Available online at www.sciencedirect.com
ScienceDirect
Veterinary Microbiology 126 (2008) 189–199
veterinary microbiology
www.elsevier.com/locate/vetmic

Genetic diversity of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks and small ruminants in The Gambia determined by restriction fragment profile analysis

Bonto Faburay [a,c,*], Frans Jongejan [c,d], Amar Taoufik [c], Ansumana Ceesay [a], Dirk Geysen [b]

[a] *International Trypanotolerance Centre, PMB 14, Banjul, The Gambia*
[b] *Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium*
[c] *Utrecht Centre for Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL Utrecht, The Netherlands*
[d] *Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa*

Received 24 March 2007; received in revised form 29 May 2007; accepted 14 June 2007

Abstract

Understanding genetic diversity of *Ehrlichia ruminantium* in host and vector populations is an important prerequisite to controlling heartwater by vaccination in traditional livestock systems in sub-Saharan Africa. We carried out a study in two phases: (i) evaluating the usefulness of the PCR-RFLP assay based on the *map*1 coding sequence of *E. ruminantium* as a discriminatory tool to characterise genetic diversity, (ii) applying the technique to field samples from *Amblyomma variegatum* ticks and small ruminants to characterise genotypic diversity of the organism in three main agroecological zones of The Gambia, Sudano-Guinean (SG), Western Sudano-Sahelian (WSS) and Eastern Sudano-Sahelian (ESS). Restriction fragment length polymorphisms were observed among different strains of *E. ruminantium* supporting the usefulness of the PCR-RFLP technique for studying genetic diversity of the organism. Restriction enzyme *map1* profile analysis indicated the presence in The Gambia of multiple genotypes (at least 11) of *E. ruminantium* with sites in the WSS and SG zones showing comparatively high number of diverse genotypes. Profiles similar to the Kerr Seringe genotype (DQ333230) showed the highest distribution frequency, being present at sites in all three agroecological zones, thereby making the strain a suitable candidate for further characterisation in cross-protection studies. An additional three genotypes showed relatively high distribution frequency and were present in all three zones making them equally important for isolation and subsequent characterisation. The study demonstrated the occurrence of mixed infections with *E. ruminantium* genotypes in ruminants and ticks.
© 2007 Elsevier B.V. All rights reserved.

Keywords: *Ehrlichia ruminantium*; *map1*; Restriction fragment length polymorphism; Genetic diversity; Genotypes; The Gambia

* Corresponding author at: Utrecht Centre for Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL Utrecht, The Netherlands. Tel.: +31 302531114; fax: +31 302546723.
E-mail address: b.fabureh1@vet.uu.nl (B. Faburay).

0378-1135/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2007.06.010

1. Introduction

Heartwater is a rickettsial disease of domestic and wild ruminants caused by *Ehrlichia* (formerly *Cowdria*) *ruminantium* (Dumler et al., 2001). The organism is transmitted by ticks of the genus *Amblyomma*; the major vector in West Africa is *Amblyomma variegatum*, which is distributed in most parts of sub-Saharan Africa and on some islands in the Caribbean (Walker and Olwage, 1987). Heartwater represents a significant obstacle to improvement of livestock production in the tropics and subtropics with mortality rates ranging from 20% to 90% in susceptible animals (Uilenberg, 1983). Small ruminants are particularly at risk from the disease.

Different genotypes of *E. ruminantium* (Allsopp et al., 1997) were found using PCR and sequencing of the V1 loop (Neefs et al., 1993) small-subunit ribosomal RNA gene and random amplified polymorphic DNA and southern blotting (Perez et al., 1997); and stocks with differing immunogenicity exist in the field (Du Plessis et al., 1989). In The Gambia, frequent cases of mortality due to heartwater has been observed in indigenous small ruminants upon translocation from the eastern part of the country to the western part and potential antigenic diversity between different stocks of *E. ruminantium* in the different locations was considered a possible cause (Faburay et al., 2005). Over the past five decades, efforts to control heartwater through development of vaccines have been considerably hampered by the presence of a wide diversity of *E. ruminantium* stocks in the field, which demonstrated phenotypic differences. It is therefore essential to have information on the variety and distribution of stocks within a target area prior to initiating or planning any large-scale vaccination or disease control programmes. At present there is no simple and reliable method for the molecular typing of different *E. ruminantium* stocks (Jongejan and Bekker, 1999). The *map1* gene of *E. ruminantium* represents an ideal target for genotypic characterisation (Allsopp et al., 1999) as it shows a high degree of sequence polymorphisms between isolates (Allsopp et al., 2001; Reddy et al., 1996), does not vary during host–tick passages (D. Martinez, unpublished) and is conserved in all *E. ruminantium* isolates from different geographic regions examined so far (Allsopp et al., 2001; Barbet et al., 1994; Van Vliet et al., 1994). In the present study, we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique (Geysen et al., 2003) on the *map1* gene to characterise genetic diversity of *E. ruminantium* in ticks and small ruminants in The Gambia. This study was carried out in two phases: (i) evaluating the usefulness of a PCR-RFLP assay of the *map1* coding sequence of *E. ruminantium* to distinguish between different isolates and (ii) characterisation of genetic diversity of *E. ruminantium* in the tick vector and small ruminant hosts at selected sites in the three principal agroecological zones of The Gambia.

