Genetic diversity of *Ehrlichia ruminantium* strains in Cameroon

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In order to investigate the extent of genetic diversity among *Ehrlichia ruminantium* strains in Cameroon, a partial fragment (800 bp) of the *E. ruminantium map*1 gene was amplified by nested polymerase chain reaction in 121 of 156 *E. ruminantium* pCS20-positive DNA samples extracted from ticks and cattle collected from two ranches. Deoxyribonucleic acid sequencing of the *map*1 gene products indicated the presence of at least 21 genotypes at the nucleotide level and 16 genotypes at the amino acid level circulating within the study sites. Some of the genotypes were identical to Antigua (U50830), Blaaukrans (AF368000) or UmBanein (U50835), whilst the others were new genotypes. Twenty-four representative sequences were deposited in GenBank and given accession numbers JX477663 – JX477674 (for sequences of tick origin) and JX486788 – JX486799 (for sequences of cattle origin). Knowledge of *E. ruminantium* strain diversity could be important in understanding the epidemiology of heartwater.

Introduction

Ehrlichia ruminantium, the causative agent of heartwater in domestic and wild ruminants, is an obligate intracellular Gram-negative and pleomorphic bacterium. It is transmitted by ticks of the genus *Amblyomma* (Dumler *et al.* 2001; Uilenberg 1983), with *Amblyomma variegatum* being reported as the most efficient vector of this pathogen (Waghela *et al.* 1991). Heartwater has been reported to be a substantial obstacle to the improvement of livestock production throughout sub-Saharan Africa (Dumler *et al.* 2001; Faburay *et al.* 2008; Uilenberg 1983). Reports of the presence of heartwater in Cameroon have been based on: molecular testing (Esemu *et al.* 2013); serology (Awa 1997); microscopic demonstration of morulae in Giemsa-stained brain impression smears of grey matter from deceased cattle (Ndi *et al.* 1998); clinical diagnosis (Merlin, Tsangueu & Ronsvoal 1986; Ndi *et al.* 1998); and the presence of the heartwater vector, *A. variegatum* (Awa 1997; Merlin *et al.* 1986; Merlin, Tsangueu & Ronsvoal 1987; Ndi *et al.* 1998; Ndip *et al.* 2004; Stachurski 2000).

In addition to tick control, immunisation is an important prevention strategy; however, a successful immunisation programme should be informed by appropriate epidemiological data, which include the genetic characteristics of the circulating strains. In Cameroon, the economic impact of heartwater in domestic ruminants is recognised, but not yet well-documented (Ndi *et al.* 1998). The control of heartwater is mainly by tick control, the main tick control strategy being hand-picking and the application of acaricides through spray races, dips or using knapsack sprayers (Ndi *et al.* 1998). So far, there has not been any report of the control of heartwater by immunisation.

Several strains of *E. ruminantium* have been reported in sub-Saharan Africa (Esemu, Ndip & Ndip 2011) and they exhibit different levels of genetic diversity. The genetic diversity within the *E. ruminantium map*1 gene has not shown any evidence of geographic clustering (Allsopp *et al.* 1997; Allsopp *et al.* 2001; Faburay *et al.* 2008). The *map*1 gene, one of the 16 paralogous genes that make up the *map*1 multigene family, has been used to understand genetic diversity among *E. ruminantium* strains (Allsopp *et al.* 2001; Martinez *et al.* 2004) and it remains the best tool for characterising genetic diversity among African, Caribbean and Madagascan strains of *E. ruminantium* (Raliniaina *et al.* 2010). Previous studies have been aimed at detecting *E. ruminantium* in *A. variegatum* ticks, based on the pCS20 polymerase chain reaction (PCR) assay, and have revealed a high degree of similarity within the pCS20 region of the *E. ruminantium* strains that were identified (Esemu *et al.* 2013). In the present study, genetic diversity among the *E. ruminantium* strains circulating between *A. variegatum* tick vector and the cattle host by analysis of the *map*1 gene was investigated.

Materials and methods

Study sites and samples

One hundred and fifty-six *E. ruminantium*-pCS20-positive DNA samples were extracted from *A. variegatum* ticks (144) and cattle (12) originating from two ranches (Société de Développement et d'Exploitation des Productions Animales [SODEPA] Dumbo ranch [SDR] and Upper Farms ranch [UFR]), as previously described by Esemu *et al*. (2013), were included in this study. Of these 156 DNA samples, 59 were from SDR (49 extracted from *A. variegatum* ticks and 10 from cattle blood) and 97 from UFR (95 extracted from *A. variegatum* ticks and 2 from cattle blood).

