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

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29 Abstract

30

31 Understanding genetic diversity of *Ehrlichia ruminantium* in host and vector
32 populations is an important prerequisite to controlling heartwater by vaccination in
33 traditional livestock systems in sub-Saharan Africa. We carried out a study in two
34 phases: i) evaluating the usefulness of the PCR-RFLP assay based on the *map1*
35 coding sequence of *E. ruminantium* as a discriminatory tool to characterise genetic
36 diversity, ii) applying the technique to field samples from *A. variegatum* ticks and
37 small ruminants to characterise genotypic diversity of the organism in 3 main
38 agroecological zones of The Gambia, Sudano-Guinean (SG), Western Sudano-
39 Sahelian (WSS) and Eastern Sudano-Sahelian (ESS). Restriction fragment length
40 polymorphisms were observed among different strains of *E. ruminantium* supporting
41 the usefulness of the PCR-RFLP technique for studying genetic diversity of the
42 organism. Restriction enzyme *map1* profile analysis indicated the presence in The
43 Gambia of multiple genotypes (at least 11) of *E. ruminantium* with sites in the WSS
44 and SG zones showing comparatively high number of diverse genotypes. Profiles
45 similar to the Kerr Seringe genotype (DQ333230) showed the highest distribution
46 frequency, being present at sites in all 3 agroecological zones, thereby making the
47 strain a suitable candidate for further characterisation in cross-protection studies. An
48 additional 3 genotypes showed relatively high distribution frequency and were present
49 in all 3 zones making them equally important for isolation and subsequent
50 characterisation. The study demonstrated the occurrence of mixed infections with *E.*
51 *ruminantium* genotypes in ruminants and ticks.

52

53 Keywords: *Ehrlichia ruminantium*; *map1*; Restriction Fragment Length Polymorphism;
54 genetic diversity; genotypes; The Gambia

55

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 *A. 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 – 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).

82 The *map1* gene of *E. ruminantium* represents an ideal target for genotypic
 83 characterisation (Allsopp et al., 1999) as it shows a high degree of sequence
 84 polymorphisms between isolates (Allsopp et al., 2001; Reddy et al., 1996), does not
 85 vary during host-tick passages (D. Martinez, unpublished) and is conserved in all *E.*
 86 *ruminantium* isolates from different geographic regions examined so far (Allsopp et
 87 al., 2001; Barbet et al., 1994; Van Vliet et al., 1994). In the present study, we used
 88 polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)
 89 technique (Geysen et al., 2003) on the *map1* gene to characterise genetic diversity of
 90 *E. ruminantium* in ticks and small ruminants in The Gambia. This study was carried
 91 out in two phases: i) evaluating the usefulness of a PCR-RFLP assay of the *map1*
 92 coding sequence of *E. ruminantium* to distinguish between different isolates; ii)
 93 characterisation of genetic diversity of *E. ruminantium* in the tick vector and small
 94 ruminant hosts at selected sites in the three principal agroecological zones of The
 95 Gambia.

96

97 **Materials and Methods**

98

99 **Study sites**

100 *Sudano-Guinean zone (SG)*

101 Sampling sites (13°43' N, 16°72' W) in the Sudano-Guinean zone were located within
 102 the 900 and 1210 mm of rainfall isohyets. Maximum daily temperatures range from
 103 26° to 32° C (Climatological Unit, Dept. of Water Resources, The Gambia). The
 104 vegetation is savannah-woodland or woodland in certain areas, with *Acacia* spp.,
 105 *Cordia* spp. and *Elaeis guineensis* predominating lowland ecologies. In some areas
 106 around the coast, the vegetation is characterized by humid tropical forest vegetation.

107

108 *Western Sudano-Sahelian zone (WSS)*

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

115

116 *Eastern Sudano-Sahelian zone (ESS)*

117 Annual precipitation at the sampling sites (13°27' W, 14°40' N) in the Eastern
 118 Sudano-Sahelian zone averages 700 mm of rainfall isohyets with maximum
 119 temperatures ranging from 30° to 40° C. The vegetation is mainly open savannah
 120 interspersed with trees, grasses and arable farmland. Towards the river, riparian
 121 woodland, including *Mitragyna inermis* and *Acacia seyal*, with scattered *Adansonia*
 122 *digitata* are interspersed with rice fields.