2. Materials and methods

2.1. Study sites

2.1.1. Sudano-Guinean zone (SG)

Sampling sites (13°43′N, 16°72′W) in the Sudano-Guinean zone were located within the 900 and 1210 mm of rainfall isohyets. Maximum daily temperatures range from 26 to 32 °C (Climatological Unit, Dept. of Water Resources, The Gambia). The vegetation is savannah-woodland or woodland in certain areas, with *Acacia* spp., *Cordia* spp. and *Elaeis guineensis* predominating lowland ecologies. In some areas around the coast, the vegetation is characterised by humid tropical forest vegetation.

2.1.2. Western Sudano-Sahelian zone (WSS)

Sampling sites (13°20′N, 16°01′W) in the Western Sudano-Sahelian zone received an average of 800 mm of rainfall isohyets with maximum daily temperatures ranging from 28 to 38 °C. The vegetation is composed of degraded savannah woodland interspersed with natural unimproved grasslands dominated by *Andropogon gayanus* and *Meriscus* spp., and also with trees and farmland. The lowland tree vegetation consists principally of low and high mangroves.

2.1.3. Eastern Sudano-Sahelian zone (ESS)

Annual precipitation at the sampling sites (13°27′W, 14°40′N) in the Eastern Sudano-Sahelian zone averages 700 mm of rainfall isohyets with maximum temperatures ranging from 30 to 40 °C. The vegetation is mainly open savannah interspersed with trees, grasses and arable farmland. Towards the river, riparian woodland,

including *Mitragyna inermis* and *Acacia seyal*, with scattered *Adansonia digitata* are interspersed with rice fields.

2.1.4. Samples and DNA extraction

Blood was collected in EDTA from extensively managed sheep and goats. The blood was introduced into plain microhaematocrit capillary tubes and centrifuged for 5 min to separate the buffycoat. The microtube was cut just beneath the buffycoat. The latter was applied to Whatman® filter paper No. 3 or 4 by bringing the tube in contact with the filter paper to allow absorption. The filter paper was allowed to dry at room temperature and then stored at −20 °C until used. DNA was extracted by the Modified Plowe extraction method using saponin-chelex (Geysen et al., 2003). Tick samples were preserved in 70% ethanol and DNA was extracted from them individually ($n = 145$ ticks) or pooled in batches 2–5 ticks ($n = 512$ ticks) using the DNeasy™ Tissue kit for isolation of genomic DNA from insects (Qiagen, Westburg, Leusden, The Netherlands). Genomic DNA from 16 reference strains of *E. ruminantium* (Table 1) was extracted from blood, tick or culture stabilates using the Qiagen® Blood Fluid Spin Protocol. The DNA extracts were stored at −20 °C until used.

2.1.5. Nested map1 PCR

All primers were designed using the Clustal alignment of 10 different *E. ruminantium map1* sequences from GenBank™ as described in Faburay et al. (2007) and various computer DNA software programmes including PC-rare (Griffais et al., 1991), Rightprimer™ version M1.2.5 (Biodisk, USA) and PrimerPremier (Biosoft international, USA). Briefly, amplification of the *map*1 gene was carried out using the following primers:

- external forward primer (ERF3) 5′-CCAGCAGGTAGTGTTTACATTAGCGCA-3′;
- external reverse (ERR1) 5′-CAAACCTTCCTCCAATTTCTATACC-3′;
- 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 5 μl DNA sample, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 pmol of each dNTP, 40 pmol of each primer and 0.4 U of Taq polymerase. After a DNA denaturing step at 94 °C for 3 min, the first round of amplification (simple PCR) using ERF3 and ERR1 was carried out using the following conditions: 40 cycles of 1 min denaturation at 92 °C, 1 min 30 s annealing at 60 °C and 2 min elongation at 72 °C and a final extension of 10 min at 72 °C. A 0.5 μl aliquot of PCR product from the first round amplification was transferred as template to a

Table 1
E. ruminantium reference strains tested in the PCR-RFLP assay

E. ruminantium strain	Geographic origin	Source of material	Reference
Kerr Seringe1	The Gambia	Culture	Faburay et al. (2005)
Senegal	Senegal	Culture	Jongejan et al. (1988)
Welgevonden	South Africa	Culture	Du Plessis (1985)
Nonile	South Africa	Blood	MacKenzie and McHardy (1984)
Kwanyanga	South Africa	Blood	MacKenzie and Van Rooyen (1981)
Burkina Faso	Burkina Faso	Ticks	Jongejan et al. (unpublished)
Lutale	Zambia	Blood	Jongejan et al. (1988)
Um Banein	Sudan	Blood	Jongejan et al. (1984)
Gardel	Guadeloupe	Culture	Uilenberg et al. (1985)
Kiswani	Kenya	Blood	Kocan et al. (1987)
Pokoase 417	Ghana	Culture	Bell-Sakyi et al. (1997)
Sankat 430	Ghana	Blood	Bell-Sakyi et al. (1997)
Nigeria (Ifé)	Nigeria	Blood	Ilemobade and Blotkamp (1978)
Kümm	South Africa	Blood	Du Plessis and Kumm (1971)
Bela vista	Mozambique	Blood	Bekker et al. (2001)
Sao Tomé	Sao Tomé	Blood	Uilenberg et al. (1982)

second round of PCR (nested PCR) at 84 °C (hot start principle) with ERF3 and ERR3 primers 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 Seringe) 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 PCR assays were visualised in 1.5% agarose gels after staining with ethidium bromide.

2.1.6. Sensitivity of nested map1 PCR

Ten-fold serial dilutions of culture-derived purified *E. ruminantium* DNA of Kerr Serigne isolate were made to test the sensitivity of the nested *map1* PCR in a previous study (Faburay et al., 2007). Also, DNA derived from *A. variegatum* ticks ($n = 145$) collected randomly from traditionally managed cattle and DNA from blood samples of traditionally managed small ruminants ($n = 150$) were tested in the simple and nested PCR to evaluate the sensitivity of the assay.

