Chapter 3

Molecular detection of *Ehrlichia ruminantium* infection in *Amblyomma variegatum* ticks in The Gambia

---

Bonto Faburay, Dirk Geysen, Susanne Munstermann, Amar Taoufik, Milagros Postigo and Frans Jongejan

Experimental and Applied Acarology DOI 10.1007/s10493-9073-2 (2007)

Abstract

In West Africa, losses due to heartwater disease are not known because the incidence/prevalence has not been well studied or documented. To develop a diagnostic tool for molecular epidemiology, three PCR-based diagnostic assays, a nested pCS20 PCR, nested *map*1 PCR and a nested reverse line blot (RLB) hybridization assay, were evaluated to determine their ability to detect infection in vector ticks, by applying them simultaneously to *A. variegatum* field ticks to detect *Ehrlichia ruminantium,* the causative agent of heartwater. The nested pCS20 PCR assay which amplified the pCS20 gene fragment showed the highest detection performance with a detection rate of 16.6 %; the nested *map*1 PCR, which amplified the gene encoding the major antigenic protein1 (*map*1 gene) showed a detection rate of 11 % and the RLB, based on the 16S rDNA sequence of anaplasma and ehrlichial species, detected 6.2 %. The RLB, in addition, demonstrated molecular evidence of *Ehrlichia ovina, Anaplasma marginale* and *Anaplasma ovis* infections in The Gambia. Subsequently, the pCS20 assay was applied to study the prevalence and distribution of *E. ruminantium* tick infection rates at different sites in five divisions of The Gambia. The rates of infection in the country ranged from 1.6 % to 15.1 % with higher prevalences detected at sites in the westerly divisions (Western, Lower River and North Bank; range 8.3 % to 15.1%) than in the easterly divisions (Central River and Upper River; range 1.6 % to 7.5 %). This study demonstrated a gradient in the distribution of heartwater disease risk for susceptible livestock in The Gambia which factor must be considered in the overall design of future upgrading programmes.

Introduction

Cowdriosis (heartwater) is a disease of significant economic importance to ruminant livestock production in sub-Saharan Africa. The disease affects domestic and wild ruminants and is caused by *Ehrlichia ruminantium* (ER). The organism is transmitted by ticks of the genus *Amblyomma*, the most widespread vector being *A. variegatum*, which is distributed in sub-Saharan Africa and in the Caribbean.

The control of heartwater depends, to a large extent, on the availability of accurate data on the epidemiology of the infection in the vector and host. Previous tests relied on the use of serological tests (Du Plessis 1993; Jongejan et al. 1989; Martinez et al. 1990; Asselbergs et al. 1993; Martinez et al. 1993), which are of limited reliability, giving false positive and negative reactions and cannot be used to detect *E. ruminantium* infection in ticks. These tests also suffered from low sensitivity. On the other hand, xenodiagnosis, by inoculation of tick homogenates into, or feeding of ticks on susceptible small ruminants (Barré et al. 1984; Andrew and Norval 1989; Birnie et al. 1985; Camus and Barre 1987, 1992; Norval et al. 1990) or mice (Du Plessis 1985) was the method of detection of *E. ruminantium* in ticks. This method is both laborious and expensive. PCR-based tests, in comparison, are highly sensitive and specific and could improve our understanding of the epidemiology of cowdriosis by their ability to detect low levels of *E. ruminantium* infection in *Amblyomma* ticks. In this study, we compared 3 PCR-based assays, nested pCS20 PCR, nested *map*1 PCR and *Anaplasma/Ehrlichia* reverse line blot, using nested primers, by applying them simultaneously to *A. variegatum* ticks to detect ER infection. In this study, the pCS20 PCR was considered the reference test, since it is considered the method of choice for the detection of *E. ruminantium* infection (Simbi et al. 2003), whereas the performance of the *Anaplasma/Ehrlichia* RLB assay (Bekker et al. 2002) with a nested approach is being comparatively evaluated for the first time on field ticks to detect *E. ruminantium* infection.

In West Africa generally, the real or potential economic losses due to the disease are not known because the incidence/prevalence has not been well studied or documented. Attempts to improve local breeds through crossing with highly producing dairy cattle or susceptible small ruminants from non-endemic regions have resulted in high mortality from heartwater. In order to upgrade the livestock industry and prioritize future research on the development of improved control measures, it is essential to provide an accurate definition of the spatial distribution of heartwater disease risk and prevalence of infection in the vector population, *A. variegatum* ticks. In this

study, we demonstrated that the nested PCR assay based on pCS20 target sequences, showed superior performance over nested *map*1 PCR and nested *Anaplasma/Ehrlichia* RLB in determining tick infection rates and considered most suitable for detection of *E. ruminantium* in *Amblyomma* ticks and useful tool for field epidemiological investigation of heartwater. We therefore applied the nested pCS20 in the second phase of this study to determine the prevalence and spatial distribution of *E. ruminantium* infection rates in *A. variegatum* ticks at selected sites in The Gambia.

Materials and Methods

Comparative evaluation of molecular assays

Tick samples and DNA extraction

One hundred and forty-five adult *Amblyomma variegatum* ticks composed of 70 males and 75 mainly flat females (with the exception of 21 semi-engorged females) were collected from indigenous cattle at various field sites in three major intervention areas of the International Trypanotolerance Centre in The Gambia (Kerr Seringe, Keneba and Bansang). The ticks were preserved in 70 % ethanol prior to extraction of DNA. The ticks were first rinsed in sterile distilled water and air-dried. DNA was extracted using protocol A of Qiagen® for isolation of genomic DNA from insects.

