

## CHAPTER THREE

### 3. THE USE OF THE PCR ASSAY TO DETERMINE THE PREVALENCE OF *COWDRIA RUMINANTIUM* IN FREE-LIVING, UNFED ADULT *AMBLYOMMA HEBRAEUM*

#### 3.1 INTRODUCTION

The advancement of our understanding of the epidemiology of heartwater has undoubtedly been hampered by the poor diagnostic tests available to determine *C. ruminantium* in free-living, unfed *Amblyomma* ticks. Estimations of the prevalence of infection rates in these free-living, unfed *A. hebraeum* are important because they reflect the true prevalence of *C. ruminantium* in the field population (Section 1.1). Previously, indirect methods (Section 1.3.2) were used to detect *C. ruminantium*, but many of these methods were cumbersome, expensive and with unknown sensitivities and specificities. In this project a molecular assay, which was specific for *C. ruminantium*, was used on an epidemiological sample which was known to be truly representative of the free-living, unfed *A. hebraeum* population.

#### 3.1.1 MOLECULAR-BASED DIAGNOSTIC TECHNIQUES FOR THE DETECTION OF *C. RUMINANTIUM*

New molecular-based techniques have been evaluated to detect *C. ruminantium* in both animals and ticks. In 1994/5, a collaborative research project was initiated between the

University of Florida and Washington State University, with the aim of identifying and characterising a diagnostic DNA probe that could be used to detect *C. ruminantium* in animals and ticks (Mahan 1995).

A DNA library was made from DNA isolated from *C. ruminantium*-infected endothelial cell cultures. Seventy-four recombinant plasmids were screened, and two were identified as possible probes. DNA from the one recombinant originated from the Kiswani strain from Kenya, and was referred to as the pCR9. The other DNA was from the Crystal Springs strain from Zimbabwe, and referred to as the pCS20 probe.

The pCS20 probe cross-reacted with strains of *C. ruminantium* from Zimbabwe, South Africa, Nigeria, the Caribbean and Kenya (Waghela *et al.* 1991). It could also detect *C. ruminantium* in infected *A. hebraeum* (Yunker *et al.* 1993), and *A. variegatum* which had been fed on febrile animals (Waghela *et al.* 1991). The use of the pCS20 probe also showed that the *C. ruminantium* infection was mainly in the midguts of the infected ticks (Yunker *et al.* 1993), as well as in infected sheep (Mahan *et al.* 1992), and carrier animals (Peter *et al.* 1995a).

Allsopp *et al.* (1999) listed ten different molecular probes which have been used to detect heartwater associated organisms. Eight of these probes have been used to detect *Cowdria* and two to detect *Ehrlichia*. They classified the probes into three main groups containing the 16S probes, the pCS20 probe and the MAP-1 probe respectively. These three groups of

probes were then evaluated to compare their ability to detect *Cowdria* in field ticks collected in the North and North West Provinces of South Africa. The pCS20 probe was found to be the most specific indicator for *Cowdria* genotypes, other than the Omatjenne stock, and showed no cross-hybridization with the *Ehrlichia* species tested. Allsopp *et al.* (1999) stated that the pCS20 probe should be the probe of choice when initially screening field samples for *Cowdria*. The MAP-1 probe currently in use at the Onderstepoort Veterinary Institute, was less sensitive and specific compared to the pCS20 probe, and did not provide genotypic information.

The DNA sequences of both the pCS20 and pCR9 probes were determined and published (Waghela *et al.* 1991). The pCS20 probe consists of 1 306-bp, and contains two Open reading frames (ORF) of 512 and 459 nucleotides. The pCR9 has a 754-bp insert and had one ORF of 243 nucleotides. Waghela *et al.* (1991) suggested that the sequence of the pCS20 probe should be used to develop a diagnostic PCR.

### **3.1.2 THE USE OF THE PCR TO DETECT *C. RUMINANTIUM***

Mahan *et al.* (1992), described the use of two oligonucleotide primers (AB 128 and AB 129), developed from the pCS20 probe and which amplified a 279-bp fragment. These primers were specific for *C. ruminantium*, and did not amplify DNA from bovine DNA or any other organisms tested. Mahan *et al.* (1992) concluded that the sensitivity and specificity of the PCR, and the availability of the pCS20 probe, could play a crucial role in the detection of

*C. ruminantium* in field ticks and carrier animals, as the rickettsaemia was so low it could not be detected by the probe alone. Peter *et al.* (1995) confirmed these results by showing that the PCR could amplify between one and ten *C. ruminantium* organisms, and did not amplify DNA from *E. canis*.

Peter *et al.* (1995) demonstrated that the PCR, using the primers of Mahan *et al.* (1992), can be used to detect *C. ruminantium* infection in geographically different strains, as well as with ticks preserved in ethanol, formalin and glutaraldehyde. Mahan *et al.* (1998b) demonstrated that the PCR can detect 92.5% of infected ticks, which was far better than the DNA probe (76.7%), or mouse inoculation assay (MIA) (8.3%).

It was clear that the combination of the PCR and the pCS20 probe was the most credible combination for the detection of *Cowdria* in free-living, unfed *A. hebraeum*. It was therefore decided that the PCR should be used to detect *C. ruminantium* in free-living, unfed *A. hebraeum* adult ticks.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 STORAGE AND TRANSPORT OF THE FREE-LIVING, UNFED TICKS**

All captured free-living, unfed ticks were kept in special, small plastic containers inside a glass humidifier (750 g glass bottle with 200 ml tap water at the bottom). The majority of the ticks were collected in summer, and it was not necessary to control the ambient temperature. They were kept alive at room temperature until they were transported to the laboratory at the UF/USAID/SADC Heartwater Research Project at the Central Veterinary

Diagnostic and Research Laboratory in Harare, Zimbabwe. At the laboratory the ticks were incubated at 28°C and 75% RH until they were dissected for the PCR work.