2.1.7. Restriction fragment length polymorphism (RFLP) analysis

PCR amplification products obtained from nested *map1* PCR of field (from ticks and small ruminants) and reference strains of *E. ruminantium* were subjected to restriction enzyme analysis with the restriction enzyme, *Alu*1 as recommended by the manufacturer (New England Biolabs®). Four microlitres of the digested sample was mixed with 2 μl of loading buffer and loaded onto a 10% polyacrylamide gel. A 100 bp ladder was included to determine the fragment size. DNA fragments were separated by horizontal electrophoresis in 1× TBE buffer at 100 V for 2 h 40 min. The gel was subjected to silver or SYBR® green (Cambrex Bio science Rockland Inc.) staining according to the manufacturer's instructions and visualised under ultra-violet illumination. The gels were photographed using a digital camera (NikonE4500, Nikon Corp.) fitted with a green filter (for SYBR green-stained gels).

2.1.8. Temporal stability of map1

To assess the reliability of the results of the RFLP, the stability of *map1* over time was examined *in vivo* and *in vitro*. *In vivo* evaluation involved a goat artificially infected with *E. ruminantium* (Kerr Seringe) and treated with oxytetracycline following manifestation of clinical symptoms. The animal consequently became a carrier, which was confirmed by examining genomic DNA extracts from whole blood by nested *map*1 PCR as described above. Thereafter, sequential DNA samples were collected at weekly intervals and subsequently analysed in the PCR-RFLP assay. In the *in vitro* evaluation, we examined the *map1* profile stability of bovine umbilical endothelial cell culture-derived *E. ruminantium* of the Senegal stock at passage levels 1, 15, 30, 72 and 81.

2.1.9. Genetic diversity studies

The aim of this part of the study was to determine the extent of genotypic diversity of *E. ruminantium* in The Gambia. Genomic DNA was extracted from adult *A. variegatum* ticks (male and female) and small ruminant (sheep and goat) whole-blood samples collected at selected sites/villages representative of the three major agroecological zones of the country (SG, WSS, ESS) described above. Table 2 summarises the number of cattle herds sampled for ticks, *A. variegatum* ticks collected, and small ruminants sampled for the study. Overall, 657 partially engorged or unfed (flat females) ticks were collected in 15 villages from selected cattle herds in the three main study areas (Table 2). These herds were located in geographically widely separated villages, and in most places, the animals travelled long distances daily in search of forage and were therefore potentially exposed to challenge with *E. ruminantium* from different areas. In addition, we made 150 genomic DNA extracts from whole blood collected from extensively managed small ruminants in the same villages as indicated in the sampling frame (Table 2). All samples were tested using the nested *map1* PCR and samples that gave specific positive amplicons were subsequently typed by RFLP analysis.

2.1.10. Gene sequencing and analysis of sequence identity

Two field samples, one from a tick and the other from a sheep, gave *map1* PCR products mixed profiles. The *map1* PCR products from these samples and also from *E. ruminantium* (Kerr Seringe) were cloned into pGEM-T Easy Vector Systems (Promega, Madison, USA). The resulting recombinant plasmids were

Table 2
Sampling frame showing number of cattle herds, *A. variegatum* ticks and small ruminants sampled in villages in the three agroecological zones (AEZ) of The Gambia

AEZ	Village	No. of cattle herds sampled for ticks	No. of ticks sampled	No. of small ruminants sampled
Sudano-Guinean	Kerr Seringe	1	40	10
	Giboro kuta	2	42	8
	Tumani Tenda	2	50	10
	Mandinaba	1	38	10
	Berefet/Somita	3	55	12
Western Sudano-Sahelian	Keneba	1	46	10
	Burong	1	50	10
	Kollikunda	1	40	10
	Mbappa Ba	2	35	10
	Mbappa Mariga	2	30	10
Eastern Sudano-Sahelian	Yorro Beri Kunda	1	40	10
	Sare Sofie	1	25	10
	MamutFana	1	25	–
	Jimballa Kerr Chendu	2	42	10
	Kulkullay	3	50	10
	Sare Demba Torro	2	50	10
Total		26	657	150

transformed into *Escherichia coli* DH5α and plated out on X-gal/IPTG plates. Positive transformants (colourless) were selected and confirmed by PCR. Confirmed transformants were subsequently cultured overnight at 37 °C in LB-Ampicillin medium and the plasmids were isolated using the GFX PCR DNA and Gel Band purification kit (Amersham Biosciences, Sweden). The purified plasmids were sent to a commercial company (BaseClear, Leiden, The Netherlands or VIB Genetic Service Facility, Antwerp, Belgium) for sequencing. Overall, six *map1* clones (er80/1, er80/2, er80/8, er80/10, L4306/8, L4306/3) were obtained from the field samples and together with the Kerr Seringe stock, were subsequently sequenced. These sequences were aligned with *map1* sequences of 13 reference strains (Table 1) obtained from GenBank, using the ClustalW method. Sequence distances or percentage identity between different strains or stocks of *E. ruminantium* was determined using MegAlign 4.00 (DNASTAR Inc.).

2.1.11. Statistical analysis

General linear model (GLM) procedure (SAS® statistical programme) was used to determine the level of significance of differences in genotypic diversity of *E. ruminantium* in the three agroecological zones. Mean frequencies of the various profiles and the significance of differences between the frequencies of the various profiles were determined by one-way analysis of variance (ANOVA) with Bonferroni-corrected *P*-value (Stata® statistical programme). Overall frequencies of the various *map1* genotypes detected in the study were determined using Excel programme (Microsoft® Corp.).