Reverse line blot hybridisation assay (RLB)

A nested PCR that amplified a 470 bp fragment of the V1 region of 16S rDNA sequence of *Anaplasma* and *Ehrlichia* species was initially carried out. The first round amplification was carried out with the forward primer AnEhF1 (5´-GGT TTT GTC AAA CTT GAG AG-3´) and the reverse primer AnEhR1 (5´-GTA TTA CCG CCG CTG CT-3´); second round amplification was carried out with the forward primer AnEhF2 (5´-AGA GTT TGA TCC TGG CTC AG-3´) and the reverse primer AnEhR2 (5´-CGA GTT TGC CGG GAC TT-3´). Reaction conditions in a 25 µl volume were as follows: 1 x PCR buffer (Promega, Leiden, The Netherlands), 3mM $MgCl_2$, 200 µM of each of the following deoxynucleoside triphosphates, dATP, dCTP, dGTP, dTTP and dUTP in second nested round, 1.25 U of Super Taq, 0.1 U of Uracil DNA glycosylase, 25 pmol of each primer, and 2.5 µl and 1 µl of DNA template for the first and second round respectively. The reactions were performed on an automated thermal cycler (I-Cycler; Bio-Rad, Richmond, Calif.). The PCR program used was 3 min at 37 °C; 10 min at 94 °C; 40 cycles of 30 sec at 94 °C, 30 sec

50 °C (55 °C for the nested/second run) and 30 sec at 72 °C. This was followed by a final extension phase of 7 min at 72 °C.

The preparation and subsequent hybridisation of the RLB membrane with species-specific probes was done as described before (Gubbels et al. 1999) with the exception that the twice 10 min post hybridisation wash step in 125 ml of 2x SSPE-0.5 % SDS after addition of the PCR products was done at 51°C.

Nested pCS20 PCR

The same tick DNA samples as above were analyzed by semi-nested pCS20 PCR assay. Previous experiments (Peter et al. 1995) already showed the pCS20 PCR detection assay using AB128 and AB129 primers to be highly specific for *E. ruminantium*. Thus we avoided determination of specificity by using AB128 and AB129 as internal primers of the nested PCR. AB129 was also used as the external reverse primer, whereas ITM130 (TCAATTGCTT AATGAAGCACT AACTCAC) was used as the new external forward primer. PCR amplification was carried out in a 25 µl volume containing 5 µl DNA sample, 50mM KCl, 10mM Tris-HCl (pH 8.3), 1.65mM $MgCl_2$, 400 pmol of each of the following deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP, 0.4U of Taq polymerase, 40 pmol of each primer. After a denaturing step of DNA at 94°C for 3 min, the first round of amplification was carried out using the following conditions: 39 cycles of 30 sec denaturation at 94 °C, 45 sec annealing at 62 °C and 1 min elongation at 72°C and a final extension of 10 min at 72°C. Half microlitre (0.5 µl) of PCR product of first round amplification was transferred as template to a second round of PCR at 84°C (hot start principle) consisting of 25 cycles of the same PCR conditions as in the first round except the annealing temperature which was set at 58°C. The PCR amplified a 280bp fragment of open reading frame 2 of the 1,306-bp pCS20 sequence.

Nested *map*1 PCR

Primers for the nested *map*1 PCR were designed using the Clustal alignment of *map*1 sequences of ten different *E. ruminantium* isolates from GenBank™ (Antigua: U50830, Ball-3: AF355200, Burkina Faso: AF368001, Crystal springs: AF125275, Gardel: U50832, Mali: AF368007, Nyatsanga: U50834, Senegal: X74250, South East Botswana: AF368015, Welgevonden: AF125274). The different primer parameters were checked by using computer DNA software programmes like PC-rare (Griffais et al. 1991), Rightprimer™ version M1.2.5 (Biodisk, USA) and PrimerPremier (Biosoft international, USA). Amplification of *map*1 was carried out using the following primers:

- external forward (ERF3) 5′-CCAGCAGGTAGTGTTTACATTAGCGCA-3′
- external reverse (ERR1) 5′-CAAACCTTCCTCCAATTTCTATACC-3′
- internal forward (ERF3)
- internal reverse (ERR3) 5′-GGCAAACATCAAGTGTTGCTGATGC-3′

Thus the external forward primer (ERF3) in the first round PCR was also maintained as the internal forward primer for the second round amplification. PCR amplification was carried out in a 25 µl volume containing the same ingredients as described for the nested pCS20 PCR. After a denaturing step of DNA at 94°C for 3 min, the first round of amplification was carried out using the following conditions: 39 cycles of 1 min denaturation at 92 °C, 1 min 30 sec annealing at 62 °C and 2 min elongation at 72°C and a final extension of 10 min at 72°C. Half microlitre (0.5 µl) of PCR product of first round amplification was transferred as template to a second round of PCR at 84°C (hot start principle) consisting of 25 cycles of the same PCR conditions as in the first round except the annealing temperature which was set at 58°C. In each PCR run, positive and negative controls were included. Positive controls were derived from *E. ruminantium* (Kerr Seringe1 isolate) DNA obtained from cell culture derived organisms and negative controls were reagent blank samples without DNA. The PCR amplified a 720-738 bp fragment of the *map*1 gene of *E. ruminantium*. Amplification products from all PCRs were visualized in 1.5 % agarose gels after staining with ethidium bromide.

Sensitivity of the pCS20 and *map1* PCRs

The sensitivity of the molecular assays, nested pCS20 PCR, nested *map*1 PCR and RLB using nested PCR, was compared by initially measuring the number of *E. ruminantium* (GenBank accession no. DQ333230) organisms contained in a given volume of genomic DNA using real-time PCR as described previously (Postigo et al., 2007). Since the *map1-1* (GenBank accession no. AY652746) is a single copy gene (Collins et al., 2005) allowing a direct estimation of the number of *E. ruminantium* organisms per sample, this gene was targeted for quantifying the number of organisms in a sample. Subsequently, 10-fold serial dilutions of culture-purified *E. ruminantium* DNA were made and tested sequentially by each of the different assays to determine their detection limits.

Determining tick infection rates

Tick sampling

To determine the distribution and prevalence of *E. ruminantium* infection in *A. variegatum* ticks in The Gambia, we carried out collection of adult male ticks and flat adult female ticks from cattle, sheep, goats, or from vegetation at 15 sites in the 5 divisions of the country, Western Division, Lower River Division, North Bank Division, Central River Division and Upper River Division (Figure 1).

