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Prevalence of *Ehrlichia ruminantium* in adult *Amblyomma variegatum* collected from cattle in Cameroon

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Abstract *Ehrlichia ruminantium*, the etiologic agent of the economically important disease heartwater, is an obligate intracellular bacterium transmitted by ticks of the genus *Amblyomma*, particularly *A. hebraeum* and *A. variegatum*. Although serologic and microscopic evidence of the presence of heartwater have been reported in ruminants in Cameroon, knowledge of *E. ruminantium* infection in the tick vector, *A. variegatum*, is lacking. In order to determine the infectivity of *A. variegatum* ticks by *E. ruminantium*, we analysed 500 un-engorged *A. variegatum* ticks collected by hand-picking from predilection sites from 182 cattle [115 ticks from 82 cattle at Société de Développement et d'Exploitation des Productions Animales (SODEPA) Dumbo ranch (SDR) and 385 ticks from 100 cattle at the Upper Farms ranch (UFR)] by amplification of the open reading frame (ORF) 2 of the pCS20 region of *E. ruminantium*. PCR amplification of the 279 bp fragment of the pCS20 region detected *E. ruminantium* DNA in 142 (28.4 %) of the 500 ticks with a higher infection rate (47/115; 40.9 %) observed in ticks from SDR and 24.7 % (95/385) of ticks collected from cattle at UFR. Twenty five randomly selected PCR products were sequenced and results indicated that some of the isolates shared homology with one another and to sequences of *E. ruminantium* in the GenBank. This report represents the first molecular evidence of *E. ruminantium* infection in *A. variegatum* ticks in Cameroon and suggests possible exposure of cattle to this pathogen in our environment.

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Introduction

Ehrlichia (previously *Cowdria*) *ruminantium* is an obligate intracellular tick-transmitted bacterium belonging to the rickettsial family Anaplasmataceace (Dumler et al. 2001). This bacterium causes heartwater (cowdriosis), a disease particularly important in cattle and small ruminants and transmitted by ticks of the genus *Amblyomma* (Uilenberg 1983). Heartwater causes considerable economic losses through death, decreased meat and milk production, unthriftiness, decreased draught power and manure, in addition to high cost of control measures (Faburay et al. 2008); it is also a major impediment to the introduction of more productive exotic breeds of ruminants in endemic areas.

The epidemiology and ecology of heartwater, including its effect on livestock in Cameroon is largely unknown. A previous serological survey suggesting endemic stability reported a mean prevalence of antibodies to *Cowdria* (*Ehrlichia*) *ruminantium* of 61–67 % and demonstrated the association of heartwater with the presence of *Amblyomma variegatum* (Awa 1997). Another study, which focused on post mortem findings and microscopy (Ndi et al. 1998), revealed a prevalence of 12.2 % while also suggesting that unreported sporadic deaths in the local ruminant populations with symptoms characteristic of heartwater were not uncommon (Ndi et al. 1998).

Amblyomma variegatum, has been consistently reported in Cameroon (Ndip et al. 2004; Ndi et al. 1998; Awa 1997; Merlin et al. 1986, 1987). Reports suggest that the tick also serves as the reservoir since the pathogen is maintained within stages trans-stadially. The humid tropical climate in Cameroon suggest that *A. variegatum* ticks could be prevalent all year round with adult populations peaking during the rainy season while the immature developmental stages are frequently encountered during the dry season (Awa 1997; Stachurski et al. 1993; Merlin et al. 1986). This suggests that heartwater transmission could be prevalent all year round.