### **3.2.2 SAMPLE PROCESSING**

Two batches of free-living, unfed ticks were processed (Table 14). The first was the survey group (A) from Rietgat (n = 150), which was processed during November and December 1996 at the UF/USAID/SADC Heartwater Research Project in Harare. The second group (B) consisted of ticks collected at the Rietgat CGA (n = 284), Kruger National Park (n = 88) and Songimvelo Game Reserve (n = 48), and were processed in the laboratory in Harare during April/May 1997.

All the tick samples (groups A and B, n = 570) were initially digested with proteinase K and extracted with phenol, chloroform, isoamyl-alcohol 25:24:1 (PCIA). However, problems were experienced with PCR inhibition within group B (n = 420), and these samples were then re-extracted using QIA amp tissue DNA extraction columns (QIAGEN method, Qiagen, Ca., USA). The extracted tick DNA samples were then frozen at -20°C until used. An aliquot of the frozen material was PCR amplified and confirmed by gel-electrophoresis. PCR amplified products were dot-blotted and hybridized to the pCS20 probe.

### **3.2.3 TICK DISSECTION**

Ticks were dissected on sterile plastic petri dishes with four to five ticks dissected on each dish and after each batch of dissections, a new dish was used. After every 2 - 3 ticks, gloves were changed. The ticks were pinned down dorsally on the dish and the back legs cut off.

The posterior tip of the ticks idiosoma was dissected with a new scalpel blade. The tick guts were then forcibly extruded with the scalpel blade and transferred to an eppendorf tube with a sterile disposable needle. The flat, free-living, unfed ticks were different to laboratory-reared ticks, as many of them had been starved for months and were very thin and at times it was difficult to get a decent sample of the midgut. Ticks which had dried out were not used, as they were difficult to dissect. Tick midguts were collected in eppendorf tubes and frozen at  $-70^{\circ}\text{C}$ . Previous workers (Waghela *et al.* 1991, Yunker *et al.* 1993) have shown that *C. ruminantium* occurs primarily in the midgut tissue of both *A. variegatum* and *A. hebraeum*.

### **3.2.4 DIGESTION OF TICK GUT MATERIAL**

The tick midguts were thawed at  $37^{\circ}\text{C}$  and  $200\ \mu\ell$  of a PCR digestion buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 M MgCl<sub>2</sub>, 0,5% Tween, 0,5% NP40) was added. Proteinase K (10 mg/ml) was added to a final concentration of  $250\ \mu\text{g}/\text{ml}$ . The tubes were then incubated at  $56^{\circ}\text{C}$  for 16 hours.

### **3.2.5 EXTRACTION OF THE DNA FROM THE DIGESTED MATERIAL**

#### **3.2.5.1 Phenol, chloroform, isoamyl-alcohol method (25:24:1)**

The DNA in the first batch of ticks (samples 1 - 150) from survey group A was extracted using the PCIA method. An equal volume ( $200\ \mu\ell$ ) of phosphate buffered saline (PBS) equilibrated phenol was added to each tube, mixed and centrifuged at 12 000 g for five minutes. The aqueous top layer was transferred to a clean tube, and  $200\ \mu\ell$  PCIA was added

to the samples, mixed and centrifuged for five minutes at 14 000 g. The aqueous top layer was then transferred to a fresh tube, and equal volumes of chloroform isoamyl alcohol (24:1) was added. After being mixed and centrifuged for five minutes at 12 000 g, the aqueous top layer was transferred to a clean tube. An aliquot from each tube (5  $\mu\text{l}$ ) was then transferred to a 0.6 ml PCR tube.

#### 3.2.5.2 Q1A amp tissue DNA extraction column

The DNA of the tick midguts in the second batch of samples, group B (refer to 3.2.2), were initially extracted with the PCIA method. Problems with PCR inhibitors were experienced and these samples were re-extracted to purify the DNA, using the Q1A amp tissue DNA extraction columns (Qiagen, CA, USA). Briefly, the tick guts were digested using the buffers and proteinase K provided in the kit. The digested material was coated onto spin columns where DNA was bound. After a number of washes, using buffers with different concentrations of salt, the DNA was eluted pure and free of inhibitors.

#### 3.2.6 POLYMERASE CHAIN REACTION

The two primers (AB 128 and AB 129) originally developed from the pCS20 probe by Mahan *et al.* (1992), were obtained from the University of Florida, Interdisciplinary Centre for Biotechnology and Research. The primers were used to amplify a 279-bp fragment of *C. ruminantium* DNA, and had the following sequences:

AB 128 5' ACTAGTAGAATTGCACAATCTAT 3'

AB 129 5' TGATAACTTGGTGC GGAAATCCTT 3'.

PCR was performed with 5  $\mu\text{l}$  of sample DNA in a total reaction volume of 50  $\mu\text{l}$ . The following concentrations of reagents were used for each 50  $\mu\text{l}$  PCR mixture: 10 mM Tris, HCl pH 8,3, 50 mM KCl, 3.0 mM  $\text{MgCl}_2$ , 0.001% w/v gelatin, 2.00  $\mu\text{M}$  of each dNTP, 0.5  $\mu\text{M}$  of each of the primers and 1.25 units of Taq polymerase.