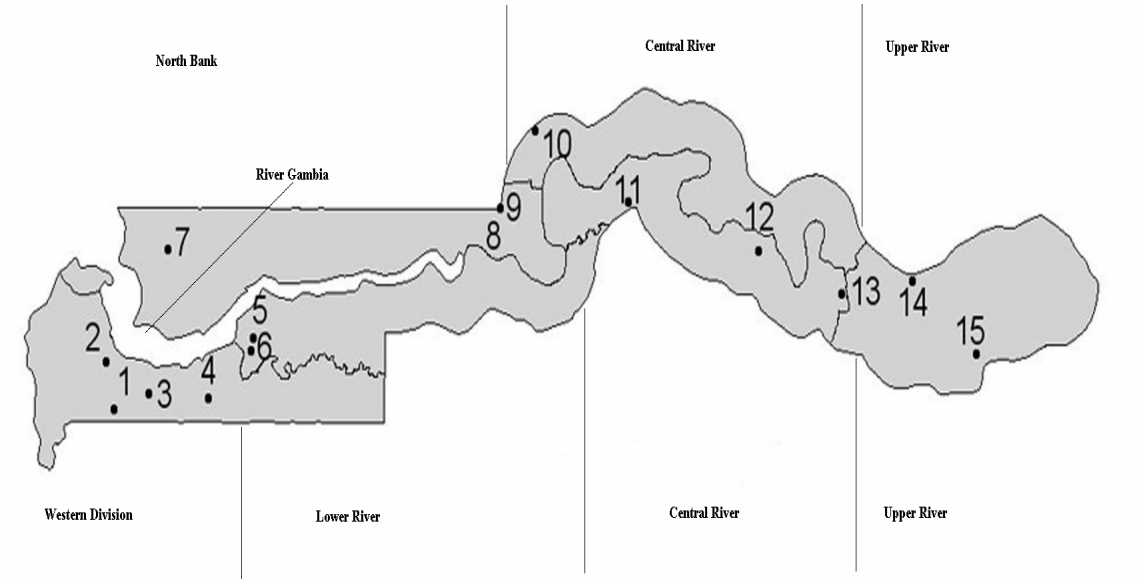

Figure 1. Map of The Gambia showing the five divisions and distribution of the sampling sites

Collection of the ticks was done from July through August 2005, the period of peak incidence of adult *A. variegatum* ticks in The Gambia. From each herd of cattle or flock of sheep or goats, one tick per animal was collected, whereas sampling from vegetation was based on a visual search and collection of unattached ticks. The ticks were preserved in 70 % ethanol until further analysis. Georeferenced coordinates of all sampling sites were recorded using the Global Positioning System (GPS) to show the distribution of the sampling sites (Figure 1). A total of 816 adult ticks composed of 428 flat females and 388 males were tested in PCR. The ticks were preserved in 70 % ethanol until processed for laboratory analysis. Based on the previous evaluation results of the nested pCS20 PCR in detecting *E. ruminantium* in *A. variegatum* ticks picked at random from local cattle at these study sites, ticks were pooled and processed in batches of 2-5 ticks or tested individually for only 9 ticks collected from vegetation at one study site (Tumani Tenda).

DNA extraction and PCR analysis

DNA was extracted from the ticks according to protocol A of Qiagen® for isolation of genomic DNA from insects. The nested pCS20 PCR was carried out as previously described.

Statistical analysis

Differences in the proportion of *E. ruminantium*-positive ticks detected by the 3 different molecular assays were compared using Krukal-Wallis one-way analysis of variance and as well as the general linear model (GLM) procedure (SAS statistical program). McNemar´s change test (chi-squared test) was used to compare the *E. ruminantium* detection performance of the nested pCS20 and nested *map*1 PCR assays. Infection rates at different sites in the five Divisions were deduced using Stata® statistical program.

Results

The detection performance of three molecular assays

All PCR-assays gave clean amplicons of the expected size and the RLB also gave clear positive signals on X-ray films of DNA extracts from *A. variegatum* ticks. No aspecific amplification was seen in the three assays, pCS20, *map*1 and 16S rDNA PCRs. The rate of detection of *E. ruminantium* in ticks by the various assays is shown in Table 1. Of the 3 diagnostic assays, the nested pCS20 PCR consistently detected the highest number of positive samples tested at all three sites. Overall, the assay detected 16.6 % (24/145) of the samples positive for *E. ruminantium*, whereas the *map*1 detected 11 % (16/145) and the reverse line blot 6.2 % (9/145). Of the 24 pCS20-positive samples, 9 (38 %) were positive by *map*1 PCR, whereas 7 tested positive (29.2 %) by RLB.

Table 1. Proportion of *E. ruminantium*-positive samples at various sites detected by 3 PCR-based assays

Site	**Proportion of positive ticks (%) detected by different assays**		
	pCS20	***map*1**	**RLB**
Kerr Seringe	9.8	5.9	5.9
Keneba	25.5	21.6	7.8
Bansang	13.6	4.5	4.5
Overall	16.6	11	6.2

A degree of disparity was observed between the assays in detecting *E. ruminantium* in some of the field samples. Two samples (#92 and #93) tested positive only by pCS20 PCR, whereas samples #85, #163 tested positive by *map*1 assay only, with all samples showing strong positive signals in the respective PCR reactions. Of the 9 samples that tested positive by RLB, 6 tested positive by the pCS20 assay, whereas 7 were positive by *map*1. The detection rates of *map*1 PCR and RLB showed better agreement especially for samples originating from Kerr Seringe (5.9 %) and Bansang (4.5 %; Table 1). The RLB detected a case of co-infection of *A. marginale* and *E. ruminantium* (tick sample # 61, lane 4; Figure 2) and also detected *E. canis/ovina* infection (sample #45, lane 10) at the Keneba study site.

