactors was detected. For example, ovine sera from Spring Valley Farm reacted more strongly with *C. ruminantium* immunoblots than ovine sera from Sandringham Farm. The prevalence of false-positive reactors in the cattle from Henderson Research Station was lower than that of the cattle from all the other farms, such as Chitara Estate, where the tick infestation was very high.

The tick species in the sampled areas were as predicted by Norval (1983). *Rhipicephalus evertsi evertsi* was the main tick species found on the farms with a high prevalence of false-positive reactors. Furthermore, the transmission tests confirmed that the *R. e. evertsi* ticks (from Spring Valley Farm) were able to transmit an

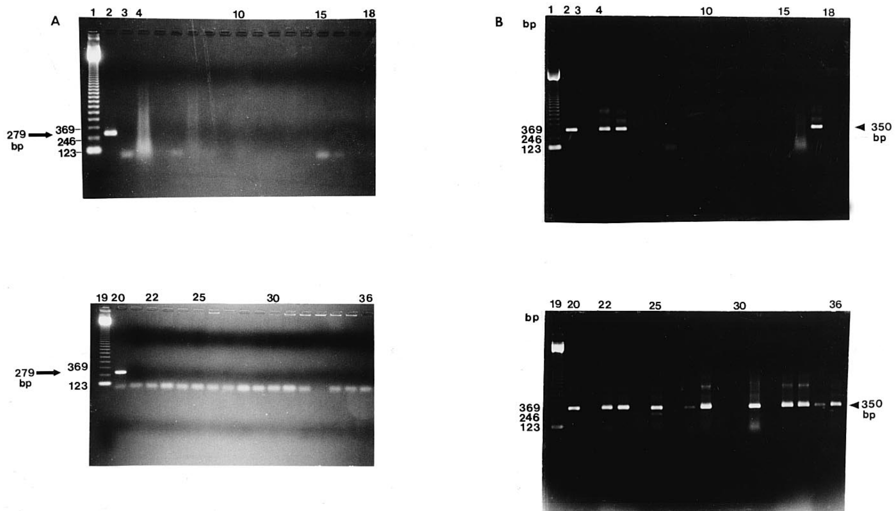


Fig. 3. PCR amplification on *R. e. evertsi* ticks from Spring Valley farm with a high prevalence of false positives. (A) PCR amplification with the *C. ruminantium*-specific primers AB128 and AB129 demonstrating that the ticks were not infected with *C. ruminantium*. Lanes 1 and 19 are the 123 bp DNA ladder. Lane 2 and 20 are positive controls. Lanes 3 and 21 are negative reagent controls. Lanes 4–18 represent ticks that were not fed on experimental sheep. Lanes 22–36 represent ticks that were fed on experimental sheep number 2525 and caused seroconversion. (B) PCR amplification with general *Ehrlichia* primers demonstrating that some of the ticks were infected with an *Ehrlichia* agent. Lanes 1 and 19 are the 123 bp DNA ladder. Lanes 2 and 20 are positive controls. Lanes 3 and 21 are negative reagent controls. Lanes 4–18 represent ticks that were not fed on experimental sheep. Lanes 22–36 represent ticks that were fed on experimental sheep number 2525 and caused seroconversion. Positive amplification was detected in lanes 4, 5, 8, 17, 22, 23, 25, 27, 28, 31, 33, 34, 35 and 36.

agent that caused seroconversion in sheep 2525, providing direct evidence that the agent responsible in Zimbabwe for cross-reactions with *C. ruminantium* was tick transmitted and also demonstrated that *R. e. evertsi* was a vector of the agent. *Rhipicephalus evertsi evertsi* ticks have been reported to transmit *Ehrlichia ovina* (Norval, 1981) and this could be the agent that is being transmitted to the sheep, rendering them serologically positive against *C. ruminantium* antigens. A serological relationship of *E. ovina* to *C. ruminantium* has been demonstrated previously (Jongejan *et al*., 1993; van Vliet *et al*., 1995). The transmission tests done using three *H. marginatum rufipes* ticks from Chitara Estate did not cause seroconversion in sheep 2420 probably because of the small number of ticks fed or the lack of infection within these ticks. Due to seasonal limitation, *H. truncatum* ticks, which formed 7.8% of the tick population at Spring Valley Farm, were not available for the transmission tests. However, *Hyalomma* ticks are also believed to transmit an *Ehrlichial* agent that serologically cross-reacts with *C. ruminantium* (Norval, 1979).

The PCR analyses conducted on the ticks sampled from Spring Valley Farm, Chitara Estate, Kent Estate and the ticks that had caused seroconversion in sheep number 2525 were all negative for *C. ruminantium* but positive for *Ehrlichia* spp. DNA sequences. These data further support the assumption that the cross-reacting agent is a member of the *Ehrlichia* spp. Further proof that the sheep were heartwater-negative came from the fact that the seroconverted sheep was fully susceptible to a challenge with cell culture-derived *C. ruminantium*. Animals that recover after infection with *C. ruminantium* are usually protected from subsequent challenge (Uilenberg, 1983). In addition, that *Amblyomma* ticks were not found on these farms also proves that heartwater was not present on these farms. The transmission of the cross-reacting agent(s) may not be limited to *R. e. evertsi* since some *H. marginatum rufipes* ticks from Chitara Estate were also positive for *Ehrlichia* by the PCR. Although we were unable to isolate the agent in this study several *Ehrlichia* species could be responsible for this serological cross-reactivity. *Ehrlichia canis*, *E. ovina*, *E. phagocytophila* and *Ehrlicha bovis* have been demonstrated to cause cross-reaction with *C. ruminantium*. Further studies need to be focused on isolation of the agent for the definition of *C. ruminantium*-specific antigens or their epitopes.

ACKNOWLEDGEMENT

This work was supported by United States Agency for International Development Grant no. LAG-1328-G-00-3030-00.

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