#### Reagents in the master mix

The reagents in the master mix were always made up as follows:

● 10 x PCR reaction buffer*	5 $\mu\text{l}$
● 25 mM $\text{MgCl}_2$ *	6 $\mu\text{l}$
● dNTP's (10 mM) (4 x 1 $\mu\text{l}$ each)	4 $\mu\text{l}$
● Primer AB 128 and AB 129 (2 x 5 $\mu\text{l}$ each)	10 $\mu\text{l}$
● Sterile (distilled) water	19.75 $\mu\text{l}$
● *Taq DNA polymerase	<u>0.25 <math>\mu\text{l}</math></u>
	Total mixture = <u>45.00 <math>\mu\text{l}</math></u>

\* Reagents bought from Perker Elmer

After adding the Taq DNA polymerase, the reagents were well mixed, and put on ice. In the sample preparation room 45  $\mu\text{l}$  of the master mix was added to each 5  $\mu\text{l}$  aliquot of tick DNA sample and 45  $\mu\text{l}$  sterile mineral oil was layered on top of the tube. Positive controls consisted of 1 ng genomic *C. ruminantium* DNA in 5  $\mu\text{l}$  distilled water, as well as 5  $\mu\text{l}$  tick DNA spiked with 1 ng of the same DNA. Distilled water (5  $\mu\text{l}$ ), as well as DNA extracted from uninfected female and male ticks served as negative controls. One cycle of

amplification consisted of denaturation at 95°C for one minute, annealing at 55°C for one minute, and extension at 72°C for two minutes. Amplification took place for 45 cycles with the final extension step at 72°C for ten minutes. Amplified DNA was then kept at 4°C until analysed.

PCR products (20  $\mu\text{l}$ ) were analysed using a 1.5% agarose gel (Gibco BRL Life Technologies, USA). The gels contained 0.4  $\mu\text{g}/\text{ml}$  of ethidium bromide (Sigma Company, USA) (10  $\text{mg}/\text{ml}$ ), and were photographed on a UV transilluminator. To determine the size of the amplicon a 1Kb molecular weight marker (Gibco, BRL, Bethesda, MD, USA) was electrophoresed together with the samples. The specificity of the PCR products were then confirmed by dot-blotting.

### **3.2.7 HYBRIDIZATION WITH THE pCS20 PROBE**

To denature the DNA the PCR product (40  $\mu\text{l}$ ) was added to a tube containing 10  $\mu\text{l}$  of 0.4 M NaOH and 50  $\mu\text{l}$  distilled water for 15 minutes at 37°C. The samples were then dot-blotted onto nylon membranes (Genescreen Plus, Du Pont), using a dot-blot vacuum manifold apparatus (Gibco, BRL, Bethesda, MD, USA). The membranes were rinsed in 2 x SSC buffer (0.3 M NaCl and 0.03 M Na citrate), exposed to UV light (312 nm) for two minutes, to cross-link the DNA to the membrane and allowed to air dry.

The method for the hybridization of the membranes was described by Mahan *et al.* (1992) and Yunker *et al.* (1993). Briefly, the probe was labelled with  $^{32}\text{P}$ -dCTP, using the multi

prime labelling kit (Boehringer Mannheim). The blotted nylon membranes containing DNA samples were pre-hybridized for six hours at 42°C in a hybridization solution (2 M Na phosphate [pH 7.2], 5 M NaCl, 25 M EDTA, deionized formamide with dextran sulphate: 25% SDS and 10 mg/ml denatured herring sperm DNA) containing 10% dextran sulphate in formamide. The pCS20 probe was denatured by boiling for ten minutes and immediately put on ice for ten minutes. It was then added to the solution and hybridized at 42°C for at least 16 hours. After hybridization, the blots were washed under stringent conditions of four washes of 15 minutes each at room temperature: one wash in 2 x SSC; one wash in 2 x SSC, 0.1% SDS; one wash in 0.5 x SSC, 0.1% SDS and the fourth wash in 0.1 SSC, 0.1% SDS. Two washes of 30 minutes each at 60°C in 0.1 x SSC, 0.1% SDS. The membranes were then exposed to X-ray films (Kodak), for 24 hours at -80°C (Mahan *et al.* 1992), and the autoradiographs were developed according to the recommended procedure of the manufacturers.

### **3.3 RESULTS**

#### **3.3.1 PCR AMPLIFICATION**

PCR amplification of the group A samples occurred without any problems, as indicated by both negative and positive controls. The group B samples were initially processed using the PCIA method for extraction of DNA. However, in some of the positive control samples containing 1 ng *C. ruminantium* DNA, positive amplification occurred (Fig. 35, lanes 2, 4, 9, 12 and 15), but no amplification occurred in the spiked tick samples containing the same DNA (lanes 7, 10, 13 and 16). This indicated that PCR inhibition by tick DNA occurred in

all these samples. The PCR inhibition was removed by purifying the samples using spin columns (QIA amp tissue DNA purification columns), after which the positive PCR amplification was restored (Fig. 36, lanes 7, 10, 13 and 16), except for a few PCIA extracted samples (Fig. 36, lanes 5 and 6). After analysing the results in Fig. 35 and 36, it was decided to re-extract the group B samples with the QIA amp tissue DNA purification columns.