Reports indicate that strains of *E. ruminantium*, show differences in pathogenicity (Allsopp et al. 2007), with genetic (Yu et al. 2007; Reddy et al. 1996) and antigenic (Perez et al. 1998; Uilenberg 1983) diversities in the isolates constituting a major constraint to the control of heartwater. Although reports indicate that a Cameroonian isolate of *E. ruminantium* has been characterized, data available is quite restrictive (Raliniaina et al. 2010; Allsopp et al. 2001; Perez et al. 1998) and the epidemiology and ecology of the pathogen, vector and exact distribution of the disease in Cameroon are still lacking. Detection of the strains of *E. ruminantium* infecting *Amblyomma* ticks could be important in developing and understanding the epidemiology of heartwater and providing data on strain diversity (Peter et al. 1999). Recently, the use of pCS20 PCR for the screening of ticks and animals for *E. ruminantium* has replaced older screening techniques (Van Heerden et al. 2004) and is now considered the most reliable and specific diagnostic test for *E. ruminantium* infections in ruminants and *Amblyomma* ticks (Faburay et al. 2007; Van Heerden et al. 2004; Simbi et al. 2003). Therefore the objective of this study was to determine the infectivity of *A. variegatum* ticks by *E. ruminantium* inorder to understand the probable role of *A. variegatum* in the transmission of *E. ruminantium*. This will extend our current knowledge on the ecology and epidemiology of *E. ruminantium* in Cameroon.

Materials and methods

Study sites

Cameroon is a sub-Saharan African country that lies in the intertropical zone (between longitudes 8° and 16° East and latitudes 1° and 13° North) with characteristic hot, moist and dry climatic conditions. The south has an equatorial climate up to latitude 6°N; while a tropical climate is observed between latitudes 6° and 13°N. There are two clear seasons: the dry season from November to March and the rainy season from April to October. For this study, two sampling sites were selected: the Upper Farms ranch (UFR) located in Buea (4°9′N, 9°13′E) with an altitude of 800 m above sea level and the SODEPA Dumbo ranch (SDR). UFR has an equatorial climate, with rainfall between 2,000 and 4,000 mm/year. The UFR is a small-scale ranch with less than 150 cattle which graze freely in the evergreen tropical rain forest. SDR is situated some 8 km west of Dumbo village (6°49′N, 10°25′E) in the Donga Mantung Division of the North West region of Cameroon. Rainfall varies between 1,700 and 3,000 mm. This area forms part of the most important grazing lands in Cameroon with an exploitable surface area of about 20,000 hectares. SDR is a large-scale commercial ranch with about 6,000 cattle. Cattle at SDR undergo acaricide treatment every 2 weeks whereas at UFR, tick control is rarely practiced (personal observation). SDR and UFR are separated by a distance of about 450 km (Fig. 1).

Tick population

Five hundred un-engorged ticks collected from 182 cattle by hand-picking between February and April 2010, were identified using basic taxonomic keys as adult *A. variegatum*. One hundred and fifteen (23 %) of these ticks were collected from 82 cattle at SDR while 385 (77 %) were collected from 100 cattle at UFR. These ticks were made up of 287 (57.4 %) males and 213 (42.6 %) females. Of the 287 males, 107 (37.3 %) were from SDR and 180 (62.7 %) from UFR. Similarly, of the 213 females, 8 (3.8 %) were from SDR and 205 (96.2 %) from UFR. *Amblyomma variegatum* ticks identified as fed or engorged were not included in the study (Table 1). Ticks were transported at room temperature to the Laboratory for Emerging Infectious Diseases, University of Buea, for identification and analysis. Ticks were surfaced sterilized by washing (3 times) in 70 % ethanol and stored at 4 °C until processing.

DNA extraction

All the 500 un-engorged ticks comprising 287 (57.4 %) males and 213 (42.6 %) females were processed for analysis. All ticks were rinsed 3 times with sterile phosphate-buffered saline (PBS) to remove residual ethanol and individually processed. DNA was extracted from whole ticks using the DNeasy Tissue Kit (Qiagen, Chatsworth, CA, USA) following the manufacturer's protocol for isolation of DNA from animal tissue. Measures to avoid cross contamination included using a new set of apparatus for maceration during the extraction process. An extraction control (sterile distilled water) was added with every batch of DNA extraction. Purified DNA was quantified using a digital spectrophotometer (SmartSpec^{TM} Plus, BIORAD) at 260 nm wavelength. To avoid DNA degradation due to frequent thawing and freezing, each DNA sample was stored in two aliquots; at −20 °C for short term and at −70 °C for long term storage.