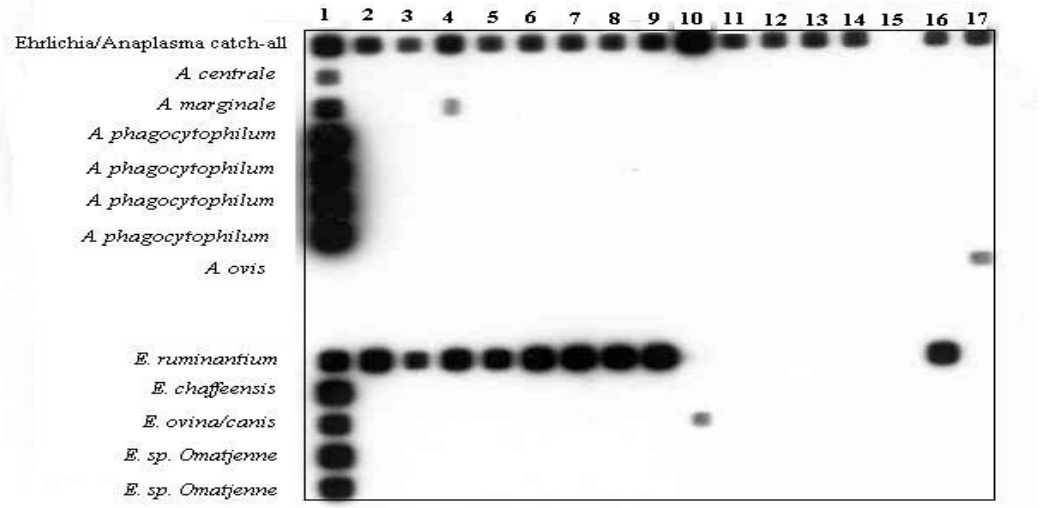

Figure 2. RLB result of the 16S rDNA PCR amplification products of *A. variegatum* field ticks. Lanes #2(19), #3(56), #5(65), #6(90), #7(118), #8(121), #9(135), = positive for *E. ruminantium*; lane #4(61) = positive for *E. ruminantium* and *A. mariginale*; lane #10(45) = positive for *E. ovina*; lane #11(81), #12(82), #13(84), #14(87) = gave only catchall signals; lane #15 = negative control; lane #16 = *E. ruminantium* positive control; lane #17 = *A. ovis* positive control (the *Ehrlichia*/*Anaplasma* probe did not work with *A. ovis*); lane #1 = *Ehrlichia*/*Anaplasma* probe; figures in parenthesis are sample identification numbers.

It also showed a positive *A. ovis* infection in a sample from the same study site (lane 17), although the species-specific oligonucleotide sequence of the latter did not show hybridization signal with the catch-all probe. Four samples (#81, #82, #84 and #87) produced only catch-all signals without any species-specific signals and were thus sequenced to determine if novel species or strain of *Ehrlichia/Anaplasma* were present. The sequence data showed they were *Ehrlichia canis*/*ovina* species with point mutations in their oligonucleotide sequences considered the reason for failure of showing hybridization signals with the *E.canis/ovis* probe.

Comparative analysis of the detection rate of pCS20 and RLB assays showed that the nested pCS20 PCR detected a significantly ($P < 0.05$) higher proportion of *E. ruminantium*-positive samples than the nested RLB. On the other hand, the Chi-squared (McNemar´s) statistic using the formula based on the number of discordant pairs (*r, s;* Table 2): $\chi^2 = (|r - s| - 1)^2 / (r + s)$, showed that the proportion of samples detected positive by the pCS20 assay did not differ significantly from that detected by the *map*1 assay ($\chi^2 = 2.23$; $P > 0.05$) with an odds ratio (*s/r*) of 2.14.

Table 2. Outcome of the detection of *E. ruminantium* infection in ticks by nested pCS20 PCR and nested *map*1 PCR assays

		*map*1		
		+	-	Total
pCS20	+	9	15*(s)*	24
	-	7*(r)*	114	121
		16	129	145

Table 3. *E. ruminantium* infection rates determined by pCS20 PCR in field-collected *A. variegatum* ticks from different sites in The Gambia

Division	Village	Site	Infection rate (%)/no. analyzed		
			Male	Female	Overall
Western Division					
	Giboro Kuta	1	15.0/20	6.7/30	10.0/50
	Tumani Tenda	3	20.0/25	10.7/28	15.1/53
	Mandinaba	2	20.0/25	8.0/25	14.0/50
	Somita	4	20.0/25	7.5/40	12.3/65
Lower River Division	Keneba	6	16.0/25	8.0/25	12.0/50
	Burong	5	14.0/50	8.0/50	11.0/100
North Bank Division	Kollikunda	7	12.0/25	5.7/35	8.3/60
	Mbappa Ba/Mariga	8/9	13.0/23	3.3/30	7.5/53
Central River Division	Yorro Beri Kunda	11	13.0/30	10.0/20	12.0/50
	Sare Sofie	10	5.0/40	6.7/15	5.5/55
	MamutFana	12	3.0/33	0/30	1.6/63
	Jimballa Kerr Chendu	13	5.4/37	0/30	4.5/67
Upper River Division	Kulkullay	15	5.0/20	0/30	2.0/50
	Sare Demba Torro	14	10.0/20	0/30	4.0/50

This statistic, however, showed a better detection performance for the nested pCS20 assay than the nested *map*1 and indicated that ticks in this sample were twice more likely to be positive to the pCS20 assay than to the *map*1. The real-time PCR showed that the undiluted genomic DNA contained 10^7 organisms of *E. ruminantium*. Sensitivity test of the 10-fold serial dilutions showed that the nested pCS20 PCR and nested *map*1 PCR have equal detection thresholds of 1 *E. ruminantium* organism in a sample. The nested RLB showed a sensitivity of 10 *E. ruminantium* organisms per sample.