123

124

125 *Samples and DNA Extraction*

126 Blood was collected in EDTA from extensively managed sheep and goats. The blood
 127 was introduced into plain microhaematocrit capillary tubes and centrifuged for 5
 128 minutes to separate the buffycoat. The microtube was cut just beneath the buffycoat.
 129 The latter was applied to Whatman® filter paper No. 3 or 4 by bringing the tube in
 130 contact with the filter paper to allow absorption. The filter paper was allowed to dry at
 131 room temperature and then stored at -20° C until used. DNA was extracted by the
 132 Modified Plowe extraction method using saponin-chelex (Geysen et al., 2003). Tick
 133 samples were preserved in 70 % ethanol and DNA was extracted from them
 134 individually ($n = 145$ ticks) or pooled in batches 2-5 ticks ($n = 512$ ticks) using the
 135 DNeasyTM Tissue kit for isolation of genomic DNA from insects (Qiagen, Westburg,

136 Leusden, The Netherlands). Genomic DNA from 16 reference strains of *E.*
 137 *ruminantium* (Table 1) was extracted from blood, tick or culture stabulates using the
 138 Qiagen® Blood Fluid Spin Protocol. The DNA extracts were stored at -20°C until
 139 used.

140

141 *Nested map1 PCR*

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

- 148 • external forward primer (ERF3) 5'-CCAGCAGGTAGTGTTACATTAGCGCA-3'
- 149 • external reverse (ERR1) 5'-CAAACCTTCCTCCAATTTCTATACC-3'
- 150 • internal reverse (ERR3) 5'-GGCAAACATCAAGTGTTGCTGATGC-3'

151 Thus the external forward primer (ERF3) in the first round PCR was also maintained
 152 as the internal forward primer for the second round amplification. PCR amplification
 153 was carried out in a 25 µl volume containing 5 µl DNA sample, 50mM KCl, 10mM
 154 Tris-HCl (pH 8.3), 1.5mM MgCl₂, 200 pmol of each dNTP, 40 pmol of each primer
 155 and 0.4 U of Taq polymerase. After a DNA denaturing step at 94°C for 3 min, the first
 156 round of amplification (simple PCR) using ERF3 and ERR1 was carried out using the
 157 following conditions: 40 cycles of 1 min denaturation at 92 °C, 1 min 30 sec
 158 annealing at 60 °C and 2 min elongation at 72°C and a final extension of 10 min at
 159 72°C. A 0.5 µl aliquot of PCR product from the first round amplification was
 160 transferred as template to a 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 *map1* gene of *E. ruminantium*. Amplification products from all PCR assays were visualised in 1.5 % agarose gels after staining with ethidium bromide.

169

170 *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.

177 *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, *Alu1* 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 1x TBE buffer at 100 V for 2h: 40min. The gel was subjected to silver or SYBR® green (Cambrex Bio science Rockland Inc.) staining according to the manufacturer's instructions and visualised under ultraviolet

illumination. The gels were photographed using a digital camera (NikonE4500, Nikon Corp.) fitted with a green filter (for SYBR green-stained gels).

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 *map1* 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.

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 summarizes 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 fifteen 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

214 long distances daily in search of forage and were therefore potentially exposed to
 215 challenge with *E. ruminantium* from different areas. In addition, we made 150
 216 genomic DNA extracts from whole blood collected from extensively managed small
 217 ruminants in the same villages as indicated in the sampling frame (Table 2). All
 218 samples were tested using the nested *map1* PCR and samples that gave specific
 219 positive amplicons were subsequently typed by RFLP analysis.