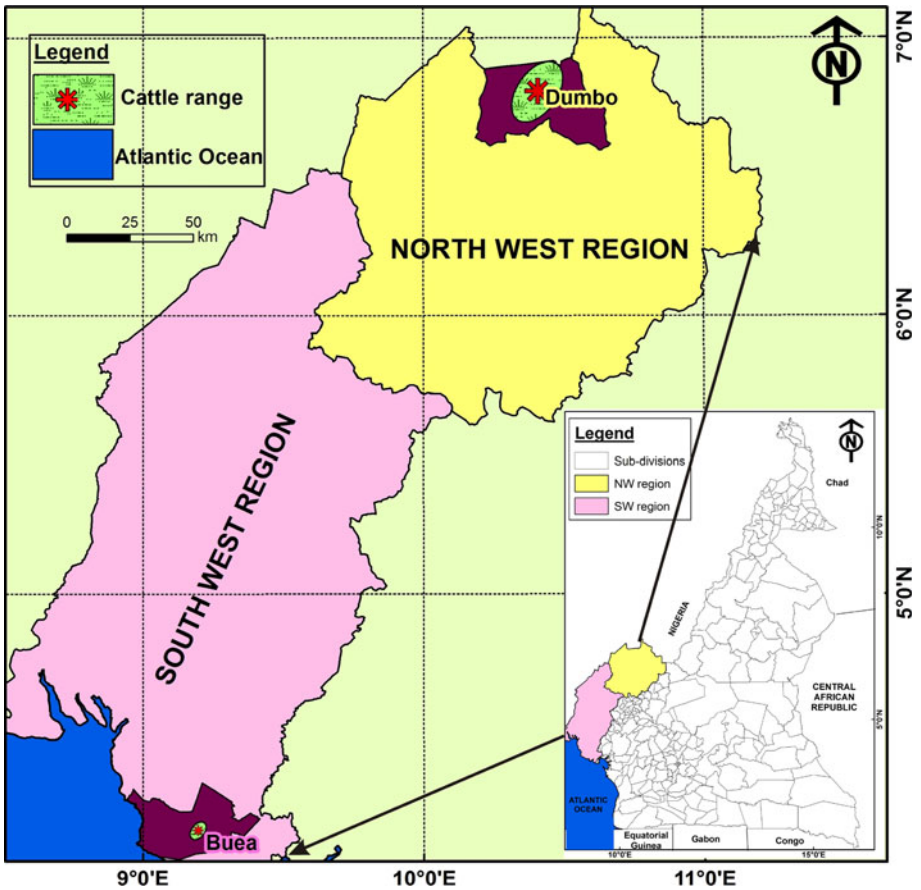

Fig. 1 Sites of *Amblyomma variegatum* tick collection in the north- and south-west regions of Cameroon

Table 1 Sampling frame showing study sites, number of cattle from which *Amblyomma variegatum* ticks were collected and total adult *A. variegatum* ticks collected

Site	Mean *A. variegatum* count/cattle	Cattle	Adult *A. variegatum* ticks				Total
			Male		Female		
			Un-engorged	Fed	Un-engorged	Engorged	
SDR	2	82	107	20	8	25	160
UFR	10	100	180	414	205	202	1,001
Total		182	287	434	213	227	1,161

pCS20 PCR

Semi nested pCS20 PCR was performed using the primer set ITM130 (TCAATTGCTTAATGAAGCACTAACTCAC) and AB129 (TGATAACTTGGTGCGGGAAATCCTT)