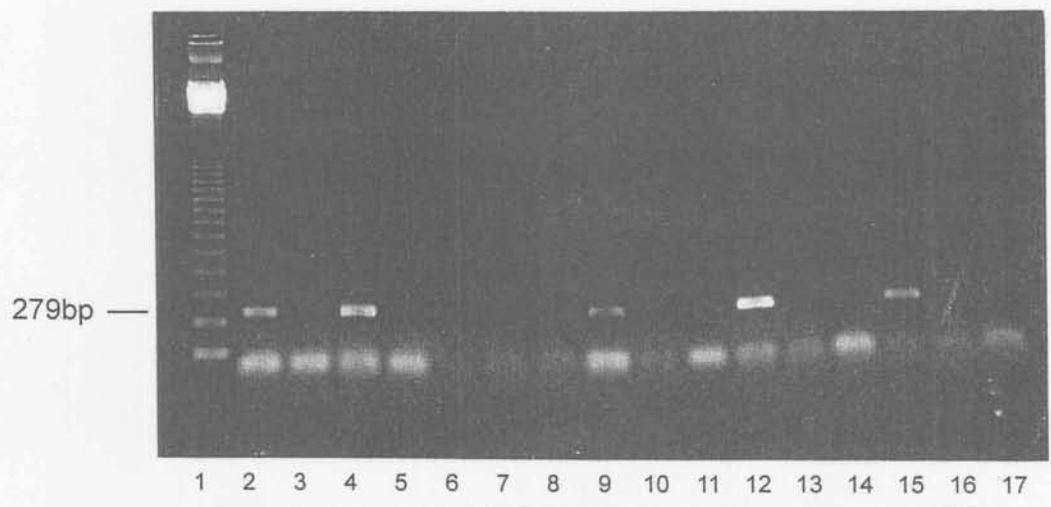


Fig. 35 Agarose gel electrophoresis indicating amplification of genomic *C. ruminantium* DNA after extraction with PCIA. Lane 1: 123-bp molecular marker (Gibco BRL Life Technologies, USA). Lanes 2, 4, 9, 12 and 15: PCR products from amplification of 1 ng *C. ruminantium* DNA. Lanes 3, 5, 8, 11, 14 and 17: negative controls. Lanes 7, 10, 13 and 16: PCR products from amplification of tick DNA spiked with 1 ng *C. ruminantium* genomic DNA.

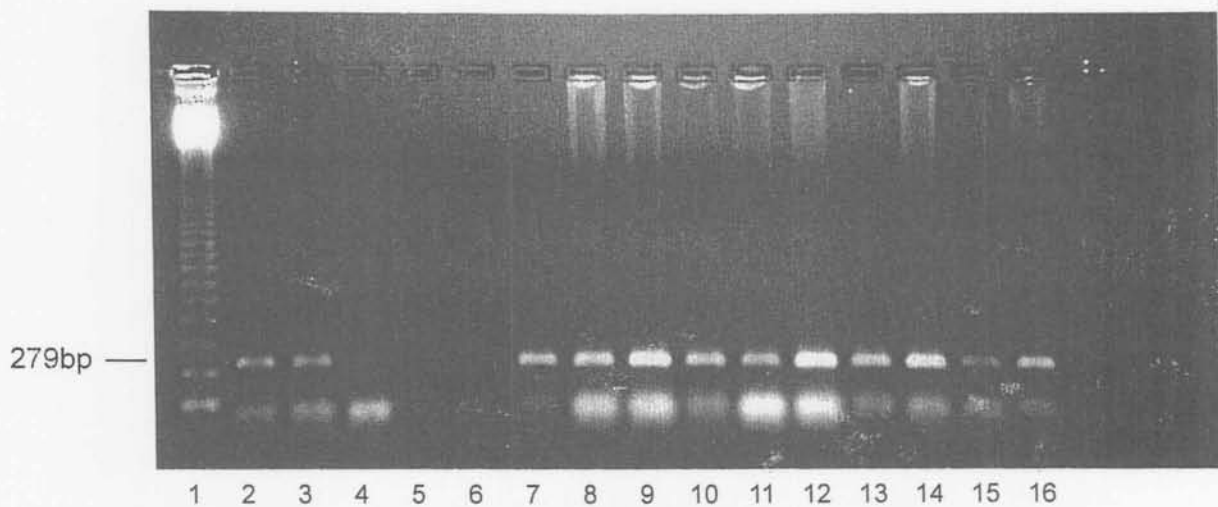


Fig. 36 Agarose gel electrophoresis indicating amplification of *C. ruminantium* DNA after purification with Q1A amp spin columns to remove inhibitors from crude tick digests. Lane 1: 123-bp molecular marker (Gibco BRL Life Technologies, USA). Lanes 2, 5, 8, 11 and 14: PCR products from amplification of 1 ng *C. ruminantium* genomic DNA (PCIA extraction only). Lanes 3, 6, 9, 12 and 15: PCR products from the amplification of 100 ng *C. ruminantium* genomic DNA (PCIA extraction only). Lane 4: PCR negative control. Lanes 7, 10, 13 and 16: PCR products purified with Q1A amp spin columns.

Figure 37 also illustrated PCR amplified DNA from *C. ruminantium* collected from ticks in the field, and extracted using the PCIA method, followed by purification with QIA amp tissue DNA columns.

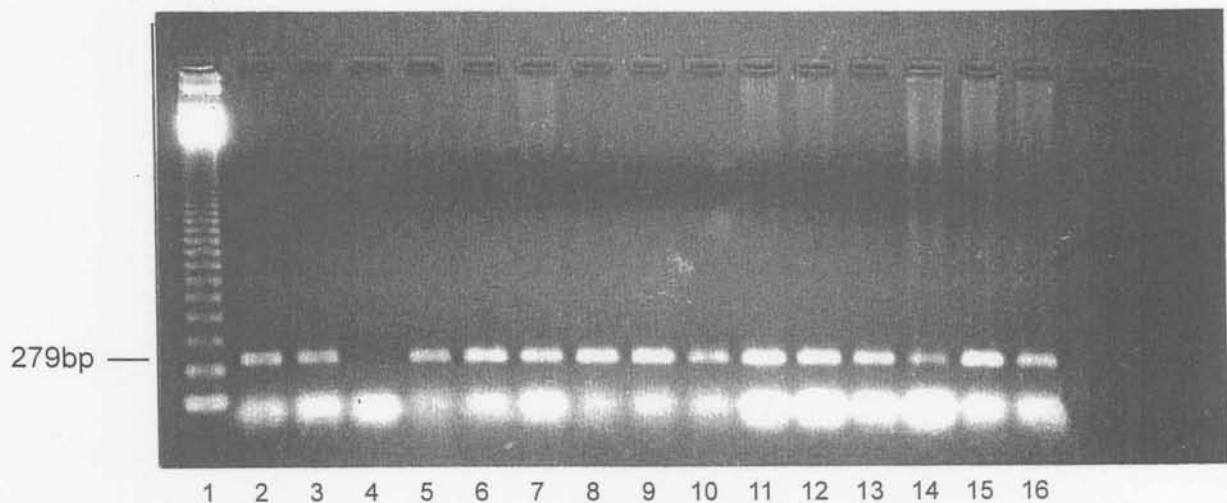


Fig. 37 Agarose gel electrophoresis showing positive and negative amplicons of *C. ruminantium* DNA extracted from field collected ticks. Lane 1: 123-bp molecular marker (Gibco BRL Life Technologies, USA). Lanes 2, 5, 8, 11 and 14: PCR products from amplification of 1 ng genomic DNA. Lanes 3, 6, 9, 12 and 15: PCR products from the amplification of 100 ng *C. ruminantium* genomic DNA. Lane 4: PCR negative control. Lanes 7, 10, 13 and 16: PCR products which had been purified with QIA amp spin columns.