220

221 *Gene sequencing and analysis of sequence identity*

222 Two field samples, one from a tick and the other from a sheep, gave *map1* PCR
 223 products mixed profiles. The *map1* PCR products from these samples and also from
 224 *E. ruminantium* (Kerr Seringe) were cloned into pGEM-T Easy Vector Systems
 225 (Promega, Madison, USA). The resulting recombinant plasmids were transformed
 226 into *Escherichia coli* DH5 α and plated out on X-gal/IPTG plates. Positive
 227 transformants (colourless) were selected and confirmed by PCR. Confirmed
 228 transformants were subsequently cultured overnight at 37 °C in LB-Ampicillin
 229 medium and the plasmids were isolated using the GFX PCR DNA and Gel Band
 230 purification kit (Amersham Biosciences, Sweden). The purified plasmids were sent to
 231 a commercial company (BaseClear, Leiden, The Netherlands or VIB Genetic Service
 232 Facility, Antwerp, Belgium) for sequencing. Overall, six *map1* clones (er80/1, er80/2,
 233 er80/8, er80/10, L4306/8, L4306/3) were obtained from the field samples and together
 234 with the Kerr Seringe stock, were subsequently sequenced. These sequences were
 235 aligned with *map1* sequences of thirteen reference strains (Table 1) obtained from
 236 GenBank, using the ClustalW method. Sequence distances or percentage identity
 237 between different strains or stocks of *E. ruminantium* was determined using
 238 MegAlign 4.00 (DNASTAR Inc.).

239

240 *Statistical analysis*

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

249

250 **Results**

251 *Sensitivity of nested map1 PCR*

252 Serial 10-fold dilutions of purified DNA showed a 100-fold increase of the sensitivity
 253 in the nested *map1* PCR compared to the simple *map1* PCR. In conjunction with real-
 254 time PCR using *map1-1* primers (Postigo et al., 2007), the nested technique was
 255 shown to have a detection threshold of 1 *E. ruminantium* organism in a sample
 256 (Faburay et al., 2007). In ticks, the simple PCR technique detected an infection rate of
 257 5.2 %, whereas the nested technique detected 11 % ($n = 145$). Similarly in small
 258 ruminants, the infection rate detected by simple PCR was 4 %, whereas it was 10.6 %
 259 by nested PCR ($n = 150$).

260

261 *Nested map1 PCR and RFLP of reference strains*

262 The PCR-RFLP protocol was validated for the sixteen *E. ruminantium* strains (Table
 263 1). In the PCR assay, the primers amplified specific amplicons from all the strains
 264 except Kümm (data not shown). The RFLP assay was able to distinguish between the
 265 different strains of *E. ruminantium* (Figure 1). Based on similarities between the
 266 individual *map1* restriction profiles, the isolates were grouped into six clusters: i) Kerr

267 Seringe 1, Sankat 430 ii) Kiswani, Um Banein iii) Pokoase 417, Senegal iv) Gardel,
 268 Lutale v) Kwanyanga , Nigeria (Ifé), vi) Burkina Faso, Welgevonden , Sao Tomé,
 269 Bela Vista, Nonile. Restriction profiles of Um Banein, Nigeria (Ifé), Bela Vista,
 270 Lutale and Nonile are not shown. Analysis of the respective *map1* restriction profiles
 271 did not show any geographical clustering among the different isolates. Also, the
 272 restriction profile of *map1* gene of *E. ruminantium* *in vivo* (Kerr Seringe stock), in the
 273 4-month observation period, and *in vitro* (Senegal stock), did not manifest any change
 274 suggesting temporal stability of the target gene in infected ruminant hosts.
 275 Additionally, *map1* gene was reported not to vary during host-tick passages (D.
 276 Martinez, unpublished).