to amplify the open reading frame (ORF) 2 of unknown function of the 1,306 bp pCS20 sequence of *E. ruminantium* DNA as the first round PCR reaction while for the second round of PCR, the AB129 was still used as the reverse primer and AB128 (ACT-AGTAGAAATTGCACAATCTAT) was used as internal forward primer (Faburay et al. 2007). For the first round of PCR amplification, DNA (5 µl approximately 250 ng of DNA) from each tick was added to individual reactions (25 µl) comprising 12.5 µl PCR master mix (2X) (IQ™ Supermix, BIO-RAD, USA), 0.5 µl (from a working solution of 20 µM) of each primer giving a final concentration of 0.4 µM and 6.5 µl of DNase/RNase free distilled water (GIBCO®, Invitrogen™, USA). The thermal cycler (MyCycler™ Thermal Cycler BIORAD, USA) was set to give optimized conditions of initial denaturation for 3 min at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 62 °C, 30 s extension at 72 °C; with a final extension of 10 min at 72 °C; hold at 4 °C. For the second round of PCR amplification, aliquots of 1 µl of PCR product from the first round PCR amplification were used as DNA template. The cycling parameters consisted of 25 cycles of similar amplification conditions used above with a modification of the annealing temperature (58 °C). Two negative controls (extraction control and control using sterile distilled water as the template) were included with each run. DNA amplification was confirmed by separating the PCR amplicons using 1.5 % (w/v) agarose gel (Fisher Biotech, Australia). The gel was stained with ethidium bromide, viewed under ultraviolet light and photographed in a Molecular Imager Gel Doc XR system (BIO-RAD, Hercules, CA, USA).

Sequence and sequence analysis

To further confirm the *Ehrlichia* species, 25 positive PCR products (11 from UFR and 14 from SDR) were randomly selected and sequenced. In order to obtain maximum data accuracy, sequencing was done on both the forward and reverse strands using the AB128 and AB129 primer set. The BLAST program (National Center for Biotechnology Information, Bethesda, MD, USA) was used for sequence analysis.

Statistical significance

Tick infection rates by sex, site and ecological zone were compared by χ^2 analysis using SPSS version 19 (Chicago, IL, USA, 2007).

Results

Amblyomma variegatum tick infection by *Ehrlichia ruminantium*

Of the 500 un-engorged *A. variegatum* ticks tested by pCS20 nested PCR, 142 (28.4 %) were infected by *E. ruminantium*. Amplification was confirmed by the presence of 279 bp PCR product (Fig. 2). Of the 142 *A. variegatum* ticks positive for *E. ruminantium* infection (Table 2), there were more males (99, 69.7 %) than females (43, 30.3 %). There was a statistically significant difference ($\chi^2 = 12.31$, $P < 0.05$, $df = 1$) between *E. ruminantium* infection in male and female *A. variegatum* ticks. A higher rate of *E. ruminantium* infection was observed with ticks from UFR than with those from SDR.

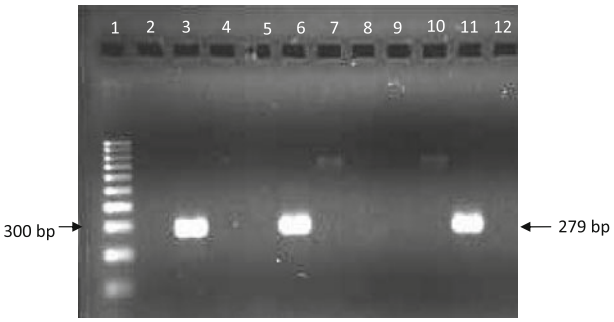

Fig. 2 Detection of amplified pCS20 PCR products. 1.5 % agarose gel electrophoretic separation of PCR products; 100 bp molecular weight marker (*lane 1*), negative controls (*lanes 2* and *12*), positive samples (*lanes 3*, *6* and *11*), and negative samples (*lanes 4, 5* and *7–10*)

Table 2 Complete results of nested pCS20 PCR for 500 un-engorged adult *Amblyomma variegatum* ticks collected from cattle in Cameroon

Site	Male (positive/ no. analysed)	Female (positive/ no. analysed)	Total (positive/ no. analysed)
SDR	42/107 (39.3 %)	5/8 (62.5 %)	47/115 (40.9 %)
UFR	57/180 (31.7 %)	38/205 (18.5 %)	95/385 (24.7 %)
Total	99/287 (34.5 %)	43/213 (20.2 %)	142/500 (28.4 %)