3. Results

3.1. Sensitivity of nested map1 PCR

Serial 10-fold dilutions of purified DNA showed a 100-fold increase of the sensitivity in the nested *map1* PCR compared to the simple *map1* PCR. In conjunction with real-time PCR using *map1-1* primers (Postigo et al., 2007), the nested technique was shown to have a detection threshold of one *E. ruminantum* organism in a sample (Faburay et al., 2007). In ticks, the simple PCR technique detected an infection rate of 5.2%, whereas the nested technique detected 11% ($n = 145$). Similarly in small ruminants, the infection rate detected by simple PCR was 4%, whereas it was 10.6% by nested PCR ($n = 150$).

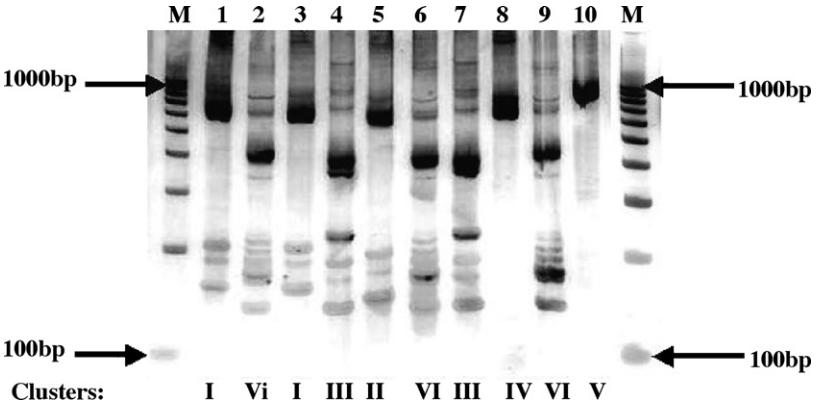

Fig. 1. RFLP of *map1* gene of *E. ruminantium* reference strains showing profile diversity and the various clusters (1 = Kerr Seringe1, 2 = Burkina Faso, 3 = Sankat 430, 4 = Pokoase 417, 5 = Kiswani, 6 = Welgevonden, 7 = Senegal, 8 = Gardel, 9 = Sao Tome, 10 = Kwanyanga) *M* = 100 bp marker.

3.2. Nested map1 PCR and RFLP of reference strains

The PCR-RFLP protocol was validated for the 16 *E. ruminantium* strains (Table 1). In the PCR assay, the primers amplified specific amplicons from all the strains except Kümm (data not shown). The RFLP assay was able to distinguish between the different strains of *E. ruminantium* (Fig. 1). Based on similarities between the individual *map1* restriction profiles, the isolates were grouped into six clusters: (i) Kerr Seringe 1, Sankat 430; (ii) Kiswani, Um Banein; (iii) Pokoase 417, Senegal; (iv) Gardel, Lutale; (v) Kwanyanga, Nigeria (Ifé); (vi) Burkina Faso, Welgevonden, Sao Tomé, Bela Vista, Nonile. Restriction profiles of Um Banein, Nigeria (Ifé), Bela Vista, Lutale and Nonile are not shown. Analysis of the respective *map1* restriction profiles did not show any geographical clustering among the different isolates. Also, the restriction profile of *map1* gene of *E. ruminantium in vivo* (Kerr Seringe stock), in the 4-month observation period, and *in vitro* (Senegal stock), did not manifest any change suggesting temporal stability of the target gene in infected ruminant hosts. Additionally, *map1* gene was reported not to vary during host–tick passages (D. Martinez, unpublished).

3.3. Genetic diversity of E. ruminantium in The Gambia

Restriction enzyme analysis of *map1* coding sequences of *E. ruminantium* in field samples from *A. variegatum* ticks and small ruminants revealed 11 different profiles (Fig. 2; Table 3). Fig. 3 shows the overall frequency of the different *map1* genotypes detected in the three agroecological zones of The Gambia, whereas statistical analysis of the mean frequencies of the various profiles is shown in Table 4. Frequencies of the various genotypes observed in the study were significantly different ($P = 0.0257$). Profile 1 showed the highest overall frequency of 25.8% (Table 3), mean and maximum frequency (Table 4) and was present at sites in all three zones with the frequency highest in the SG zone (Table 3). Profile 6 showed the second highest overall frequency (15.1%) followed by profiles 3 and 11, the latter two showing the same frequency (14%). These profiles were similarly present in all three agroecological zones. Comparison of differences in the frequencies between profile 1 and the rest of the profiles ranged from the borderline of significance ($P = 0.060$) to not significant ($P = 1.000$) (Table 4). Differences in genotypic diversity observed at sites in the three agroecological zones were statistically not significant ($P = 0.182$). Sites in the SG and WSS zones showed a diversity of eight and nine different profiles, respectively; whereas sites in the ESS zone showed five different profiles (Table 3). Profiles 2 and 5 were only seen in the SG zone, whereas profiles 8, 9 and 10 were detected only in the WSS zone. The profiles of a number of *map1* genotypes detected in this study were identical to the restriction profiles of some of the reference strains of *E. ruminantium* distributed throughout heartwater-endemic regions in sub-Saharan