277

278 *Genetic diversity of E. ruminantium in The Gambia*

279 Restriction enzyme analysis of *map1* coding sequences of *E. ruminantium* in field
 280 samples from *A. variegatum* ticks and small ruminants revealed 11 different profiles
 281 (Figure 2; Table 3). Figure 3 shows the overall frequency of the different *map1*
 282 genotypes detected in the three agroecological zones of The Gambia, whereas
 283 statistical analysis of the mean frequencies of the various profiles is shown in Table 4.
 284 Frequencies of the various genotypes observed in the study were significantly
 285 different ($P = 0.0257$). Profile 1 showed the highest overall frequency of 25.8 %
 286 (Table 3), mean and maximum frequency (Table 4) and was present at sites in all 3
 287 zones with the frequency highest in the SG zone (Table 3). Profile 6 showed the
 288 second highest overall frequency (15.1 %) followed by profiles 3 and 11, the latter
 289 two showing the same frequency (14 %). These profiles were similarly present in all 3
 290 agroecological zones. Comparison of differences in the frequencies between profile 1
 291 and the rest of the profiles ranged from the borderline of significance ($P = 0.060$) to
 292 not significant ($P = 1.000$)(Table 4). Differences in genotypic diversity observed at

293 sites in the three agroecological zones were statistically not significant ($P = 0.182$).
 294 Sites in the SG and WSS zones showed a diversity of 8 and 9 different profiles
 295 respectively; whereas sites in the ESS zone showed 5 different profiles (Table 3).
 296 Profiles 2 and 5 were only seen in the SG zone, whereas profile profile 8, 9 and 10
 297 were detected only in the WSS zone. The profiles of a number of *map1* genotypes
 298 detected in this study were identical to the restriction profiles of some of the reference
 299 strains of *E. ruminantium* distributed throughout heartwater-endemic regions in sub-
 300 Saharan Africa and the Caribbean (Figure 1). For example, profile 1 was identical to
 301 Sankat and Kerr Seringe, profile 2 to Kiswani and Um Banein, profile 3 to Gardel,
 302 Lutale, profile 4 to Kwanyanga and Nigeria (Ifé), profile 5 to Senegal and Pokoase,
 303 and profile 10 to Welgevonden, Nonile, Burkina Faso, Sao Tomé and Bela Vista.
 304 As mentioned above, the PCR amplified a 720-738 bp fragment of the *map1* gene of
 305 *E. ruminantium*. Thus the sum of restriction bands, counted against a 100bp scale, of
 306 any RFLP profile of 1400bp and above was considered to be mixed. One sample
 307 derived from a tick (in Kerr Seringe) and one from a sheep (in Keneba) showed mixed
 308 RFLP profiles (data not shown); the *map1* PCR products derived from these samples
 309 were subsequently cloned and sequenced as described previously. Four *map1* clones
 310 (er80/1, er80/10, er80/2, er80/8,) were derived from the tick sample suggesting mixed
 311 infection with 4 *E. ruminantium* genotypes, and the sample from the sheep showed 2
 312 *map1* clones (er/L4306/3, er/L4306/8) suggesting co-infection with 2 *E. ruminantium*
 313 genotypes. The *map1* coding sequences of Kerr Seringe strain and the six clones,
 314 er80/1, er80/10, er80/2, er80/8, er/L4306/3 and er/L4306/8, derived from the field
 315 samples, were submitted to GenBank and assigned accession nos. DQ333230,
 316 EF627980, EF627981, EF627982, EF627983, EF627984 and EF627985, respectively.
 317

318
 319 *Analysis of percentage identity of map1 sequences*
 320 Analysis of percentage identity of *map1* coding sequences derived from the clones
 321 and *E. ruminantium* reference strains showed no evidence of geographic clustering.
 322 Despite being geographically distant, the *map1* coding sequence of Kerr Seringe1
 323 (Gambia) showed high percentage identity (98.6 %) to Sankat 430 (Ghana), whereas
 324 Pokoase (Ghana) showed genetic relatedness to Senegal (99 %). Despite originating
 325 from sites less than 30km apart (Bell-Sakyi et al., 2004), the two Ghanaian isolates
 326 (Sankat 430 and Pokoase 417) were relatively genetically distant showing a percent
 327 homology of 92.6 %. Similarly, Kerr Seringe and Senegal strains, although
 328 originating from closely related geographical areas with similar bioclimatic
 329 environment, were found to be genetically distant (90.4 %). Of the four clones
 330 derived from the tick, 3 (EF627982, EF627983, EF627981) showed high percentage
 331 identity to the Senegal/Pokoase 417 strains suggesting close genetic similarity to these
 332 strains (98.6 – 98.7 % identity); EF627980 showed high percentage identity (98.8 %) to Nonile. Of the 2 clones derived from the sheep co-infection, EF627984 showed
 333 94.2 % identity to Kerr Seringe/Sankat 430 strains and EF627985 was identical to
 334 (97.8 – 99.2 %) to the Gardel/Lutale strains.
 335