The 142 *E. ruminantium*-positive ticks were collected from 52 cattle (28.6 %, mean positive tick count/cattle of 2.7; range 1–19 positive ticks) from the two (SDR and UFR) study sites. Ninety five (95/500, 19.0 %) positive ticks from 21 cattle (mean positive tick count/cattle of 4.5; range 1–19 positive ticks) were recorded for UFR as against 47 (47/500, 9.4 %) positive ticks from 31 cattle (Mean positive tick count/cattle of 1.5; range 1–5 positive ticks) for SDR. As many as 19 (14 males and 5 females) positive ticks (out of the 22 analysed) were recorded from one cattle (UFR21C) from UFR while the highest number of positive ticks recorded from one cattle (SDR32C) in SDR, was 5 out of the 10 analysed. More cattle (31/182, 17.0 %) with *E. ruminantium* positive ticks were recorded from SDR than UFR (21/182, 11.5 %; Table 3).

There was a statistically significant difference ($\chi^2 = 11.42$, $P < 0.05$, $df = 1$) between ecological zones for the prevalence of *A. variegatum* tick infection by *E. ruminantium*, with respective values of 40.9 % (47/115) in SDR and 24.7 % (95/385) in UFR.

Nineteen cattle were infested with at least two *E. ruminantium* infected ticks each while the remaining 33 cattle were infested with one *E. ruminantium* infected tick each.

DNA sequence analysis

The DNA sequence identities between the 25 *E. ruminantium* isolates analysed in this study ranged from 98.3 to 100 %. Except for the DNA sequence of CMR Buea 34 which had 98.3 % identity with the other isolates from UFR, all DNA sequences from UFR had 100 % identity with each other and with 8 isolates from SDR. CMR Buea 34 has 98.3 % identity with the other isolates from UFR. The DNA sequences of 6 *E. ruminantium* isolates (CMR Dumbo 5, CMR Dumbo 7, CMR Dumbo 16, CMR Dumbo 17, CMR Dumbo 28 and CMR Dumbo 29) from SDR had 100 % homology with each other and with one isolate, CMR Buea 34, from UFR. The sequence differences within the genes of the isolates in this study, which did not share 100 % homology, were all substitutions at four nucleotide positions.

Table 3 Distribution of *Amblyomma variegatum* ticks with detectable *Ehrlichia ruminantium* DNA in the study sites, cattle and *E. ruminantium* isolate identities and GenBank accession numbers

Cattle identity	Study site	Un-engorged adult *A. variegatum* sampled	No. of ticks with *E. ruminantium* DNA	No. of positive samples sequenced	*E. ruminantium* isolate identity	GenBank accession no.
UFR8C	UFR	5	1			
UFR10C	UFR	2	1			
UFR12C	UFR	6	1			
UFR13C	UFR	11	6			
UFR14C	UFR	24	12	1	CMR Buea 20	JQ039914
UFR21C	UFR	22	19	1	CMR Buea 34	JQ039915
UFR28C	UFR	3	1			
UFR30C	UFR	6	3	1	CMR Buea 38	JQ039916
UFR49C	UFR	15	10	1	CMR Buea 46	JQ039917
UFR54C	UFR	4	1			
UFR55C	UFR	10	5	1	CMR Buea 47	JQ039918
UFR61C	UFR	6	6	1	CMR Buea 48	JQ039919
UFR62C	UFR	4	1			
UFR63C	UFR	4	1			
UFR71C	UFR	5	1			
UFR75C	UFR	2	1			
UFR82C	UFR	14	9	1	CMR Buea 49	JQ039920
UFR85C	UFR	13	10	1	CMR Buea 52	JQ039921
UFR90C	UFR	4	4	1	CMR Buea 54	JQ039922
UFR92C	UFR	3	1	1	CMR Buea 55	JQ039923
UFR93C	UFR	1	1	1	CMR Buea 57	JQ039924
SDR8712C	SDR	1	1			
SDR32C	SDR	5	3	1	CMR Dumbo 5	JQ039925
SDR78C	SDR	2	1			
SDR203C	SDR	2	2	1	CMR Dumbo 7	JQ039926
SDR81C	SDR	1	1			
SDR244C	SDR	2	2	1	CMR Dumbo 10	JQ039927
SDR70C	SDR	1	1	1	CMR Dumbo 16	JQ039928
SDR27C	SDR	5	1	1	CMR Dumbo 17	JQ039929
SDR8594C	SDR	3	1	1	CMR Dumbo 20	JQ039930
SDR005C	SDR	4	3	1	CMR Dumbo 22	JQ039931
SDR91C	SDR	2	1			
SDR11C	SDR	1	1	1	CMR Dumbo 24	JQ039932
SDR226C	SDR	2	1	1	CMR Dumbo 25	JQ039933
SDR04C	SDR	2	2			
SDR8405C	SDR	2	2	1	CMR Dumbo 26	JQ039934
SDR218C	SDR	1	1	1	CMR Dumbo 28	JQ039935
SDR42C	SDR	1	1			
SDR217C	SDR	5	3			
SDR217**C	SDR	4	3	1	CMR Dumbo 29	JQ039936