### 3.3.2 PCR RESULTS FROM THE RIETGAT CGA

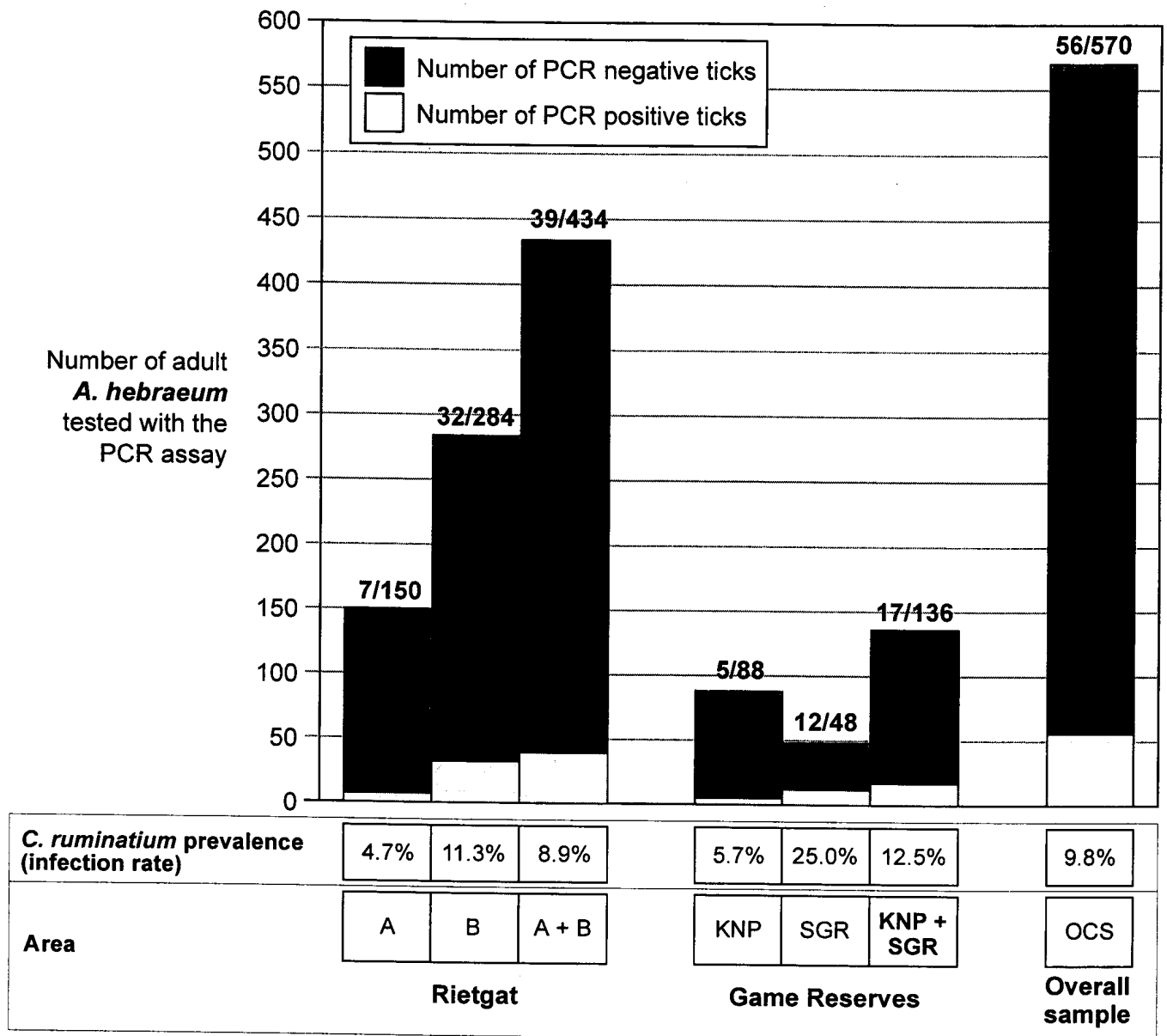
In order to get a preliminary indication of the heartwater prevalence at the Rietgat CGA (n = 150), a sample of free-living, unfed adult *A. hebraeum* were initially tested with PCR. Seven out of one hundred and fifty (4.7%) of these ticks was positive for *C. ruminantium* by PCR (Table 14 and Fig. 38), and confirmed by dot-blotting. In the second batch of adult ticks (group B) collected at the Rietgat CGA (n = 284), 32 (11.3%) were positive for *C. ruminantium*. In the total overall combined sample (OCS) of ticks collected at Rietgat (n = 434) 39 were positive, giving an overall infection rate of 8.9% (Table 14 and Fig. 38).