Nested *map*1 polymerase chain reaction

Nested *map*1 PCR was performed for the 156 DNA samples using the following primers: Map1NT (5'-CTCG TAAG AAGT GCGT TAAT-3') as the external forward primer; Map1CT1 (5'-TTAA AATA CAAA CCTT CCTC C-3') as the external reverse primer; Map1LP (5'-CTTG GTGT GTCC TTTT CTGA-3') as the internal forward primer and Map1CT2 (5'-CCTT CCTC CAAT TTCT ATAC C-3') as the internal reverse primer (Martinez *et al*. 2004). The individual reaction mixture for the first round of PCR was made up of template deoxyribonucleic acid (DNA) (5 µL), 12.5 µL PCR master mix (2X) (TopTaq™ Master Mix, Qiagen, Hilden, USA) and 0.5 µL of each primer from a working solution of 20 µM (final concentration of 0.4 µM), and nuclease-free PCR water to make up 25 µL total individual reaction volume. Deoxyribonucleic acid amplification was carried out in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, USA) under the following cycling conditions: for the first round of PCR reaction, initial denaturation was performed at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C for 45 s), primer annealing (53 °C for 45 s) and primer extension (72 °C for 45 s). The final extension was at 72 °C for 10 min and the reaction was stopped by cooling to 4 °C until the samples were collected. For the second round of PCR amplification, aliquots of 1 µL of PCR product from the first round of PCR amplification were used as the DNA template. The PCR amplification conditions for the second round were optimised as follows: initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation (94 °C for 1 min), primer annealing (57 °C for 1 min) and primer extension (72 °C for 1 min). The final extension was at 72 °C for 10 min and holding at 4 °C until the samples were collected. The expected PCR product size after the second round of PCR amplification was 800 bp. Each batch of PCR run had one negative control, in which sterile distilled water was used as the template. The PCR products were analysed on a 1% agarose gel (AGTC Bioproducts, Florida, USA).

Sequencing and sequence analysis of *map*1 PCR products

To determine the extent of genetic diversity of *E. ruminantium* strains from *A. variegatum* ticks and cattle in Cameroon, amplified *map*1 PCR products were sequenced from both ends with the nested *map*1 PCR primers (Inqaba Biotec, South Africa). Twenty-four high-quality sequences of the positive *map*1 PCR products, 12 (six from SDR and six from UFR) of tick origin and 12 (ten from SDR and two from UFR) of cattle origin were selected for analysis and aligned with ClustalW (Hall 1999). The BLAST program (National Center for Biotechnology Information, Bethesda, MD) (Morgulis *et al*. 2008) was used for similarity matches between the *E. ruminantium* strains reported in the present study and reference strains deposited in GenBank. Similarity matches between *E. ruminantium* strains reported in the present study were determined in a pair-wise sequence alignment using BioEdit version 7.0.9 (Hall 1999). Phylogenetic relationships were determined with MEGA6 (Tamura *et al*. 2013). All sequences were deposited in GenBank.

Results

Polymerase chain reaction amplification of *Ehrlichia ruminantium* DNA from *Amblyomma variegatum* ticks and cattle

Amplified nested PCR products of the expected size (800 bp) were detected in 109 (75.7%) of the 144 *E. ruminantium* pCS20-positive tick DNA samples that were tested. The *map*1 gene was amplified from all of the 12 (100%) *E. ruminantium* pCS20-positive cattle DNA samples. All PCR products were separated in a 1% agarose gel, stained with ethidium bromide, visualised and photographed in a Molecular Imager Gel Doc XR system (BIO-RAD, Hercules, CA, USA).

Genetic diversity of *Ehrlichia ruminantium* in Cameroon

Sequence analysis of *map*1 PCR products revealed several genotypes of *E. ruminantium* circulating in *A. variegatum* ticks and the cattle host in the study sites. Alignment of the *map*1 sequences of all the strains revealed deletions and insertions concentrated within three variable regions. Single nucleotide polymorphisms (SNPs) were dispersed throughout the DNA sequences. Similarly, alignment of the MAP1 amino acid sequences of all the strains revealed deletions and insertions concentrated within three variable regions (Figure 1).

Based on nucleotide sequence identity, the *E. ruminantium* strains sequenced in this study were placed into different groups (Table 1) with some groups having more than one genotype. Identical nucleotide sequences were obtained for only three pairs among the 24 strains sequenced: CMR Buea 34 and CMR Dumbo 48 (group 1), CMR Buea 89 and CMR Dumbo 52 (group 3) and CMR Buea 20 and CMR Dumbo 7 (group 5).

