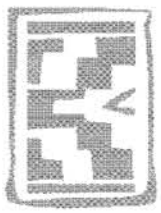

Comparing the detection of exposure to *Ehrlichia ruminantium* infection on a heartwater-endemic farm by the pCS20 polymerase chain reaction assay and an indirect MAP1-B enzyme linked immunosorbent assay

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ABSTRACT

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Detection of heartwater is not always easy especially because all the serological assays so far available either have poor sensitivity or specificity. The indirect MAP-1B ELISA has been reported to be the most specific test for heartwater, although it does also detect antibodies to some closely related ehrlichial agents. This study was undertaken to compare two methods for the detection of heartwater infection caused by the ehrlichial agent *Ehrlichia (Cowdria) ruminantium*. Fifteen cattle on a heartwater-endemic farm infested with high numbers of *Amblyomma hebraeum* ticks, and hence exposure to *E. ruminantium* infection were monitored over an 8-week period by pCS20 PCR and an indirect MAP-1B ELISA. Infection was detected by pCS20 PCR in most animals with the highest number of positives (60%) in week 6 of the study. Similarly, exposure to *E. ruminantium* was detected by indirect MAP-1B ELISA in some animals, with the highest number of seropositives (27%) at weeks 2–6 of the study. The data demonstrated a fluctuating rickettsaemia in cattle in a heartwater-endemic area. Comparison of the two tests indicated that the pCS20 PCR assay was more reliable because it detected more infections than the indirect MAP-1B ELISA and would therefore be the method of choice for detection of *E. ruminantium* infection.

Keywords: *Amblyomma hebraeum*, *Ehrlichia (Cowdria) ruminantium*, heartwater, indirect MAP-1B Elisa, pCS20 PCR, rickettsaemia

INTRODUCTION

Improved diagnosis of infection with *Ehrlichia (Cowdria) ruminantium*, the causative agent of heartwater in domestic and certain wild ruminants, has resulted from the development of a specific and sensitive pCS20 polymerase chain reaction (PCR) assay based on the DNA sequence of the pCS20 DNA probe (Waghela, Rurangirwa, Mahan, Yunker, Crawford, Barbet, Burridge & McGuire 1991; Mahan, Waghela, McGuire, Rurangirwa, Wassink & Barbet 1992; Peter, Deem, Simbi, Barbet, Norval, Kelly & Mahan 1995; Peter, Barbet, Alleman, Simbi, Burridge & Mahan 2000). The pCS20 DNA sequence cross-reacts with all strains of *E. ruminantium* tested so far (Mahan *et al.* 1992; Peter *et al.* 2000). It was found to be the most sensitive for detection of *E. ruminantium* when compared to other probes

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such as the *map1* gene or 16S ribosomal DNA probes (Allsopp, Hattingh, Vogel & Allsop 1999).

Serological tests have long suffered from a lack of specificity due to cross-reactions with other ehrlichial agents (Du Plessis, Bezuidenhout, Brett, Camus, Jongejan, Mahan & Martinez 1993; Mahan, Tebele, Mukwedeya, Semu, Nyathi, Wassink, Kelly, Peter & Barbet 1993; Van Vliet, Van der Zeust, Camus, Mahan, Martinez & Jongejan 1995). However, the indirect MAP1-B ELISA has increased sensitivity over other serological tests (Van Vliet *et al.* 1995).

The indirect MAP1-B ELISA and pCS20 PCR tests have been evaluated and used to detect infections in experimentally infected or clinically ill domestic ruminants (Mahan, Peter, Simbi & Burridge 1998a; Mahan, Semu, Peter & Jongejan 1998b; Peter, Perry, O'Callaghan, Medley, Mlambo, Barbet & Mahan 1999b; Semu, Peter, Mukwedeya, Barbet, Jongejan & Mahan 2001) and in wild animal carriers (Peter, Anderson, Burridge & Mahan 1998; Peter, Anderson, Burridge, Perry & Mahan 1999a).