### 3.3.3 PCR RESULTS FROM THE SOUTH AFRICAN GAME RESERVES (KRUGER NATIONAL PARK AND SONGIMVELO GAME RESERVE)

Eighty-eight of the total of 447 *A. hebraeum* adult ticks collected at the KNP, were tested with the PCR and five of these were positive (5.7% prevalence) for *C. ruminantium* (Table 14, Fig. 38). The 88 ticks were all from the first KNP collection trip in 1992 (Table 12). Five adult *A. marmoreum* ticks were also collected off a tortoise, but none of these ticks were positive for *C. ruminantium* (0/5 = 0%). A total of 48 adult ticks were collected at SGR, and of these 12 were positive for *C. ruminantium*, giving an infection rate of 25%. A combined total of 136 *A. hebraeum* adults ticks were collected in the two game reserves (KNP and SGR), 17 were positive, and which gave an infection rate of 12.5% for the game reserve ticks (Table 14, Fig. 38).

TABLE 14 Results of the PCR for *Cowdria ruminantium* on the free-living, unfed adult *A. hebraeum* ticks (n = 570), collected from the different regions in South Africa

AREA	NUMBER OF TICKS COLLECTED	PERCENTAGE POSITIVE FOR <i>C. ruminantium</i> (INFECTION RATE)
<u>RIETGAT CGA</u>		
* Survey Group A	n = 150	4.7% ( 7/150)
* Survey Group B	<u>n = 284</u>	<u>11.3%</u> (32/284)
<b>TOTAL</b>	<b>n = 434</b>	<b>8.9%</b> (39/434)
<u>GAME RESERVES</u>		
* Kruger National Park (KNP)	n = 88	5.7% ( 5/88)
* Songimvelo Game Reserve (SGR)	n = 48	25.0% (12/48)
<b>TOTAL:</b>	<b>n = 570</b>	<b>9.8%</b> (56/570)
● Five adult <i>A. marmoreum</i> were also tested with the PCR and all proved negative.		



**Rietgat CGA**

A = Survey group A  
 B = Group B  
 A + B = Combined group A + B

**Games Reserves**

KNP = Kruger National Park  
 SGR = SGR Songimvelo Game Reserve  
 KNP + SGR = Combined Game Reserve Sample

**OCS (Overall Combined Sample)**

OCS = Rietgat CGA + KNP + SGR

Fig. 38 PCR positive and negative adult *A. hebraeum* assayed at the different collection sites. The *C. ruminantium* prevalence (infection rate) is also illustrated.

### 3.3.4 HYBRIDIZATION RESULTS

Seven dot-blot plans, representing all 570 PCR reactions done at the UF/USAID/SADC Laboratory in Harare, were recorded (dot-plot plans 1 to 7). An example of one of these dot-blot plans (Fig. 39a) and the corresponding auto-radiograph (Fig. 39b), is illustrated below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12 ●
B	13	14	15	16	17	18	19	20	21	22	23 ●w	24
C	25	26	27 ●	28	29	30	31	32	33	34	35	36
D	37	38	38	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65 ●	66	67	68	69	70	71	72
G												
H	FUN	FUN	FUN	MUN	MUN	MUN	NEG				T/S ●	POS ●

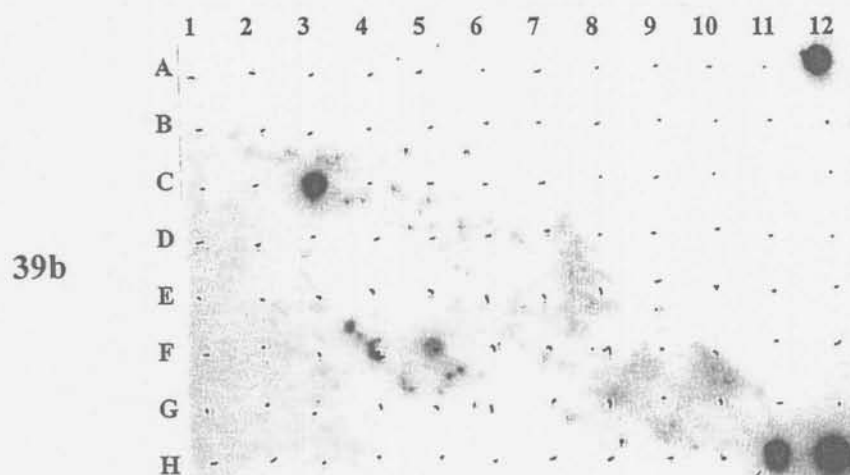


Fig. 39a and b Dot-blot plan 1 of the DNA of tick midgut samples 1 - 72 from the Survey Group A (Rietgat CGA). Corresponding auto-radiograph of the samples 1 - 72, after the hybridization with the pCS20 DNA probe. **Negative control** samples were FUN (female uninfected ticks) at H/1, H/2 and H/3; MUN (male uninfected ticks) at H/4, H/5 and H/6, and negative blank sample with no DNA at H/7. **Positive control** tick DNA samples, spiked with 1 ng genomic *C. ruminantium* DNA tick samples (T/S), and 1 ng *C. ruminantium* DNA (Positive) were at H/11 and H/12, respectively. The three strongly positive hybridization signals of tick midgut samples are indicated as ● at A12, C3 and F5 (Fig. 39b), and represent tick numbers 12, 27 and 65. A fourth weak positive signal, ●w, is at B/11 from tick 23.

### 3.4 DISCUSSION

The overall infection rate of the ticks collected at the Rietgat CGA was 8.9% (Fig. 38). This indicated that nearly one in ten adult ticks, collected at this CGA, was infected with *C. ruminantium*. This infection rate was higher than many of the previous surveys for *C. ruminantium* prevalence in South Africa (Table 2), (Du Plessis 1981; Du Plessis & Malan 1987; Du Plessis *et al.* 1992 and Peter *et al.* 1999); Madagascar (Uilenberg 1971); Senegal (Gueye *et al.* 1993) and the Caribbean (Camus & Barré 1987b). The results from the Rietgat CGA were more in line with some of the higher values recorded in Zimbabwe (Table 2), (Norval 1981; Norval *et al.* 1990; Peter 1997). The infection rates recorded during this project were considerably higher than many of the vector-borne diseases illustrated in Table 1. Surveys done for Rift Valley fever (Jupp 1994), African swine fever (Plowright *et al.* 1994), *Babesia bigemina* (Mahoney & Mirre 1971), *Babesia bovis* (Mahoney & Mirre 1971), East Coast fever (Leitch & Young 1981), and Lyme disease (Burgdorfer *et al.* 1979) all report prevalence levels below two per cent.