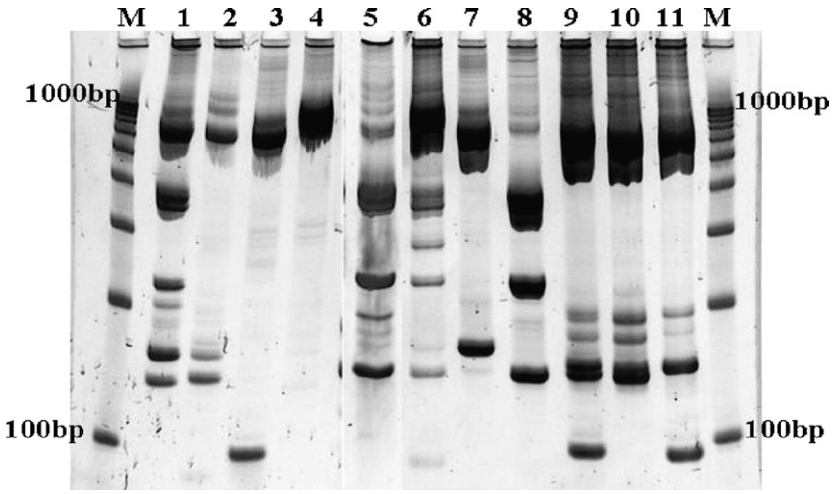

Fig. 2. RFLP of *map1* showing the diversity of *E. ruminantium* profiles detected at various sites in The Gambia (profiles Nos. 1–11), *M* = 100 bp marker.

Africa and the Caribbean (Fig. 1). For example, profile 1 was identical to Sankat and Kerr Seringe, profile 2 to Kiswani and Um Banein, profile 3 to Gardel, Lutale, profile 4 to Kwanyanga and Nigeria (Ifé), profile 5 to Senegal and Pokoase, and profile 10 to Welgevonden, Nonile, Burkina Faso, Sao Tomé and Bela Vista.

As mentioned above, the PCR amplified a 720–738 bp fragment of the *map1 g*ene of *E. ruminantium*. Thus, the sum of restriction bands, counted against a 100 bp scale, of any RFLP profile of 1400 bp and above was considered to be mixed. One sample derived from a tick (in Kerr Seringe) and one from a sheep (in Keneba) showed mixed RFLP profiles (data not shown); the *map1* PCR products derived from these samples were subsequently cloned and sequenced as described previously. Four *map1* clones (er80/1, er80/10, er80/2, er80/8,) were derived from the tick sample suggesting mixed infection with four *E. ruminantium* genotypes, and the sample from the sheep showed two *map1* clones (er/L4306/3, er/L4306/8) suggesting co-infection with two *E. ruminantium* genotypes. The *map1* coding sequences of Kerr Seringe strain and the six clones, er80/1, er80/10, er80/2, er80/8, er/L4306/3 and er/L4306/8, derived from the field samples, were

Table 3
Outcome of RFLP restriction profile analysis of *map1* from *A. variegatum* ticks and small ruminants in different agroecological zones (AEZ) of The Gambia

Profile no.	Frequency (%)	No. of profiles per agroecological zone		
		Sudano-Guinean	Western Sudano-Sahelian	Eastern Sudano-Sahelian
1	25.8	14	6	4
2	2.2	2	–	–
3	14.0	5	4	4
4	10.0	6	3	–
5	4.3	4	–	–
6	15.1	7	2	5
7	8.6	1	1	6
8	2.2	–	2	–
9	3.2	–	3	–
10	1.1	–	1	–
11	14.0	3	7	3
Total number of profiles per AEZ		8	9	5

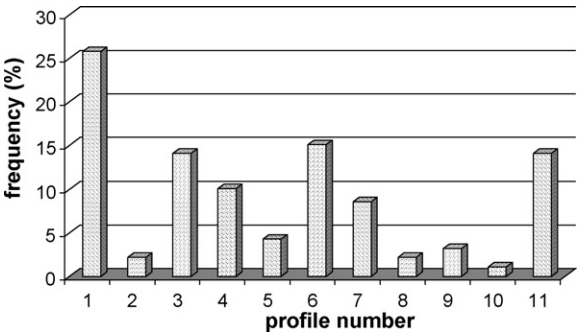

Fig. 3. Overall frequency of the various *map1* genotypes of *E. ruminantium* at various sites detected in the study.

submitted to GenBank and assigned accession nos. DQ333230, EF627980, EF627981, EF627982, EF627983, EF627984 and EF627985, respectively.

3.4. Analysis of percentage identity of map1 sequences

Analysis of percentage identity of *map1* coding sequences derived from the clones and *E. ruminantium* reference strains showed no evidence of geographic clustering. Despite being geographically distant, the *map1* coding sequence of Kerr Seringe1 (Gambia) showed high percentage identity (98.6%) to Sankat 430 (Ghana), whereas Pokoase (Ghana) showed genetic relatedness to Senegal (99%). Despite originating from sites less than 30 km apart (Bell-Sakyi et al., 2004), the two Ghanaian isolates (Sankat 430 and Pokoase 417) were relatively genetically distant showing a percent homology of 92.6%. Similarly, Kerr Seringe and Senegal strains, although originating from closely related geographical areas with similar bioclimatic environment, were found to be genetically distant (90.4%). Of the four clones derived from the tick, three (EF627982, EF627983, EF627981) showed high percentage identity to the Senegal/Pokoase 417 strains suggesting close genetic similarity to these strains (98.6–98.7% identity); EF627980 showed high percentage identity (98.8%) to Nonile. Of the two clones derived from the sheep co-infection, EF627984 showed 94.2% identity to Kerr Seringe/Sankat 430 strains and EF627985 was identical to (97.8–99.2%) to the Gardel/Lutale strains.