Distribution of tick infection rates

The prevalence of *E. ruminantium* infection in *A. variegatum* ticks from the 15 sites ranged from 1.6 % to 15.1 % (Table 3). Higher rates of infection were detected at sites in the westerly divisions of the country (Western, Lower River and North Bank), with prevalence ranging from 8.3 % (Kollikunda in North Bank Division), to 15.1 % (Tumani Tenda in Western Division. Sites in easterly divisions (Upper River and Central River) showed lower rates of infection (range 1.6 % to 7.5 %; with the exception of a 'hot spot' site of 12 % in Yorro Beri Kunda). Comparatively, higher rates of infection were detected in male ticks (5 % to 20 %) than in the females (0 % to 10.7 %). Among the tick samples collected from vegetation at four sites in Western Division, North Bank and Central River Divisions, the detected rates of infection ranged from 0 % (n = 13) to 9.5 % (n = 21).

Discussion

In this study, we initially evaluated the performance of 3 PCR-based molecular diagnostic assays, the nested pCS20 PCR, nested *map*1 PCR and RLB (using nested PCR approach) by applying them simultaneously to *A. variegatum* tick samples to detect *E. ruminantium* infection. The samples originated from three study sites (Kerr Seringe, Keneba and Bansang) where heartwater disease is known to occur (Faburay et al. 2005). The nested pCS20 PCR, in comparison to the nested *map*1 assay and the reverse line blot, detected the highest proportion of samples positive

for *E. ruminantium* infection at all three sampling sites. Statistically, there was no significant difference ($P > 0.05$) between overall rates of detection by the pCS20 and *map*1 assays and results of the 10-fold serial dilutions, to assess sensitivity, demonstrated equal detection limit for both assays. In contrast, the reverse line blot detected a significantly lower ($P < 0.05$) proportion of *E. ruminantium*-positive samples and also showed lower sensitivity. The three different assays used primers that amplified different gene or nucleotide sequence targets in *E. ruminantium*, which have varying levels of sequence conservation. For example, the pCS20 assay primers amplified open reading frame 2 of unknown function of the 1,306-bp pCS20 sequence, the *map*1

assay primers amplified the nucleotide sequence of major antigenic protein 1 gene that belongs to a *map*1 multigene family that codes for an antigenic outer-membrane protein of *E. ruminantium* (MAP1); this *map*1 gene is present in all isolates of *E. ruminantium* originating from different geographic regions (van Vliet et al. 1994; Allsopp et al. 2001). The RLB primers amplified the 16S rRNA gene; this gene is widely used for bacterial characterisation (Olsen et al. 1986). The superior performance of the pCS20 assay was attributed to a great extent to a possible higher degree of conservation of the pCS20 target-nucleotide sequence in *E. ruminantium* than either the *map*1 or 16S rRNA gene sequences. The *map*1 gene sequences have been reported to exhibit sequence polymorphisms between *E. ruminantium* isolates (Reddy et al. 1996), which could possibly explain the lower rate of detection by the assay in comparison to the pCS20 assay. In a comparative evaluation of PCR-based diagnostic tests for *E. ruminantium* using a normal PCR approach, the pCS20 assay showed higher sensitivity than assays based on 16S rDNA or the *map*1 gene (Allsopp et al. 1999). Furthermore, the RLB combines a PCR amplification followed by a hybridisation step designed to achieve increased sensitivity over PCR alone. Although the test showed a reliable performance in detection and molecular characterisation of *Theileria* and *Babesia* spp. (Oura et al. 2003; Schnittger et al. 2003; authors' unpublished data), in this study it showed comparatively lower sensitivity for detecting *E. ruminantium* infection in *A. variegatum* ticks. Indeed, the *Anaplasma/Ehrlichia* RLB (using simple PCR) has been shown to detect *E. ruminantium* primarily in experimentally infected sheep and most efficiently during the clinical phase of infection (Bekker et al. 2002). Also in a related experiment (authors' unpublished data), the current assay (RLB with nested 16S PCR) detected 30.8 % (4/13) of the samples positive for *E. ruminantium* in field-exposed carrier animals all of which tested positive by the nested pCS20 PCR. Similarly, in this study, not all samples that tested positive in the nested 16S PCR showed a hybridisation signal with *E. ruminantium* probe (data not shown) suggesting the need for further optimisation of the assay for detection of *E. ruminantium* in field samples for application in epidemiological investigation of hearwater.

The advantage of applying RLB in field epidemiological studies is the ability to detect multiple parasites and discover novel species of parasites (Nijhof et al. 2003). In this study, a case of co-infection of *A. marginale* and *E. ruminantium* (Figure 2, lane 4) was detected in *A. variegatum* tick at one of the study sites (Keneba). This observation is highly plausible, as a range of vectors including ticks and biting flies is known to transmit *A. marginale*. The assay also detected *Ehrlichia canis/ovina* (Figure 2, lane 10) as well as *A. ovis* (lane 17) in the same tick species in the same study area. The detection of *A. marginale*, *E. ovina* and *A. ovis* in *A. variegatum* ticks at this site provided the first molecular evidence of the presence of these parasites and could be an

indication of their possible presence in the resident ruminant livestock populations. Interestingly, it is impossible, clinically, to differentiate fatal ovine ehrlichiosis from heartwater in areas where *Amblyomma variegatum* ticks are present without a microscopic search (Scott 1990). Moreover, in a longitudinal monitoring of mortality in local dwarf sheep and dwarf goats belonging to the International Trypanotolerance Centre (ITC) open-nucleus pure breeding programme in the same study area, a substantial proportion of heartwater-suspected (by apparent manifestation of clinical symptoms) mortalities could not be confirmed by microscopic examination of brain smears (Mattioli and Faburay unpublished data). These findings suggest the need for further investigation to determine the extent and role of these infections in the resident ruminant livestock population.

The evident disparity between the molecular assays in detecting *E. ruminantium* DNA in some samples in this study was attributed to the relative low sensitivity of the *Anaplasma/Ehrlichia* RLB with respect to *E. ruminantium,* as well as to the presence of polymorphisms in the respective primer annealing sites. Indeed, the *map*1 gene of *E. ruminantium* has been reported to exhibit a high degree of sequence polymorphism (Reddy et al. 1996; Allsopp et al. 2001), whereas the lack of amplification by pCS20 primers (AB128/AB129 primers, Mahan et al. 1992) could be attributed to the design of these primers in a comparatively less conserved region of the pCS20 sequence (Van Heerden et al. 2004). Similar findings were also reported in a recent study (Allsopp et al. 2007), in which the pCS20 PCR failed to give amplicons from ticks from which *E. ruminantium* 16S sequences were obtained; this finding was attributed to the presence of polymorphisms in one or both of the pCS20 nested amplification primer target sites thus preventing effective hybridization and chain extension. The same authors (Allsopp et al., 2007) reported the detection of pCS20 and 16S sequences in animals and non-*Amblyomma* tick species in a heartwater-free area in South Africa (Northern Cape) and suggested the presence of a non-pathogenic variant of *E. ruminantium* in this area.