The exact reasons for the higher infection rates in this project are difficult to explain, but it is clear that there are a wide range of results from even within one country. In South Africa, Du Plessis & Malan (1987) recorded prevalence values up to 30%, whilst Peter *et al.* (1999) found only 1.7% of the ticks infected. The consequences of the high infection rates in the free-living, unfed *A. hebraeum* adults at the Rietgat CGA have important repercussions for the endemic stability of heartwater on the communal grazing. Clearly, the free-living, unfed *A. hebraeum* will be important reservoirs of infection for *C. ruminantium*, and the disease will be maintained by a smaller population of infected ticks. Another consequence of the

higher infection rate in free-living ticks would be the direct influence this parameter would have on the epidemiology model of Yonow *et al.* (1998). The high infection rate in the ticks, coupled with the high tick burdens on the cattle (Tice 1997), would produce endemic stability to heartwater in the cattle. A very positive aspect of the higher infection rate in the ticks at the Rietgat CGA would be the increased endemic stability to TBD on the communal grazing. This would be a consequence of the higher exposure to infected ticks, and the farmers would not have to dip their cattle on such a regular basis as a result of the endemic stability to the TBD.

The overall prevalence of 5.7% *C. ruminantium* in the unfed, free-living ticks found at the KNP, was the first documented evidence of the existence of *C. ruminantium* in free-living, unfed *A. hebraeum* ticks collected in a game reserve where no cattle hosts were present. Five *A. marmoreum* adult ticks were also collected from a tortoise in the KNP, but none of these ticks were positive for *C. ruminantium* by PCR. At SGR the 25% prevalence rate was very high, but this area was only visited on one occasion, and it was not possible to revisit to collect more ticks. The prevalence of 25% was by far the highest in our survey, and a larger sample size would have given a more accurate result. It is important not to put too much emphasis on these results which came from a very small sample size (n = 48). Nevertheless, one in four ticks in this game reserve was infected with *C. ruminantium*.

The findings that *C. ruminantium* was present in the ticks collected in the KNP and SGR, was an important epidemiological result, because at neither game reserve was there any contact between the wild and the domestic animals. In Africa wildlife and domestic animals

frequently share grazing areas, but in these game reserves this was not possible. A sylvatic life cycle for *C. ruminantium* has been suspected on the basis of demonstrated infections in wild animals (Norval *et al.* 1992; Peter *et al.* 1998a). The presence of *C. ruminantium* in the free-living, unfed *A. hebraeum* in these two game reserves in this study, adds credibility to the idea of a sylvatic cycle for *C. ruminantium*.

The high infection rate in the *A. hebraeum* adults collected in the two game reserves, was to be expected as *C. ruminantium*, *Amblyomma* ticks and wild animals have a long association, and were probably the original hosts of *Cowdria*. A number of different wild animals have been implicated as reservoirs of heartwater (section 1.3.1.5), and some of these hosts were present in the KNP and SGR, and carried heavy burdens of *A. hebraeum*. These wild animal species, such as buffalo, giraffe and kudu, must play an important role in transporting the ticks, and circulating the *Cowdria* organisms to infect the free-living, unfed *A. hebraeum*. This could lead to the high infection rates seen in this population of ticks. Buffalo have been shown to be asymptomatic carriers of *C. ruminantium* for as long as 161 days. It is clear therefore that these buffalo carriers would be able to infect sylvatic free-living, unfed *A. hebraeum* in the game reserves, and increase the prevalence of *Cowdria* in the ticks.

The PCR used during the project worked well during the investigation, and the results of the combined sample of free-living, unfed *A. hebraeum* collected with the AAAP/CO<sub>2</sub> trap, and *C. ruminantium* detected with the PCR/pCS20 probe, produced the most creditable field data

to date. PCR inhibition was, however, one problem experienced during the investigation, and Peter (1995) stated that his initial PCR's, performed on unextracted DNA of tick tissues, were unsuccessful, and this inhibition was removed by extracting with PCIA. Blood is often an inhibitory substance which PCR laboratories have to deal with on a regular basis. McCusker, Dawson, Noone, Gannon, & Smith (1992) heated the blood to 95°C prior to the addition of the PCR components and this eliminated the inhibitory effects.

Diaz, Nussenzweig & Gonzales (1992) noted that some PCR inhibitors were present in whole human blood, and these inhibitors include heparin, which was used as an anti-coagulant to collect the sample. Gage, Gilmore, Karstens, & Schwan (1992) found that haemoglobin, and especially, the breakdown products such as haematin, are very inhibitory to the PCR reaction. In this project, free-living, unfed ticks would obviously have less haemoglobin products in the midgut than feeding ticks collected off hosts. Stich *et al.* (1993) were aware of the potential problems of PCR inhibitions by haemoglobin products, and chose rather to collect haemolymph from the ticks than use midgut samples.

Kelly, Dasch, Chan & Ho (1994) described the PCIA method of DNA extraction as tedious and potentially hazardous. They also experienced problems with PCR inhibition whilst extracting *Rickettsia tsutsugamushi* from chiggers fleas, and they used a resin to increase the yield of DNA with better results.

Peter (1995) states that organic solvent DNA extractions are hazardous and tedious, and are prone to cross-contamination and damage to or loss of target DNA through frequent sample transfers. He suggested that rapid DNA extraction techniques that remove inhibitors without multiple tube changes should be investigated. These included solid DNA binding media such as filters, resins or selective protein precipitation (Peter, 1995).