336

337 **Discussion**

338 The use of restriction endonuclease digestion of target genes (Brindley et al., 1993;
 339 Gasser et al., 1994; Geysen et al., 2003) or bacterial chromosomes (De Villiers et al.,
 340 2000), with infrequently cutting restriction endonucleases, has produced restriction
 341 profiles that give reliable fingerprints for strain- and species-specific identification.
 342 We used the PCR-RFLP targeting the polymorphic *map1* gene to characterise
 343 potential genetic diversity of *E. ruminantium* in ticks and small ruminants at selected

344 sites in different agroecological zones (SG, WSS, and ESS) of The Gambia. The
 345 nested PCR amplification approach considerably increased the sensitivity of the assay
 346 resulting in an improved rate of specific amplification of target *E. ruminantium* DNA
 347 in ticks and notably carrier small ruminants for subsequent analysis by RFLP. Using
 348 *AluI* restriction endonuclease, we were able to distinguish amongst West African and
 349 Southern African isolates (Figure 1). The technique distinguished between the four
 350 West African isolates examined, Kerr Seringe, Senegal, Sankat 430 and Pokoase 417.
 351 In contrast, De Villiers et al. (2000) used *SmaI* and *KspI* restriction enzymes in
 352 separate digestions on whole genomic DNA and were unable to distinguish between
 353 the three West African isolates, Senegal, Sankat 430 and Pokoase 417. The reason for
 354 their failure to distinguish between the *E. ruminantium* isolates may be due to
 355 digestion of whole genomic DNA instead of a specific amplified gene target, *map1*, as
 356 in the present study.

357
 358 Comparison of the various restriction profiles detected in tick and animal samples
 359 originating from the 3 agroecological zones showed remarkable diversity. Overall, 11
 360 *map1* genotypes were detected in the study (Figure 2; Table 3). Frequencies of the
 361 various profiles in the study areas were significantly different ($P = 0.0257$). Profile #
 362 1, which was identical to the Gambian Kerr Seringe strain, appeared to be the most
 363 widely distributed, with the highest overall, mean and maximum frequency (Figure 3;
 364 Table 3, Table 4), and was present in all three study zones of the country (Table 3).
 365 Two and three *map1* genotypes were identified only in the SG and WSS zones
 366 (towards the coastal area) respectively (Table 3), supporting the hypothesis that
 367 susceptible livestock translocated from sites in the far eastern part of the country
 368 (ESS) to the western part towards the coast are at greater risk of exposure to challenge

369 with genotypically different stocks of *E. ruminantium*, which could potentially differ
 370 immunologically thereby resulting in mortalities due to lack of cross-protection. In a
 371 recent vaccination experiment involving sheep, the attenuated Senegal isolate which
 372 conferred full protection against the local Kerr Seringe isolate on-station was able to
 373 confer only 75 % ($n = 12$) protection in a field trial in a limited geographical area in
 374 the SG zone (authors' unpublished results). The three case-fatalities were caused by
 375 an *E. ruminantium* stock found to be genotypically different from the Kerr Seringe
 376 strain confirmed by RFLP analysis of brain samples (authors' results, in preparation
 377 for publication). Frequent cases of mortality due to heartwater have been observed in
 378 small ruminants upon translocation from the eastern part of the country to coastal
 379 Gambia (SG and westerly part of WSS zone). Although lack of immunity indicated by
 380 significantly low seroprevalence among small ruminant populations in the eastern part
 381 of the country has been reported to contribute to these mortalities (Faburay et al.,
 382 2005), antigenic disparities between stocks (Jongejan et al., 1988, 1991), which may
 383 reflect, to some extent, genotypic differences among *E. ruminantium* stocks in the
 384 different ecological localities, could also be an important cause. In Burkina Faso, the
 385 lack of protective immunity confirmed in cross-protection studies in considerably
 386 limited geographic area was attributed to diversity (5 *map1* genotypes detected in the
 387 area) between strains (Martinez et al., 2004).