In heartwater-endemic areas where tick control is limited and numbers of *Amblyomma* spp. ticks, the vectors of *E. ruminantium*, are high, animals are continually being challenged. Most cattle in endemically stable areas are persistently infected carriers (O'Callaghan, Medley, Peter & Perry 1998). It was estimated that cattle are exposed to new infections every 5–20 days on one such a farm, which is the site of this study (O'Callaghan *et al.* 1998). A tick infection rate of 10.2 % was demonstrated on this farm (Peter *et al.* 1999b), which, in conjunction with a heavy tick burden, resulted in a continual challenge to resident cattle.

Previously, cattle sera from heartwater-endemic farms analysed by indirect MAP1-B ELISA yielded the low seropositive rate of 33 % (Mahan *et al.* 1998b) despite the continual tick challenge. There is evidence of a general down-regulation of antibody responses to *E. ruminantium* antigens despite repeated exposure to infected ticks (Semu *et al.* 2001). Consequently, antibody responses to MAP1-B antigen, as detected by the indirect MAP1-B ELISA, may be negative while the organisms are detectable by PCR.

In this study, the detection of infection or exposure to *E. ruminantium* in cattle on a heartwater-endemic farm by both the pCS20 PCR and MAP1-B indirect ELISA is compared. This farm had been previously evaluated for *E. ruminantium* infection dynamics by Peter *et al.* (1999b) and O'Callaghan *et al.* (1998).

MATERIAL AND METHODS

Study site

Fifteen cattle (Mashona breed) were identified randomly on a farm, Vlakfontein Estates (18°47' S, 30°40' E), in Chivhu, a heartwater-endemic area in the highveld of Zimbabwe. The cattle were resident on the farm since birth and hence continually exposed to heartwater. This site was used previously by Peter *et al.* (1999b) in a study to determine the prevalence of *E. ruminantium* infection in *Amblyomma* ticks. Tick control on this farm was minimal. Mortality in resident cattle due to heartwater was low, even though there were high levels of tick infestations. It was estimated that cattle were exposed to new infections every 5–20 days (O'Callaghan *et al.* 1998). This study began in July 1998, which is towards the end of the generally cold season of Zimbabwe and going into the warm season, when ticks are abundant and actively attaching to animals. Whole blood was collected from each animal for testing by pCS20 PCR and sera for testing by indirect MAP1-B ELISA every 2 weeks for a period of 2 months. On the day of bleeding, the animals were examined for ticks, and total counts of adults and nymphs of *A. hebraeum* were recorded.

pCS20 PCR analysis

Blood was collected in 10 mℓ EDTA anticoagulant tubes, and buffy coat cells were separated and treated with a saponin lysis buffer (0.22 % sodium chloride, 0.015 % saponin, 1 mM EDTA) to remove red blood cells. DNA was extracted using the QIAmp blood kit (Qiagen Inc, Valencia, CA, USA). PCR was performed as previously described (Peter *et al.* 1995) using a 5 µℓ sample in a final reaction volume of 50 µℓ containing 10 mM Tris Cl pH 8.3, 50 mM KCl, 0.001 % gelatin, 3.0 mM $MgCl_2$, 0.5 µm each of primer 1 (AB128-5'-ACTAGTAGAAATTGCACAATCTAT-3'), primer 2 (AB129-5'-TGATAACTTGGTGCGGGAAATCCTT-3'), 200 µm (each) deoxynucleotide triphosphate and 1.25 U *Taq* polymerase (Perkin Elmer Cetus Corp., Norwalk, CT, USA). The reactions were performed in a thermal cycler (Perkin Elmer Cetus Corp., Norwalk, CT, USA) for 45 cycles. Each cycle consisted of a denaturation step of 1 min at 94 °C, an annealing step of 1 min at 55 °C and an extension step of 2 min at 72 °C. This was followed by an automatic extension for 10 minutes at 72 °C and soaking at 4 °C at the end of the 45 cycles. In each PCR run, positive and negative controls were included. Positive controls were derived from *E. ruminantium* DNA obtained

from cell culture derived organisms and negative controls were reagent blank samples without DNA.