To overcome the PCR inhibition, the QIA amp spin column was first used in the mid 1990's. This purification requires no solvents such as phenol, chloroform or alcohol, and involves very little handling of the DNA. In this procedure, DNA bound to the column is washed and therefore there is removal of residual contaminants, including PCR inhibitors (Qiagen Handbook, 1996).

In conclusion, the PCR of the UF/USAID/SADC Heartwater project was able to detect the very low numbers of *C. ruminantium* positive ticks found in the field at three different areas in South Africa. The results of this survey might have been more accurate if this purification method had been used from the start of the project. The PCR was time-consuming and expensive, but the results were credible as the PCR detects only *C. ruminantium* in the free-living, unfed ticks. The recent work of Mahan *et al.* (1998) also confirmed that the PCR was far more specific than the DNA probe or the MIA.

## CHAPTER FOUR

### 4. GENERAL DISCUSSION AND CONCLUSIONS

In Africa the frequent association of *Amblyomma* ticks with wild hosts, means that the eradication of heartwater in ruminants from this continent is simply not feasible. Therefore the first step towards the improvement of the management of heartwater, is to collect more information on the epidemiology and the economic impact of this disease. With more data on *Cowdria* infection rates in the vector populations, it should be possible to determine more accurate levels of endemic stability. Ultimately, this data should be fed into epidemiological and economic models for *Cowdria* to assist farmers to determine their levels of endemic stability to heartwater.

Our early work at Rietgat and the surrounding areas (Tice 1997; Tice *et al.* 1998), involved extensive data collection on the population dynamics of all the ticks found in this region, including *A. hebraeum*, and a serological survey was run concurrently with the tick survey (Tice *et al.* 1998). The overall objective of our study was to advise farmers on the levels of endemic stability to TBD in their domestic animals.

The main aims of this specific project were, firstly to develop a reliable AAAP/CO<sub>2</sub> trap in order to collect free-living, unfed *A. hebraeum* ticks at various field sites in South Africa, and secondly to determine the infection rates of *C. ruminantium* in a statistically relevant sample of these ticks with a *C. ruminantium*-specific PCR assay.

This project has shown that the AAAP/CO<sub>2</sub> trap can be used on a sustained basis to collect adult and nymphal *A. hebraeum*. The survey at the Rietgat CGA demonstrated that there was a definite seasonal response to the trap, with peak collection of adult *A. hebraeum* in late spring to early summer. Many different ecological factors can affect the efficacy of the AAAP/CO<sub>2</sub> trap, and these must be taken into consideration when planning the collection of these ticks. The AAAP/CO<sub>2</sub> trap can also be used to locate new population clusters of *A. hebraeum*, and this was demonstrated at the East London site. There were few disadvantages of this type of AAAP/CO<sub>2</sub> trap, but difficulties in obtaining dry ice in certain areas of Africa could be problematic. If the traps were left unattended then ticks would be frozen by the dry ice, and some means of stopping the ticks getting to the dry ice needs to be constructed.

The PCR was chosen as the diagnostic method because it is fast, extremely sensitive and very specific for *C. ruminantium*. The PCR does not cross-react with any *Ehrlichia* species, and could also be used to detect the very low rickettsaemia present in the free-living, unfed *A. hebraeum* ticks. Peter *et al.* (1999) were the first group to use the combination of a *Cowdria*-specific PCR on AAAP/CO<sub>2</sub> collected ticks, and this same technology has now been used successfully on ticks collected at a communal grazing area, as well as two game reserves.

At the Rietgat CGA, the 8.9% prevalence of *C. ruminantium* in the ticks was higher than many other surveys in southern Africa, with the exception of the study of Norval *et al.* (1990) in Zimbabwe. Previous surveys at Rietgat (Tice 1997) have shown that at least ten *A. hebraeum* ticks were always present on the cattle throughout the year, but clinical heartwater was not observed. The tick burdens and the *C. ruminantium* prevalence in the free-living ticks at Rietgat far exceeded the numbers required to establish endemic stability to heartwater (Du Plessis *et al.* 1992; Yonow *et al.* 1998), and it was clear therefore, that at the Rietgat CGA endemic stability to *C. ruminantium* was present.

Wild animals play a pivotal role in the epidemiology of heartwater in Africa, and their role is only just beginning to be unravelled. The results of this project indicated that the ticks found in the game reserves had a higher infection rate than those in the CGA. This was particularly noticeable in the SGR, where nearly 1:4 of the ticks were infected with *C. ruminantium*, and these results support the viewpoint that *C. ruminantium* is widely circulating in wildlife hosts.

To conclude, this was the first time that a large, statistically-relevant field sample of free-living, unfed adult *A. hebraeum* ticks, collected with the AAAP/CO<sub>2</sub> trap from a variety of different ecological areas, have been processed with a PCR to detect the prevalence of *C. ruminantium*. The data from this project should be epidemiologically more relevant than

previous surveys, and it is hoped that this will contribute to our understanding of heartwater epidemiology, especially the dynamics of the endemic stability of this important disease.

Future research in this field should be aimed at investigating ways of improving the collection methods for nymphal *A. hebraeum*. This was shown to be possible at the Rietgat CGA and at East London, but there was no success at the other sample areas. A survey to determine the possible, seasonal incidence of free-living, nymphal *A. hebraeum* needs to be conducted at one or more of these areas. The PCR could also be used on the free-living, unfed nymphal *A. hebraeum* collected with the AAAP/CO<sub>2</sub> trap, to determine the prevalence of *C. ruminantium* in the nymphal stage of the life cycle of *A. hebraeum*. Data on the prevalence levels in free-living nymphs would contribute important information to the models on heartwater, and this would, ultimately, lead to a better understanding of heartwater epidemiology.

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