388
 389 Differences in genotypic diversity of profiles between the three agroecological zones
 390 was statistically not significant ($P = 0.182$). However, sites in the WSS and SG zones
 391 showed comparatively high number of diverse *map1* genotypes (Table 3). This was
 392 attributed, principally, to the effect of introduction of *E. ruminantium* carrier animals
 393 from other parts of the country and beyond its borders. For example, one of the study

394 sites in the WSS zone is host to an ITC station for an Open-nucleus Ruminant Pure
 395 Breeding Programme characterised by regular introduction of breeding stock from
 396 diverse geographical areas, while the largest centre for trade in ruminant livestock in
 397 the country is located in the SG zone, which encompasses the coastal area.
 398 Furthermore, profiles identical to that of the Kerr Seringe strain, isolated from a goat
 399 in the SG zone, were also identified at sites in the WSS and ESS zones. This led us to
 400 postulate that the Kerr Seringe strain may have been introduced from the eastern part
 401 of the country following the route of livestock trade from east to west in The Gambia.

402

403 Analysis of the *mapI* restriction profile of *E. ruminantium* reference strains did not
 404 reveal any geographical clustering. However, the clustering based on restriction
 405 profile similarity was able to show diversity within West African as well Southern
 406 African isolates indicating the usefulness of the RFLP method as a tool for
 407 characterising genotypic diversity of *E. ruminantium* in the field. Although *mapI* was
 408 shown to be a stable molecular marker for identification of *E. ruminantium* strains,
 409 clusters generated by the RFLP technique did not correlate with cross protection
 410 amongst strains of *E. ruminantium* supporting previous reports that *mapI* gene
 411 identity between strains may not be a predictor of cross-immunity (Martinez et al.,
 412 2004). For instance, the Senegal isolate belonged to a different cluster from Kerr
 413 Seringe. The attenuated strain of the former, however, provided complete protection
 414 in sheep against lethal needle challenge using the Kerr Seringe isolate (unpublished
 415 results).

416

417 In conclusion, the use of nested PCR in conjunction with RFLP proved to be a reliable
 418 tool to characterise genetic diversity of *E. ruminantium* in the field. This study

419 showed that multiple genotypes of *E. ruminantium* exist in the field and specifically in
 420 The Gambia, which indicates the likelihood of a similar level of antigenic diversity
 421 and therefore constitutes a major reason for heartwater vaccine failures. This suggests
 422 the need for additional *E. ruminantium* strain isolation and characterisation especially
 423 in cross-protection trials. The fact that the RFLP profile identical to the Kerr Seringe
 424 isolate showed the highest frequency and was present in all 3 agroecological zones of
 425 the country makes this strain a prime candidate for further evaluation in cross-
 426 immunity studies. In addition to the Kerr Seringe strain, three other genotypes showed
 427 significant frequency distribution and should also be considered important candidates
 428 for isolation and subsequent characterisation. In general, the information derived from
 429 this study should contribute to the design and development of appropriate control
 430 measures (vaccination) and strategies against heartwater in The Gambia.

431

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Legends

Tables

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

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

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

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

Figure Legends

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

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

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

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

<i>E. ruminantium</i> 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 & McHardy, 1984
Kwanyanga	South Africa	Blood	MacKenzie & Van Rooyen, 1981
Burkina Faso	Burkina Faso	Ticks	Jongejan et al., 1995 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	Illemobade, 1976
Kümm	South Africa	Blood	Du Plessis & Kümm, 1971
Bela vista	Mozambique	Blood	Bekker et al., 2001
Sao Tomé	Sao Tomé	Blood	Uilenberg et al., 1982

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

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

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

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

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

^aP 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;

Figure 1

Figure 2

Figure 3