4. Discussion

The use of restriction endonuclease digestion of target genes (Brindley et al., 1993; Gasser et al., 1994; Geysen et al., 2003) or bacterial chromosomes (De Villiers et al., 2000), with infrequently cutting restriction endonucleases, has produced restriction profiles that give reliable fingerprints for strain- and species-specific identification. We used the PCR-RFLP targeting the polymorphic *map1* gene to characterise potential genetic diversity of *E. ruminantium* in ticks and small ruminants at selected sites in different agroecological zones (SG, WSS and ESS) of The Gambia. The nested PCR amplification approach

Table 4
Statistical analysis of mean frequencies of the various *map1* RFLP profiles from small ruminants and *A. variegatum* ticks

Profile no.	Mean frequency ± S.D.	Minimum frequency	Maximum frequency	Bonferroni-corrected *P*-value[a]
1	8.00 ± 5.29	4	14	
2	0.67 ± 1.15	0	2	0.089
3	4.33 ± 0.58	4	5	1.000
4	3.00 ± 3.00	0	6	1.000
5	1.33 ± 2.31	0	4	0.194
6	4.67 ± 2.52	2	7	1.000
7	2.67 ± 2.89	1	6	0.871
8	0.67 ± 1.15	0	2	0.089
9	1.00 ± 1.73	0	3	0.131
10	0.33 ± 0.58	0	1	0.060
11	4.33 ± 2.31	3	7	1.000

[a] *P*-values depict comparison of differences between the frequency of profile 1 and individual frequencies of the various profiles; *P*-value of 0.05 or less is significant.

considerably increased the sensitivity of the assay resulting in an improved rate of specific amplification of target *E. ruminantium* DNA in ticks and notably carrier small ruminants for subsequent analysis by RFLP. Using *Alu*I restriction endonuclease, we were able to distinguish amongst West African and Southern African isolates (Fig. 1). The technique distinguished between the four West African isolates examined, Kerr Seringe, Senegal, Sankat 430 and Pokoase 417. In contrast, De Villiers et al. (2000) used *Sma*I and *Ksp*I restriction enzymes in separate digestions on whole genomic DNA and were unable to distinguish between the three West African isolates, Senegal, Sankat 430 and Pokoase 417. The reason for their failure to distinguish between the *E. ruminantium* isolates may be due to digestion of whole genomic DNA instead of a specific amplified gene target, *map1*, as in the present study.

Comparison of the various restriction profiles detected in tick and animal samples originating from the three agroecological zones showed remarkable diversity. Overall, 11 *map1* genotypes were detected in the study (Fig. 2; Table 3). Frequencies of the various profiles in the study areas were significantly different ($P = 0.0257$). Profile # 1, which was identical to the Gambian Kerr Seringe strain, appeared to be the most widely distributed, with the highest overall, mean and maximum frequency (Fig. 3; Tables 3 and 4), and was present in all three study zones of the country (Table 3). Two and three *map1* genotypes were identified only in the SG and WSS zones (towards the coastal area), respectively (Table 3), supporting the hypothesis that susceptible livestock translocated from sites in the far eastern part of the country (ESS) to the western part towards the coast are at greater risk of exposure to challenge with genotypically different stocks of *E. ruminantium*, which could potentially differ immunologically thereby resulting in mortalities due to lack of cross-protection. In a recent vaccination experiment involving sheep, the attenuated Senegal isolate which conferred full protection against the local Kerr Seringe isolate on-station was able to confer only 75% ($n = 12$) protection in a field trial in a limited geographical area in the SG zone (authors' unpublished results). The three case-fatalities were caused by an *E. ruminantium* stock found to be genotypically different from the Kerr Seringe strain confirmed by RFLP analysis of brain samples (authors' results, in preparation for publication). Frequent cases of mortality due to heartwater have been observed in small ruminants upon translocation from the eastern part of the country to coastal Gambia (SG and westerly part of WSS zone). Although lack of immunity indicated by significantly low seroprevalence among small ruminant populations in the eastern part of the country has been reported to contribute to these mortalities (Faburay et al., 2005), antigenic disparities between stocks (Jongejan et al., 1988, 1991), which may reflect, to some extent, genotypic differences among *E. ruminantium* stocks in the different ecological localities, could also be an important cause. In Burkina Faso, the lack of protective immunity confirmed in cross-protection studies in considerably limited geographic area was attributed to diversity (five *map1* genotypes detected in the area) between strains (Martinez et al., 2004).

Differences in genotypic diversity of profiles between the three agroecological zones was statistically not significant ($P = 0.182$). However, sites in the WSS and SG zones showed comparatively high number of diverse *map1* genotypes (Table 3). This was attributed, principally, to the effect of introduction of *E. ruminantium* carrier animals from other parts of the country and beyond its borders. For example, one of the study sites in the WSS zone is host to an ITC station for an Open-nucleus Ruminant Pure Breeding Programme characterised by regular introduction of breeding stock from diverse geographical areas, while the largest centre for trade in ruminant livestock in the country is located in the SG zone, which encompasses the coastal area. Furthermore, profiles identical to that of the Kerr Seringe strain, isolated from a goat in the SG zone, were also identified at sites in the WSS and ESS zones. This led us to postulate that the Kerr Seringe strain may have been introduced from the eastern part of the country following the route of livestock trade from east to west in The Gambia.