Thus, although the pCS20, *map*1 and 16S PCRs have been used to detect *E. ruminantium* infection in ticks or animals (Mahan et al. 1998; Peter et al. 1995, 2000; Allsopp et al. 1999; Kock et al. 1995; Allsopp et al. 2007), this is the first time the 3 molecular assays, pCS20, *map*1 and RLB, designed in a nested approach for increased sensitivity, have been applied to vector populations, *A. variegatum* field ticks, to evaluate their comparative performance in detecting *E. ruminantium* infection. This study demonstrated that the nested pCS20 PCR is a more sensitive diagnostic tool for the detection of *E. ruminantium* infection than either the nested *map*1 PCR or nested *Anaplasma/Ehrlichia* RLB. This finding, coupled with its high specificity (Peter et al.

2000), makes the assay most suitable for detection of *E. ruminantium* infection in heartwater epidemiology.

The present study demonstrated that the nested pCS20 PCR is most suitable for detection of *E. ruminantium* infection in *A. variegatum* ticks. We therefore applied this molecular assay, in the second part of this study, to describe the prevalence and distribution of *E. ruminantium* infection in the vector population, *A. variegatum* ticks, at 15 different sites in five divisions of The Gambia, Western Division, Lower River Division, North Bank Division, Central River Division and Upper River Division. The overall infection rates in the ticks varied strongly between the 15 sites and ranged between 15.1 % (in Tumani Tenda in Western Division) and 1.6 % (in MamutFana Central River Division) (Table 3). Higher rates of infection were detected at sites in the westerly divisions of the country (Western, Lower River and North Bank) (Figure 3), with prevalence ranging from 7.5 % (Mbappa Ba/Mariga in North Bank Division) to 15.1 % (Tumani Tenda in Western Division). In contrast, sites in the easterly divisions (Central River and Upper River) showed significantly lower rates of infection (range 1.6 % to 5.5 %) with the exception of a Yorro Beri Kunda considered a `hot spot` for heartwater, thereby indicating, in general, a comparatively higher disease risk for naïve/susceptible livestock from non or less endemic areas upon introduction to sites in the westerly divisions. This is consistent with the findings of our previous study (Faburay et al. 2005), of the existence in The Gambia, of a gradient in risk of heartwater disease for susceptible livestock species with risk increasing from the eastern part of the country to the western part towards the coast (Figure 3). Varying levels of heartwater disease risk across sites in Africa have been reported (Bell-Sakyi et al. 2004; Koney et al. 2004; Awa et al.1997; Bekker et al. 2001; Gueye et al., 1993a,b; Faburay et al. 2005; Peter et al. 1999, 2000). Although these risk levels were based principally on serological assessments, we postulate, generally, the existence of a high degree of correlation between seroprevalence of heartwater and *E. ruminantium* tick infection rates as indicated in the present study. Furthermore, this finding suggests that the strategy of introducing more productive exotic ruminant livestock (cattle or small ruminants) for crossbreeding or milk production in support of the Gambia Government policy of promoting peri-urban agriculture targeting towns located in the westerly divisions of the country could result, potentially, in increased heartwater mortalities without the necessary disease control or preventive measures. Interestingly, the high rate (about 30 %) of mortality, associated with heartwater, recorded in F1 (N'Dama x Holstein) calves of the Gambia Government-ITC peri-urban F1 dairy scheme (Sanyang et al. unpublished information) in the coastal area of The Gambia, where comparatively high *E. ruminantium* tick infection rates were recorded in the present study, seems to support this observation. Furthermore, higher rates of infection were

detected in male ticks (3 % to 20 %) than in the female ticks (0 % to 10.7 %). Although we collected most of our ticks from livestock individually, the infection rate in the females examined in this study appeared to be more representative of the real picture due to their short attachment period prior to the start of engorgement (1 to 2 days) and in effect limited exposure to infection. The effect of intrastadial infection has been reported to be more important in male ticks, which can feed for prolonged periods, weeks to months, (Jordaan et al. 1981) without losing infection during feeding. On the other hand, the rate of infection in *A. variegatum* ticks collected from vegetation at four of the sampling sites, which ranged from 0 % (n = 13) to 9.5 % (n = 21), may reflect estimates of the vector infection reservoir although from a relatively small sample size. This is the first study in West Africa that applied a molecular method (PCR) to determine *E. ruminantium* infection rates in the vector population, *A. variegatum*. Although the study was carried out in The Gambia, it nonetheless provided an indication of the potential levels of heartwater disease risk in similar bioclimatic environments in West Africa. A similar study to determine *E. ruminantium* tick infection rates was carried out in a closely related bioclimatic environment in northern Senegal but using the method of inoculating sheep with ground ticks (Gueye et al. 1993). The study detected a comparatively lower infection rate in adult *A. variegatum* ticks of 1.2 %, which result was attributed to the lower sensitivity of the method of xenodiagnosis applied compared to the more sensitive PCR technique (Mahan et al. 1998) used in the present study.

In conclusion, the use of pCS20 PCR in determining field tick infection rates is an important application of this test and the prevalence estimates here provide valuable information for the analysis of heartwater transmission dynamics (O`Callaghan et al. 1998). The study demonstrated the existence of a gradient of risk for livestock with risk increasing from the eastern part of the country towards the western coastal region. This gradient is positively correlated with the distribution of seroprevalence of *E. ruminantium* in extensively managed small ruminants in the country (Figure 3). The results of this survey combined with the previous work (Faburay et al. 2005) can provide an important insight in the formulation of disease control policies thus creating a stronger basis for the development of well-targeted control measures for upgrading the productivity of the livestock industry in The Gambia.