The PCR products were denatured with 0.4 M sodium hydroxide and directly dot-blotted onto nylon membrane (GenescreenPlus®, NEN Life Science Products, Boston, MA, USA) using a hybridot apparatus (Life Technologies, Betheseda, MD, USA). The membranes were hybridized with a random prime-labelled (Roche Molecular Diagnostics, Randburg, South Africa) [^{32}P]dCTP (Amersham Pharmacia Biotech, Little Chalfont, UK) pCS20 DNA probe as previously described (Mahan *et al.* 1992; Peter *et al.* 1995; 2000), washed and exposed onto X-ray film (Biomax) (Eastman Kodak, NY, USA).

Indirect MAP1-B ELISA

The indirect MAP1-B ELISA was performed as previously described (Mahan *et al.* 1998b, Semu *et al.* 2001). ELISA plates (Greiner America Inc., Wilmington, DE, USA) were coated with recombinant MAP1-B antigen (3 µg/mℓ in 0.05 M sodium carbonate buffer) by incubation at 37 °C for 1 h and stored overnight at 4 °C. The plates were blocked for 15 min at 37 °C with PBSTM (phosphate buffered saline pH 7.3 with 0.1 % tween 20 and 3 % non-fat dry milk (Protifar, Nutricia, Zoetermeer, The Netherlands). The plates were washed and reacted for 1 h at 37 °C with cattle serum samples (in duplicate) diluted at 1/100 in PBSTM. The plates were washed three times with water. Horseradish peroxidase-conjugated anti-bovine IgG antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) were used to detect specific antibodies and developed with an ABTS substrate (Sigma Chemical Co., St Louis, MO, USA). Colour development was measured by reading optical densities using dual wavelengths of 405 nm and 492 nm. The cut-off value was calculated as two times the percent positivity of the negative control serum value to the positive control serum (Van Vliet *et al.* 1995; Semu *et al.* 2001)

RESULTS

Tick counts

The total numbers of adult and nymphal *A. hebraeum* ticks counted on each animal were higher at the end of the 8-week period than at the beginning of the study (Table 1). For example, 60 ticks were initially counted on bovine #1 with the number increasing to 88 after 2 weeks and finally to 226 after 8 weeks. Though the tick infestations showed a gradual increase, the numbers fluctuated over the study period. Bovines 707, 709 and 710 had high tick infestation, averaging more than 200 ticks per animal throughout the study (Table 1). The *E. ruminantium* infection rate in the *A. hebraeum* ticks was not determined in this study, but this was evaluated previously on this farm by Peter *et al.* (1999b) and was then determined to be 10.2 %.

pCS20 PCR and indirect MAP1-B ELISA analysis

The data in Table 1 demonstrate that *E. ruminantium* was detected by the pCS20 PCR in most cattle during the 8-week period of the study. However, two cattle remained PCR-negative throughout the study. The highest number of PCR-positive cattle (60 %) was in week 6 of the study. In contrast, the highest number of positive cattle (27 %) by the indirect MAP1-B ELISA was determined to be in weeks 2 and 6 of the study. The cattle that were positive by indirect MAP1-B ELISA had low antibody titres (data not shown). A summary of the comparison of pCS20 PCR and indirect MAP1-B ELISA data is shown in Table 2. The pCS20 PCR assay more reliably detected *E. ruminantium* infection than the indirect MAP1-B ELISA and there was no direct correlation between the results of these two tests.

DISCUSSION

The present study compares two methods of detection of *E. ruminantium* infections in cattle on a heartwater-endemic farm in Zimbabwe. The pCS20 PCR has previously been used to detect infections in cattle and goats from a heartwater-endemic area where 26.7 % of 21- to 24-month-old cattle and 23.3 % of goats were positive (Mahan *et al.* 1998a). In this study, the infection rate varied (0–60 %) over the 8-week study period using the pCS20 PCR assay. A tick infection rate for this farm was previously determined to be 10.2 % (Peter *et al.* 1999b). However, high tick infestations on each animal were observed and, according to O'Callaghan *et al.* (1998), the cattle were repeatedly exposed to new infections every 5–20 days. Therefore, it was assumed that all cattle were infected several times during their lifetime and during this study. Despite the repeated exposures, the rickettsaemic levels in the peripheral blood varied, demonstrating that *E. ruminantium* rickettsaemia fluctuated and sometimes it was not possible to detect infection. The data also confirmed that one-time testing of an animal may not reflect its actual infection status, espe-