Analysis of the *map1* restriction profile of *E. ruminantium* reference strains did not reveal any geographical clustering. However, the clustering based on restriction profile similarity was able to show diversity within West African as well as Southern African isolates indicating the usefulness of the RFLP method as a tool for characterising genotypic diversity of *E. ruminantium* in the field. Although *map1* was shown to be a stable molecular marker for identification of *E. ruminantium* strains, clusters generated by the

RFLP technique did not correlate with cross-protection amongst strains of *E. ruminantium* supporting previous reports that *map1* gene identity between strains may not be a predictor of cross-immunity (Martinez et al., 2004). For instance, the Senegal isolate belonged to a different cluster from Kerr Seringe. The attenuated strain of the former, however, provided complete protection in sheep against lethal needle challenge using the Kerr Seringe isolate (unpublished results).

In conclusion, the use of nested PCR in conjunction with RFLP proved to be a reliable tool to characterise genetic diversity of *E. ruminantium* in the field. This study showed that multiple genotypes of *E. ruminantium* exist in the field and specifically in The Gambia, which indicates the likelihood of a similar level of antigenic diversity and therefore constitutes a major reason for heartwater vaccine failures. This suggests the need for additional *E. ruminantium* strain isolation and characterisation especially in cross-protection trials. The fact that the RFLP profile identical to the Kerr Seringe isolate showed the highest frequency and was present in all three agroecological zones of the country makes this strain a prime candidate for further evaluation in cross-immunity studies. In addition to the Kerr Seringe strain, three other genotypes showed significant frequency distribution and should also be considered important candidates for isolation and subsequent characterisation. In general, the information derived from this study should contribute to the design and development of appropriate control measures (vaccination) and strategies against heartwater in The Gambia.

Acknowledgements

This work was supported by The European Development Fund, Service Contract No. REG/6061/006, Rothamsted International (UK), Epigenevac project under contract no. INCO-CT-2005-003713 and ICTTD-2 concerted action project under contract no. ICA4T-2000-30006, through the INCO-DEV programme of the European Commission, the Utrecht Scholarship Programme of Utrecht University, The International Foundation for Science in Sweden in partnership with COMSTECH. The authors are grateful to the field staff of ITC and the Department of Livestock Services, The Gambia, for their support in field sampling. We appreciate the assistance of Dr. Reginald De Deken, Dr. Cornelis Bekker, Lieve Vermeiren and Jacob De Witte. We are most grateful to Dr. Lesley Bell-Sakyi for the critical and insightful comments. Bonto Faburay holds a Rothamsted International African Fellowship Award.

References

Allsopp, M.E.T.P., Hattingh, C.M., Vogel, S.W., Allsopp, B.A., 1999. Evaluation of 16S, *map*1 and pCS20 probes for the detection of *Cowdria* and *Ehrlichia* species. Epidemiol. Infect. 122, 323–328.

Allsopp, M.T., Dorfling, C.M., Milliard, J.C., Bensaid, A., Haydon, D.T., Van Heerden, H., Allsopp, B.A., 2001. *Ehrichia 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.

Allsopp, M.T.E.P., Visser, E.S., Du Plessis, J.L., Vogel, S.W., Allsopp, B.A., 1997. Different organisms associated with heartwater as shown by analysis of 16S ribosomal RNA gene sequences. Vet. Parasitol. 71, 283–300.

Barbet, A.F., Semu, S.M., Chigagure, N., Kelly, P.J., Jongejan, F., Mahan, S.M., 1994. Size variation of the major immunodominant protein of *Cowdria ruminantium*. Clin. Diagn. Lab. Immunol. 1, 744–746.

Bekker, C.P.J., Vink, D., Lopes Pereira, C.M., Wapenaar, W., Langa, A., Jongejan, F., 2001. Heartwater (*Cowdria ruminantium* infection) as a cause of postrestcoking mortality of goats in Mozambique. Clin. Diagn. Lab. Immunol. 8, 843–846.

Bell-Sakyi, L., Koney, E.B.M., Dogbey, O., Walker, A.R., 2004. *Ehrlichia ruminantium* seroprevalence in domestic ruminants in Ghana. I. Longitudinal survey in the Greater Accra Region. Vet. Microbiol. 100, 175–188.

Bell-Sakyi, L., Koney, E.B.M., Dogbey, O., Abbam, J.A., Aning, K.G., 1997. Isolation and *in vitro* cultivation in Ghana of *Cowdria ruminantium*, the causative agent of heartwater. In: Koney, E.B.M., Aning, K.G. (Eds.), Proceedings of the W.A.C.V.A/G.V.M.A. Conference. Ministry of Food and Agriculture, Accra, pp. 46–51.

Brindley, P.J., Gazzineli, R.T., Denkers, E.Y., Davis, S.W., Dubey, J.P., Belfort Jr., R., Martins, M.C., Silvera, C., Jamra, L., Waters, A.P., Sher, A., 1993. Differentiation of *Toxoplasma gondii* from closely related coccidian by riboprint analysis and a surface antigen gene polymerase chain reaction. Am. J. Trop. Med. Hyg. 48, 447–456.

De Villiers, E.P., Brayton, K.A., Zweygarth, E., Allsopp, B.A., 2000. Macrorestriction fragment profiles reveal genetic variation of *Cowdria ruminantium* isolates. J. Clin. Microbiol. 38, 1967–1970.

Du Plessis, J.L., Van Gas, L., Olivier, J.A., Bezuidenhout, J.D., 1989. The heterogenicity of *Cowdria ruminantium* stocks: cross immunity and serology in sheep and pathogenicity to mice. Onderstepoort J. Vet. Res. 56, 195–201.