Table 3 continued

Cattle identity	Study site	Un-engorged adult *A. variegatum* sampled	No. of ticks with *E. ruminantium* DNA	No. of positive samples sequenced	*E. ruminantium* isolate identity	GenBank accession no.
SDR119C	SDR	1	1			
SDR72C	SDR	1	1			
SDR004C	SDR	1	1			
SDR006C	SDR	1	1	1	CMR Dumbo 31	JQ039937
SDR8609C	SDR	1	1			
SDR8445C	SDR	1	1			
SDR132C	SDR	10	5	1	CMR Dumbo 33	JQ039938
SDR143**C	SDR	1	1			
SDR90C	SDR	3	1			
SDR142C	SDR	1	1			
SDR108C	SDR	1	1			
SDR65C	SDR	2	1			
Total			142	25		

The gene sequence identities between these Cameroon isolates of *E. ruminantium* and some known *E. ruminantium* sequences deposited in the GenBank varied from 97.4 to 99.6 %. CMR Buea 34 isolate had 97.4 % identity with Kiswani (DQ655712), 97.8 % with Ball3 (AY236059) and Welgevonden (AY236058), and 99.1 % with Senegal (AY236066). Likewise, CMR Buea 20 isolate had 97.4 % identity with Senegal, 99.1 % with Kiswani and 99.6 % with Ball3 and Welgevonden.

The sequences of the *E. ruminantium* ORF2 of pCS20 region deposited in the GenBank were given accession numbers JQ039914 to JQ039938 (Table 3).

Discussion

Ehrlichia ruminantium has been reported to cause significant economic losses to the livestock sector in sub-Saharan Africa, especially in small ruminants and has significantly hindered the establishment of exotic cattle breeds. Amongst the several *Amblyomma* ticks known to transmit the pathogen, only *A. variegatum* has so far been reported in Cameroon (Ndi et al.1998). This study provides the first molecular evidence of *E. ruminantium* infection in *A. variegatum* ticks in Cameroon and serves as an indication of its possible presence in the resident ruminant livestock populations.

Many methods have been employed for detection of *E. ruminantium* in cattle and ticks, these include the reverse line blot assay, the *map1*, 16S DNA and nested pCS20 PCR assays (Faburay et al. 2007; Bekker et al. 2002). In this study, we employed the nested pCS20 PCR assay due to its reported higher sensitivity (Martinez et al. 2004) and high degree of sequence conservation (Allsopp et al. 2007; Faburay et al. 2007; Peter et al. 2000). Based on this assay, *E. ruminantium* DNA was detected in 28.4 % of the *A. variegatum* ticks analysed.