TABLE 1 Tick count data and detection of *Ehrlichia ruminantium* infection in cattle on the heartwater-endemic farm by PCR and MAP-IB ELISA

Animal no.	Results by week of study														
	Week 0			Week 2			Week 4			Week 6			Week 8		
	TC	PCR	MAP1-B	TC	PCR	MAP1-B	TC	PCR	MAP1-B	TC	PCR	MAP1-B	TC	PCR	MAP1-B
1	60	–	–	88	–	–	107	–	–	96	–	+	226	–	–
2	38	–	–	65	–	+	73	+	–	110	+	+	141	+	+
3	47	–	–	93	+	–	67	–	+	94	+	+	120	–	–
4	59	–	–	76	–	–	66	–	–	77	+	–	102	–	–
701	48	–	–	67	+	–	92	–	–	107	+	+	139	–	+
702	58	–	–	53	–	–	68	–	–	53	–	–	66	–	–
703	79	–	+	90	–	+	131	+	+	197	+	–	215	–	–
704	85	–	–	87	–	–	99	–	–	150	–	–	182	+	–
705	50	–	–	71	–	–	89	–	–	103	–	–	160	+	–
707	165	–	–	220	–	–	210	–	–	132	+	–	211	+	–
709	125	–	–	177	–	–	142	–	–	135	+	–	133	–	–
710	175	–	–	215	–	–	220	–	–	148	+	–	217	–	–
786	ND	–	ND	34	–	+	87	–	+	65	+	–	118	+	–
789	ND	–	ND	87	–	–	108	–	–	93	–	–	142	+	–
1 459	35	–	–	91	–	+	103	+	ND	130	–	–	145	–	–

MAP1-B MAP1-B ELISA result
TC *Amblyomma hebraeum* tick counts (adults and nymphs)
ND Test not done
PCR Polymerase chain reaction result

TABLE 2 Summary of PCR and MAP1-B ELISA results obtained from testing the cattle

Weeks	PCR		MAP1-B ELISA	
	Positive/total	Percent (%)	Positive/total	Percent (%)
0	0/15	0	1/13	8
2	2/15	13	4/15	27
4	3/15	20	3/14	21
6	9/15	60	4/15	27
8	6/15	40	2/15	13

cially if it is negative for that particular test. Two cattle were negative by PCR throughout this study. Bovine 1 was positive by indirect MAP1-B ELISA on week 6, whereas Bovine 702 was negative by both tests. It is possible that some animals self-cure and develop a sterile immunity (such as bovine 702). However, bovine 1 was positive by indirect MAP1-B ELISA on week 6 which indicated that the animal was exposed to *E. ruminantium* but its DNA was not detected by pCS20 PCR assay. This could have resulted from a rickettsaemia level that was too low to be detectable by pCS20 PCR assay.

The MAP1-B ELISA has a high sensitivity of detecting antibodies to *E. ruminantium* when testing primary infection sera (Semu *et al.* 2001). However, in studies where laboratory- and field-infected cattle were repeatedly exposed to *Amblyomma* ticks, the assay did not always indicate exposure to infection (Semu *et al.* 2001). It was observed that during the first 3 weeks of primary infection, the indirect MAP1-B ELISA and pCS20 PCR assay results correlated. Several weeks later, some cattle tested negative by indirect MAP1-B ELISA but were positive for *E. ruminantium* infection by pCS20 PCR. Semu *et al.* (2001) proposed that there was a down-regulatory effect on the production of antibodies against *E. ruminantium* during persistent infections that would explain the low seropositivity that occurred at the farm during this study period. This study and others reported previously support the conclusion that serology alone is not reliable in detecting exposure to *E. ruminantium* infection. Therefore, we recommended that the pCS20 PCR assay be applied on consecutive weekly or biweekly animal samples for more accurate detection of *E. ruminantium* infection.

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