These results indicated the presence of more *E. ruminanium* genotypes in SDR than in UFR. Of the 21 genotypes at the nucleotide level, 13 were present only in SDR, whilst five were present only in UFR. Three genotypes were present in both SDR and UFR. Ten genotypes were detected in

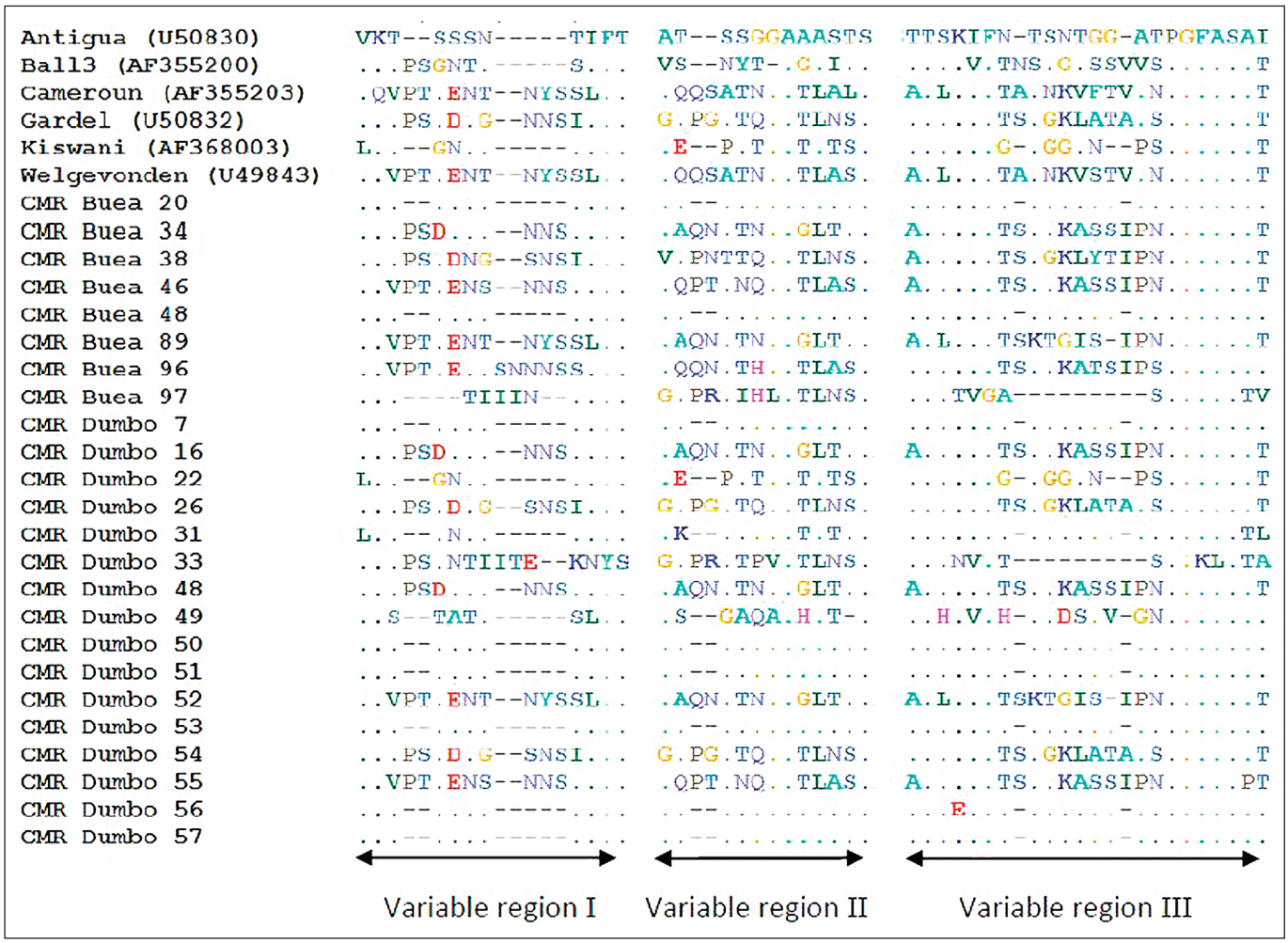


FIGURE 1: Comparison of the amino acid sequences at the three variable regions of the MAPI coding sequences of *Ehrlichia ruminantium* strains from Cameroon and six reference strains (accession number put in parentheses).

TABLE 1: Classification of the Cameroonian *Ehrlichia ruminantium* strains based on the percentage identities† at the nucleotide level and the number of genotypes in each group.