Acknowledgment

This work was supported by The European Development Fund, Service Contract No REG/6061/006, Epigenevac project under contract number no. INCO-CT-2005-003713 and ICTTD-2 concerted action project under contract no. ICA4T-2000-30006, through the INCO-DEV program of the European Commission, the Utrecht Scholarship Programme of Utrecht University and The Rural Finance and Community Initiatives project (IFAD, World Bank, The Gambia). The support of Dr. Cornelis Bekker and Jacob De Witte is highly appreciated. We appreciate the support of the field staff of ITC. Bonto Faburay holds a Rothamsted International African Fellowship Award.

References

1. **Allsopp MT, Dorfling CM, Milliard JC, Bensaid A, Haydon DT, Van Heerden H, Allsopp BA** (2001) *Ehrlichia ruminantium* major antigenic protein gene (map1) variants are not geographically constrained and show no evidence of having evolved under positive selection pressure. J Clin Microbiol **39:**4200-4203.

2. **Allsopp METP, Hattingh CM, Vogel SW, Allsopp BA** (1999) Evaluation of 16S, *map*1 and pCS20 probes for the detection of *Cowdria* and *Ehrlichia* species. Epidemiol Infect **122:**323-328.

3. **Allsopp MTEP, Van Strijp MF, Farber E, Josemans AI, Allsopp BA** (2007) *Ehrlichia ruminantium* variants which do not cause heartwater found in South Africa. Vet Microbiol **120**:158-166.

4. **Andrew HR, Norval RAI** (1989) The career status of sheep, cattle and African buffalo recovered from heartwater. Vet Parasitol **34:**261-266.

5. **Asselbergs M, Jongejan F, Langa A, Neves L, Afonso S** (1993) Antibodies to *Cowdria ruminantium* in Mozambican goats and cattle detected by immunoflourescence using endothelial cell culture antigen. Trop Anim Health Prod **25:**144-50.

6. **Awa DN** (1997) Serological survey of heartwater relative to the distribution of the vector *Amblyomma variegatum* and other tick species in north Cameroon. Vet Parasitol **68:**165-173.

7. **Barré N, Camus E, Birnie E, Burridge MJ, Uilengerg G, Provost A** (1984) Setting up a method for surveying the distribution of cowdriosis (heartwater) in the Caribbean. In: *Proc. XIIIth World Congr. Dis. Cattle*, Durban, South Africa, **1:**536-541

8. **Bell-Sakyi L, Koney EBM, Dogbey O, Walker AR** (2004) *Ehrlichia ruminantium* seroprevalence in domestic ruminants in Ghana. I. Longitudinal survey in the Greater Accra Region. Vet Microbiol **100:**175-188.

9. **Bekker CPJ, Vink D, Lopes Pereira CM, Wapenaar W, Langa A, Jongejan F** (2001) Heartwater (*Cowdria ruminantium* infection) as a cause of postrestocking mortality of goats in Mozambique. Clin Diagn Lab Immunol **8**:843-846.

10. **Bekker CPJ, De Vos S, Taoufik A, Sparagano OAE, Jongejan F** (2002) Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. Vet Microbiol **89:**223-238.

11. **Birnie EF, Burridge MJ, Camus E, Barre N** (1985) Heartwater in the Caribbean: isolation of *Cowdria ruminantium* from Antigua. Vet Rec **116:**121-123.

12. **Camus E, Barre N** (1987) Epidemiology of heartwater in Guadeloupe and in the Caribbean. Onderstepoort J Vet Res **54:**419-426.

13. **Camus E, Barre N** (1992) The role of *Amblyomma variegatum* in transmission of heartwater with special reference to Guadeloupe. Ann NY Acad Sci **653:**33-41.

14. **Collins NE, Liebenberg J, De Villiers EP, Brayton KA, Louw E, Pretorious A, Farber EF, Van Heerden H, Josemans A, Van Kleef M, Steyn MC, Van Strijp MF, Zweygarth E, Jongejan F, Maillard JC, Berthier D, Botha M, Joubert F, Corton CH, Thomson NR, Allsopp MT, Allsopp BA** (2005) The genome of the heartwater agent *Ehrlichia ruminantium* contains multiple tandem repeats of actively variable copy number. PNAS **102:**838-843.

15. **Du Plessis JL** (1985) A method for determining the *Cowdria ruminantium* infection rate of *A. hebraeum*: effects in mice injected with tick homogenates. Onnderstepoort J Vet Res **52:**55-61.

16. **Du Plessis JL, Bezuidenhout JD, Brett MS** (1993) The sero-diagnosis of heartwater: a comparison of five tests. Rev Elev Méd vét Pays trop **46:**123-29.

17. **Faburay B, Munstermann S, Geysen D, Bell-Sakyi L, Ceesay A, Bodaan C, Jongejan F** (2005) Point seroprevalence survey of *Ehrlichia ruminantium* infection in small ruminants in The Gambia. Clin Diagn Lab Immunol **12**:508-512.

18. **Griffais R, Andre PM, Thibon M** (1991) K-tuple frequency in the human genome and polymerase chain reaction. Nucleic Acids Res **19**:3887-91.

19. **Gubbels JM, De Vos AP, Van Der Weide M, Viseras J, Schouls LM, De Vries E, Jongejan F** (1999) Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridisation. J Clin Microbiol **37:**1782-1789.

20. **Gueye A, Mbengue M, Diouf A** (1993) Epidemiology of Cowdria infection in Senegal. I. Study of the transmission and infection level in *Amblyomma variegatum* (Fabricius, 1794) in the Niayes region. Rev Elev Méd vét Pays trop **46:**441-447.

21. **Gueye A, Mbengue Mb, Dieye Th, Diouf A, Seye M, Seye MH** (1993a) La cowdriose au Sénégal: quelques aspects epidemiologiques. Rev Elev Méd vét Pays trop **46:**217-221.