Considering that this is the first study to detect *E. ruminantium* in Cameroonian ticks, we sequenced 25 (11 from UFR and 14 from SDR) of the 142 *E. ruminantium* amplicons

obtained (Table 3) in order to characterize the strains of *E. ruminantium* circulating in Cameroon. Sequencing results and sequence comparison with known sequences in the GenBank database indicated that these strains exhibit a high degree of similarity among themselves and with other strains circulating in Africa and the Caribbean. This high degree of homology could be due to the fact that the pCS20 region of *E. ruminantium* is a conserved region and is not a good indicator of diversity within the species.

The humid climate at UFR (rainfall between 2,000 and 4,000 mm/year) which favours tick habitation may explain the reason why more cattle at UFR were infested with ticks when compared to those at the SDR. However, this observed higher degree of infestation was not related to infection in ticks. While at SDR, the infection rate was as high as 40 %, only 24 % of ticks from UFR were infected with *E. ruminantium*. This suggests that although the tick control measure at SDR is sufficient to keep small numbers of ticks on these animals, it probably does not have any influence on the levels of infectivity of the ticks since the 2 weeks interval between dips may allow for re-infestation with infected ticks from vegetation or sufficient for an un-infected re-infesting tick to attach and become infected during feeding. In addition, in contrast to the UFR where restricted on-farm cattle grazing is practiced, SDR practices extensive grazing on vegetation which exposes cattle to *A. variegatum* infested mongooses, birds etc. (Barré et al. 1988). Also, due to the high number of herds, it is difficult to carry out simultaneous acaricide treatment; therefore, there is a high possibility of herd mixing which facilitates tick attachment, feeding and introduction of tick borne pathogens such as *E. ruminantium*. The use of acaricides weekly and the simultaneous treatment of herds or large blocks of adjacent herds of cattle and releasing them at the same time may significantly reduce the tick re-infestation rate and subsequently tick infection.

Of the 142 *A. variegatum* ticks positive for *E. ruminantium* infection, more males (99, 69.7 %) than females (43, 30.3 %) were infected (Table 2). Higher *E. ruminantium* infection rates in *A. variegatum* males than females have been reported elsewhere (Vachiery et al. 2008; Faburay et al. 2007; Peter et al. 2000). This observation was attributed to the fact that male ticks may have more exposure to *E. ruminantium* infection due to intrastadial and prolonged feeding without losing infection during feeding (Jordaan and Baker 1981). This is in contrast to female ticks who only attach for 1–2 days prior to the start of engorgement thereby limiting their exposure to infection (Jordaan and Baker 1981). In this light, some authors have postulated that the infection rates observed in the female *A. variegatum* ticks is a representative of the real picture of *E. ruminantium* infection in cattle because their short attachment period prior to the start of engorgement limits their exposure to re-infection from the cattle (Jordaan and Baker 1981).

This study suggests a high infestation of cattle with *E. ruminantium* infected *A. variegatum*, and highlights the risk of heartwater disease for susceptible livestock in the resident ruminant population. From these results, one would expect a high number of clinical cases of heartwater in the resident ruminant population; however, fewer cases were reported (personal communication). Therefore, the high seroprevalence rates previously reported in Cameroon in addition to our current findings of high *E. ruminantium* in ticks against the background of low clinical cases suggest endemic stability against heartwater.

This study was limited in coverage and may not give a true indication of the distribution of *E. ruminantium* in *A. variegatum* ticks in Cameroon. Therefore, it necessitates an assessment of a country wide survey to understand the epidemiology of this important veterinary pathogen. This will be an important step towards setting up feasible control programs. Future directives could also be aimed at understanding the susceptibility of small ruminants from non-endemic areas to heartwater and determining the extent and role

of these infections in the resident populations. This will provide an insight in the formulation of disease control policies thus creating a stronger basis for the development of well-targeted control measures for upgrading the productivity of the livestock industry which can be applicable in other countries in the central African sub-region due to the several ecological zones in Cameroon that cut across the region.

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