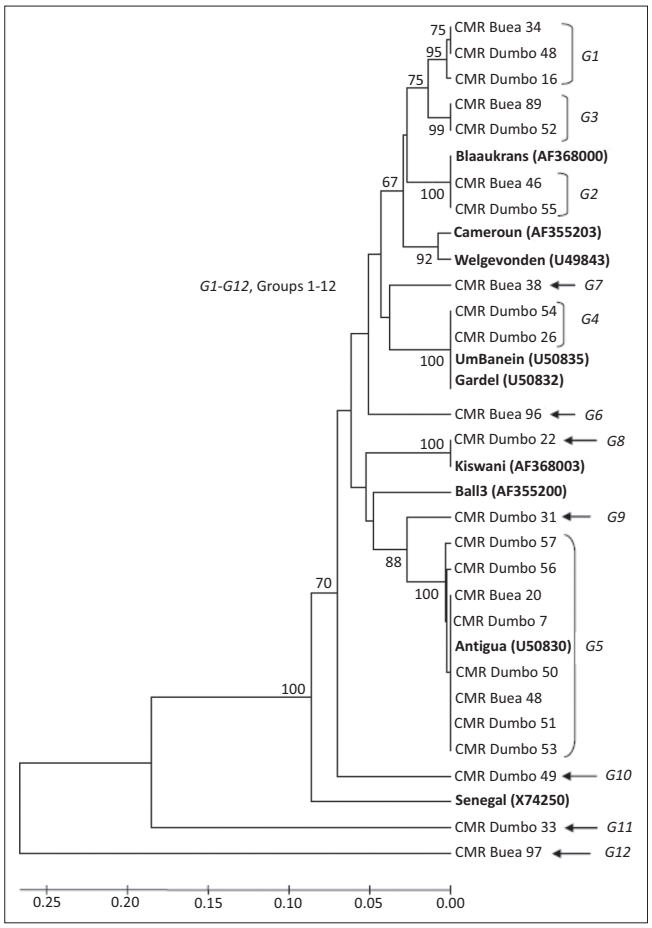
Group	*Ehrlichia ruminantium* strain(s)	Nucleotide sequence identity (%)	Genotypes	
			Nucleotide level	Amino acid level
1	CMR Buea 34 , CMR Dumbo 16, CMR Dumbo 48	99.5–100.0	2	2
2	CMR Buea 46, CMR Dumbo 55	99.1	2	2
3	CMR Buea 89, CMR Dumbo 52	100.0	1	1
4	CMR Dumbo 26, CMR Dumbo 54	99.2	2	1
5	CMR Buea 20, CMR Buea 48, CMR Dumbo 7, CMR Dumbo 50, CMR Dumbo 51, CMR Dumbo 53, CMR Dumbo 56, CMR Dumbo 57	99.0–100.0	7	3
6	CMR Buea 96	-	1	1
7	CMR Buea 38	-	1	1
8	CMR Dumbo 22	-	1	1
9	CMR Dumbo 31	-	1	1
10	CMR Dumbo 49	-	1	1
11	CMR Dumbo 33	-	1	1
12	CMR Buea 97	-	1	1
Total	-	-	**21**	**16**

†, *Ehrlichia ruminantium* strains in each group have at least 99.0% identity. Similarity matches determined in a pair-wise sequence alignment using BioEdit version 7.0.9.

cattle, whilst eight were detected in *A. variegatum* ticks. Three identical genotypes were detected in both *A. variegatum* ticks and cattle.

Some of the *E. ruminantium map*1 genotypes were identical to different strains reported elsewhere in African and Caribbean countries, such as Antigua (U50830) from the Caribbean island, Blaaukrans (AF368000) from South Africa and UmBanein (U50835) from Sudan.

The phylogenetic tree of Cameroonian *E. ruminantium* strains with some reference strains at the amino acid level revealed clustering of some strains. All strains in group 5 clustered with Antigua, strains in group 2 clustered with Blaaukrans,



Source: Phylogenetic tree obtained with MEGA 6 software (Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S., 2013, 'MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0.', *Molecular Biology and Evolution* 30, 2725–2729. http://dx.doi.org/10.1093/molbev/mst197

FIGURE 2: Phylogenetic tree based on the partial amino acid sequences of MAP1 of 24 *Ehrlichia ruminantium* strains reported in this study, and seven reference strains (bold and accession numbers put in parentheses).

group 4 clustered with UmBanein and Gardel and group 8 clustered with Kiswani (Figure 2). The partial sequences of the *map*1 gene of the *E. ruminantium* strains analysed in the present study were deposited in GenBank and given accession numbers JX477663 – JX477674 (for sequences of tick origin) and JX486788 – JX486799 (for sequences of cattle origin).