22. **Gueye A, Martinez D, Mbengue Mb, Dieye Th, Diouf A** (1993b) Epidemiologie de la cowdriose au Sénégal. II. Resultats de suivis sero-epidemiologiques. Rev Elev Méd vét Pays trop **46:**449-454.

23. **Jongejan F, Thielemans MJC** (1989) Identification of an immunodominant antigenically conserved 32-kilodalton protein from *Cowdria ruminantium*. Infect Immun 57:3243-3246.

24. **Jordan JO, Baker JAF** (1981) Survival rate on the host and mating capacity of *Amblyomma hebraeum* (Koch) male ticks, p. 115-117. In G.B. Whitehead and J.D. Gibson (ed.), Tick biology and control. Tick Research Unit, Rhodes University, Grahamstown, South Africa.

25. **Kock ND, Van Vliet AHM, Charlton K, Jongejan F** (1995). Detection of *Cowdria ruminantium* in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean wild ungulates. J Clin Microbiol **33:**2501-2504 .

26. **Koney EBM, Dogbey O, Walker AR, Bell-Sakyi L** (2004) *Ehrlichia ruminantium* seroprevalence in domestic ruminants in Ghana. II. Point prevalence survey. Vet Microbiol **103:**183-193.

27. **Mahan SM, Waghela, SD, McGuire, TC, Rurangirwa FR, Wassink LA, Barbet AF** (1992**)** A cloned DNA probe for *Cowdria ruminantium* hybridsed with eight heartwater strains and detects infected sheep. J Clin Microbiol **30:**981-986.

28. **Mahan SM, Peter TF, Simbi BH, Burridge MJ** (1998) PCR detection of *Cowdria ruminantium* infection in ticks and animals from heartwater-endemic regions of Zimbabwe. An NY Acad Sci **849:**85-87.

29. **Martinez D, Swinkels J, Camus, E, Jongejan F** (1990) Comparison de trois antigens pour le diagnostic de la cowdriose par immunofluorescence indirecte. Rev Elev Méd vét Pays trop **43:**159-166.

30. **Martinez D, Coisne S, Sheikboudou C, Jongejan F** (1993) Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by indirect ELISA. Rev Elev Méd vét Pays trop **46:**115-120.

31. **Nijhof AM, Penzhorn BL, Lynen G, Mollel JO, Morkel P, Bekker CPJ, Jongejan F** (2003) *Babesia bicornis* sp. nov. and *Theileria bicornis* sp. nov.: tick-borne parasites associated with mortality in the black rhinoceros. J Clin Microbiol **41:**2249-2254.

32. **Norval RAI, Andrew HR, Yunker CE** (1990) Infection rates with *Cowdria ruminantium* of nymphs and adults of the bont tick collected in the field in Zimbabwe. Vet Parasitol **36:**277-282.

33. **O' Callaghan CJ, Medley GF, Peter TF, Perry BD (1998)** Investigating the epidemiology of heartwater (*Cowdria ruminantium* infection) by means of transmission dynamics model. Parasitology **117:**49-61.

34. **Olsen GJ, Lane DJ, Giovannoni SJ, Pace NR, Stahl DA** (1986) Molecular evolution: a ribosomal RNA approach. Ann Rev Microbiol **40:**337-365.

35. **Oura CAL, Bishop RP, Wampande EM, Lubega GW, Tait A** (2003) Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. Int J Parasitol **34:**603-613.

36. **Peter TF, Deem SL, Barbet AF, Norval RAI, Simbi BH, Kelly PJ, Mahan SM** (1995) Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. J Clin Microbiol **33:**166-172.

37. **Peter TF, Bryson NR, Perry BD, O'Callaghan CJ, Medley GF, Smith GE, Mlambo G, Horak IG, Burridge MJ, Mahan SM** (1999) *Cowdria ruminantium* infection in ticks in Kruger National Park. Vet Rec **15:**304-307.

38. **Peter TF, Barbet AF, Alleman AR, Simbi BH, Burridge MJ, Mahan SM (2000)** Detection of the agent of heartwater, *Cowdria ruminantium*, in *Amblyomma* ticks by PCR: Validation and Application of the assay to field ticks. J Clin Microbiol **38:**1539-1544.

39. **Postigo M, Taoufik A, Bell-Sakyi L, De Vries E, Morrison WI, Jongejan F.** Differential expression of the major antigenic protein 1 multigene family of *Ehrlichia ruminantium* in ticks and *in vitro* cultures. Vet Microbiol doi: 1016/j.vetmic. 2007.01.019.

40. **Reddy GR, Sulsona CR, Harrison RH, Mahan SM, Burridge MJ, Barbet AF** (1996) Sequence heterogeneity of the major antigenic protein 1 genes from *Cowdria ruminantium* isolates from different geographical areas. Clin Diagn Lab Immunol **154:**73-79.

41. **Schnittger L, Yin H Qi B, Gubbels MJ, Beyer D, Niemann S, Jongejan F, Ahmed JS** (2003) Simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants by reverse line blotting. Parasitol Res **92:**189-196.

42. **Scott GR** (1990) Ovine ehrlichiosis, p. 248-250. *In* Sewell MMH, Brocklesby, DW (ed). Animal diseases in the tropics, 7th ed Bailliere Tindall

43. **Simbi BH, Peter TF, Burridge MJ, Mahan SM** (2003) Comparing the detection of exposure to *Ehrlichia ruminantium* infection on a heartwater-endemic farm by the pCS20 polymerase chain reaction and an indirect MAP1-B enzyme linked immunosorbent assay. Onderstepoort J Vet Res **70:**231-235.

44. **Van Heerden H, Steyn HC, Allsopp MTEP, Zweygarth E, Josemans AI, Allsopp BA** (2004) Characterisation of the pCS20 region of different *Ehrlichia ruminantium* isolates. Vet Microbiol **101:**279-291.

45. **Van Vliet AHM, Jongejan F, Van Kleef M, Zeijst BAMVD** (1994) Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. Infect Immunol **62:**1451-1456.