Du Plessis, J.L., 1985. A method for determining the *Cowdria ruminantium* rate of *Amblyomma hebraeum*: effects in mice infected with tick homogenates. Onderstepoort J. Vet. Res. 52, 55–61.

Du Plessis, J.L., Kumm, N.A.L., 1971. The passage of *Cowdria ruminantium* in mice. J. S. Afr. Vet. Med. Assoc. 42, 217–221.

Dumler, J.S., Barbet, A.F., Bekker, C.P.J., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganisation of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonymous of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. 51, 2145–2165.

Faburay, B., Geysen, D., Munstermann, S., Taoufik, A., Postigo, M., Jongejan, F., 2007. Molecular detection of *Ehrlichia ruminantium* infection in Amblyomma variegatum ticks in The Gambia. Exp. Appl. Acarol. doi:10 1007/s10493.

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.

Gasser, R.B., Chilton, N.B., Hoste, H., Stevenson, L.A., 1994. Species identification of trichostrongyle nematodes by PCR-linked RFLP. Int. J. Parasitol. 24, 291–293.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Vet. Parasitol. 110, 171–180.

Griffais, R., Andre, P.M., Thibon, M., 1991. K-tuple frequency in the human genome and polymerase chain reaction. Nucleic Acids Res. 19, 3887–3891.

Ilemobade, A.A., Blotkamp, J., 1978. Heartwater in Nigeria. II. The isolation of *Cowdria ruminantium* from live and dead animals and the importance of routes of inoculation. Trop. Anim. Health Prod. 10, 39–44.

Jongejan, F., Bekker, C.P.J., 1999. *Cowdria ruminantium*: recent developments in diagnostic methods, molecular characterization and vaccines. In: Raoult, P., Brouqui, P. (Eds.), Rickettsiae and rickettsial diseases at the turn of the third millennium. Elsevier, Paris, pp. 373–386.

Jongejan, F., Thielemans, M.J.C., Briere, C., Uilenberg, G., 1991. Antigenic diversity of *Cowdria ruminantium* isolates determined by cross-immunity. Res. Vet. Sci. 51, 24–28.

Jongejan, F., Uilenberg, G., Franssen, F.F.J., Gueye, A., Nieuwenhuijs, J., 1988. Antigenic differences between stocks of *Cowdria ruminantium*. Res. Vet. Sci. 44, 186–189.

Jongejan, F., Morzaria, S.P., Omer, A.S., Hashim, M.A., 1984. Isolation and transmission of heartwater (*Cowdria ruminantium* infection) in Blue Nile Province. Sud. Vet. Res. Commun. 8, 141–145.

Kocan, K.M., Morzaria, S.P., Voigt, W.P., Kiarie, J., Irvin, A.D., 1987. Demonstration of colonies of *Cowdria ruminantium* in midgut epithelial cells *Amblyomma variegatum*. Am. J. Vet. Res. 48, 356–360.

MacKenzie, P.K.I., McHardy, N., 1984. The culture of *Cowdria ruminantium* in mice: significance in respect of the epidemiology and control of heartwater. Prev. Vet. Med. 2, 227–237.

MacKenzie, P.K.I., Van Rooyen, R.E., 1981. The isolation and culture of *Cowdria ruminantium* in albino mice. In: Proceedings of an International Congress on Tick Biology and Control. Rhodes University, Grahamstown, pp. 47–52.

Martinez, D., Vachiery, N., Starchurski, F., Kandassamy, Y., Raliniaina, M., Aprelon, R., Gueye, A., 2004. Nested PCR for detection and genotyping of *Ehrlichia ruminantium*: use in genetic diversity analysis. Ann. N.Y. Acad. Sci. 1026, 106–113.

Neefs, J.M., van de Peer, Y., de Rijk, P., Chapell, S., de Wachter, R., 1993. Compilation of small ribosomal subunit RNA structures. Nucleic Acids Res. 21, 3025–3049.

Perez, J.M., Martinez, D., Debus, A., Sheikboudou, C., Bensaid, A., 1997. Detection of genomic polymorphisms among isolates of the intracellular bacterium *Cowdria ruminantium* by random amplified polymorphic DNA and southern blotting. FEMS Microbiol. Lett. 1, 73–79.

Postigo, M., Taoufik, A., Bell-Sakyi, L., Vries, E.D., Morrison, W.I., Jongejan, F., 2007. Differential expression of the major antigenic protein 1 multigene family of *Ehrlichia ruminantium* in ticks and in vitro cultures. Vet. Microbiol. 122, 298–305.

Reddy, G.R., Sulsona, C.R., Harrison, R.H., Mahan, S.M., Burridge, M.J., Barbet, A.F., 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.

Uilenberg, G., Camus, E., Barre, N., 1985. A strain of *Cowdria ruminantium* isolated in Guadeloupe (French West Indies). Rev. Elev. Méd. vét. Pays Trop. 38, 34–42.

Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection): current status. Adv. Vet. Sci. Comp. Med. 27, 427–480.

Uilenberg, G., Corten, J.J., Dwinger, R.H., 1982. Heartwater (*Cowdria ruminantium* infection) on Sao Tome. Vet. Q. 4, 106–107.

Van Vliet, A.H.M., Jongejan, F., Van Kleef, M., Van Der Zeijst, B.A.M., 1994. Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. Infect. Immun. 62, 1451–1456.

Walker, J.B., Olwage, A., 1987. The tick vectors of *Cowdria ruminantium* (Ixodoidae, Ixodidae, genus *Amblyomma*) and their distribution. Onderstepoort J. Vet. Res. 54, 353–379.