Discussion

Several strains of *E. ruminantium*, the causative agent of heartwater, have been reported throughout the endemic regions of Africa and the Caribbean (Esemu *et al*. 2011). The control of heartwater by immunisation will potentially be hampered by genetic diversity among these strains (Allsopp *et al*. 1999; Allsopp *et al*. 2001; Faburay *et al*. 2008; Martinez *et al*. 2004; Reddy *et al*. 1996). Consequently, the effectiveness of any future heartwater vaccine will probably be dependent on the strains incorporated into the vaccine cocktail. Recently, a high prevalence of *E. ruminantium* pCS20-positives in DNA samples extracted from *A. variegatum* ticks (Esemu *et al*. 2013) was reported in Cameroon; however, information on the extent of genetic diversity among the *E. ruminantium* strains in Cameroon remains to be established. The present study, therefore, aimed to bridge this knowledge gap by genotyping the *E. ruminantium* strains detected in Cameroon in order to add to the epidemiological information currently available and provide data that may be useful for any future heartwater control programme. Analysis of the *map*1 gene was therefore the method of choice because other authors have reported its use in demonstrating genetic diversity within strains from Africa and the Caribbean (Allsopp *et al*. 1999; Allsopp *et al*. 2001; Faburay *et al*. 2008; Martinez *et al*. 2004).

The present study is the first report of genetic diversity among *E. ruminantium* strains in Cameroon. Out of the 156 *E. ruminantium* strains analysed in the present study, 121 (77.6%) gave specific positive amplicons of the *map*1 gene. The nested *map*1 PCR assay failed to amplify 35 (22.4%) of the pCS20 positive *E. ruminantium* strains. All of the *E. ruminantium* strains that had *map*1-negative results were associated with weak positive pCS20 signals. Within the limited geographical area considered in the present study, there were at least 21 genotypes at the nucleotide level and 16 genotypes at the amino acid level, indicating the presence of silent mutations within the *map*1 gene. Elsewhere, several strains of *E. ruminantium* have been reported in limited geographical areas (Faburay *et al*. 2008; Martinez *et al*. 2004).

Notable deletions and insertions were observed at three variable regions corresponding to the three variable regions delineated by Reddy *et al*. (1996). Also, numerous SNPs resulting in both synonymous and non-synonymous mutations were observed throughout the nucleotide sequence of all strains. The results of the present study are therefore in line with results reported elsewhere (Reddy *et al*. 1996).

The genotypes were compared with previously published *map*1 sequences deposited in GenBank. Interestingly, the Cameroonian strains had affinity with both African and Caribbean strains, forming several clusters with strains from Africa and the Caribbean. Even though the MAP1 protein is immunodominant, it does not stimulate a protective response in sheep and goats (Van Kleef, Neitz & De Waal 1993). In addition, if the protein did stimulate protection, it would be expected to be under selection pressure and this has been shown not to be the case (Allsopp *et al*. 2001). The reason for the observed genetic diversity of *map*1 is unknown, but it is most unlikely to be directly related to the ability of different strains to stimulate cross-protective immunity. The genetic diversity reported within the *map*1 gene can, nevertheless, be used as a marker for *E. ruminantium* strain differences. This great diversity may be as a result of more than one introduction of *E. ruminantium* in Cameroon from *E. ruminantium*-infected ticks and ruminants. At the amino acid level, six genotypes showed 100% identity with Antigua, two with Blaaukrans and two with UmBanein. This affinity may be due to multiple introductions of *E. ruminantium*-infected ticks and/or ruminants from other parts of the country, and even beyond, to the study sites.

The amino acid phylogram (Figure 2) indicated that there was no clustering in the *E. ruminantium* strains from Cameroon and the results from the present study support the report of Allsopp *et al*. (2001), which indicated clearly that there is no geographical distribution clustering among *map*1 variants.

Future studies with more strains from more locations in Cameroon may provide additional insight into the diversity of *E. ruminantium*. Understanding the diversity of *E. ruminantium* strains in *A. variegatum* ticks and cattle is essential for epidemiological and control purposes.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

S.N.E. (University of Buea) performed the experiments, participated in the sequence alignment and analysis, and drafted the manuscript. R.N.N. (University of Buea) conceived the study, provided materials for the molecular genetic studies, participated in the sequence alignment and analysis, and revised the manuscript. L.M.N. (University of Buea) as the principal investigator conceived, designed and coordinated the study, provided materials for the molecular genetic studies, participated in the sequence alignment and analysis, and revised the manuscript. All authors read and approved the final manuscript.

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