OPEN ACCESS International Journal of Applied Biology

ISSN : 2580-2410
eISSN : 2580-2119

Molecular characterization of *Anaplasma* and *Ehrlichia* microorganisms in bovine populations of the Western Highland Agro-Ecological Zone of Cameroon

Ngangnang Ghislain Roméo [1]*, Aktas Münir [2], Ulucesme Mehmet Can [2], Keptcheu Tchankwe Désiré [1] , Fonteh Anyangwe Florence[3], Vincent Khan Payne[1]

[1] Department of Animal Biology, Faculty of Science, University of Dschang, Dschang, Cameroon.
[2] Department of Parasitology, Faculty of Veterinary Medicine, University of Firät, Elazig, Turkey.
[3] Department of Animal Production, Faculty of Agronomy and Agricultural Sciences, University of Dschang, Dschang, Cameroon.

Abstract

Rickettsial bacteria are important tick-transmitted microorganisms causing disease and death in cattle, sheep, goats and dogs in the area where tick vectors are found, becoming a major problem for improvement of animal production in the endemic areas. The study carried out in the Western Highlands of Cameroon was aimed at highlighting *Anaplasma* and *Ehrlichia* species in apparently healthy cattle. A total number of 162 blood samples were collected from cattle and screened via nested-PCR based Reverse Line Blot hybridization (RLB) assay for detection of rickettsial bacteria. Four species of these microorganisms were identified with an overall prevalence of 44.44%, *Anaplasma marginale* (41.35%) being the most prevalent species followed by *Anaplasma* sp. '*Omatjenne*' (15.43%), *Anaplasma centrale* (8.64%) and *Ehrlichia* ruminantium (3.08%). Single infection (24.69%) was more frequent among the four types of mix infection observed with a significant difference. Parasite association was most found between A. *marginale* + *Anaplasma* sp. 'Omatjenne' (11.11%). Female cattle (44.79%) were more infected than males3.93%) but without significant difference while, yearling cattle (50%) were statistically more infected than adults (44.07%). The high prevalence and diversity of rickettsial organisms identified is evidence that disease and their vectors, the *Amblyomma* and *Rhipicephalus* (formerly *Boophilus*) ticks might be widespread in the Western Highlands of Cameroon. However, these findings with veterinary significance suggest the dire need for further research on the presence of other vectors apart from *Amblyomma* sp. and *Rhipicephalus* sp. in Cameroon.

Article History
Received 11 November 2021
Accepted 30 December 2021

Keyword
Anaplasma; Ehrlichia; rickettsial bacteria; Molecular characterization; RLB; Western Highlands of Cameroon

CONTACT : NGANGNANG GHISLAIN ROMÉO ngaghirom1@yahoo.fr

Introduction

Rickettsial diseases of economic importance are ehrlichiosis and anaplasmosis, a tick-borne disease caused by obligate intracellular bacteria in the genera *Ehrlichia* and *Anaplasma* respectively. They are emerging tick-borne pathogens in humans and other wild or domesticated animals worldwide. Infections caused by these pathogens are deadly if left untreated (Iweriebor *et al.,* 2017). These organisms are widespread in nature and are usually maintained in cycles between ticks and reservoir hosts, which can sometimes remain infected for long periods. For many years, *Ehrlichia* and *Anaplasma* species have been known to cause illness in pets and livestock where the consequences of exposure vary from asymptomatic infections to severe, potentially fatal illness.

Throughout the tropics, an estimated 600 million cattle are exposed to anaplasmosis and babesiosis. In the 1970s, McCallon, (1973) estimated that the disease caused annual losses of over 300 million US dollars to the American cattle industry. In 1989, over a million cattle in eleven countries of Eastern, Central and Southern Africa were estimated to have died of tick-borne diseases. The economic cost in livestock losses and funding for control and research programs was estimated at US$168 million that year **(**ILRAD, 1991). Furthermore, Mukhebi *et al*., (1999) estimated that the national annual loss due to cowdriosis in Zimbabwe could attain 5.6 million USD and more recently, Tanzania was estimated to lose 47.3 million USD solely due to the direct costs of bovine anaplasmosis (Kivaria, 2006; Kenneil, 2015). The distribution of these diseases follows the presence of the vector *Amblyomma* among them *Amblyomma variegatum* is the most important species which is widely distributed in the sub-Saharan Africa including Cameroon. The control of disease involves controlling the tick vector, establishing endemic stability, performing immunization by infection and treatment, and preventing the disease by regular administration of prophylactic antibiotics. Most of these methods are subject to failure for various epidemiological reasons, and serious disease outbreaks could occur (Dinkisa, 2018). A relative little information is available about these rickettsial bacteria in Cameroon and need to be updated. In the current study, a reverse line blot assay (RLB) was performed in order to identify *Anaplasma* and *Ehrlichia* species circulating amongst cattle in the third agro-ecological zone of Cameroon.

Materials and Methods

Study Area

The Region considered as the Western Highlands is the third Agro-Ecological Zone (AEZ) of Cameroon (IRAD, 2008). It comprises the two Administrative Regions of West and North West, due to their common biotic and abiotic characteristics. It lies between Latitudes 5° and 7° North and Longitude 9° and 11° East of the Equator. With a size of 31,180 km^2, they cover 1/16 of the total land area of the country. Altitudes range from around 300 to 3 000 m above sea level. The climate of this region is the tropical humid type with two seasons, the dry and rainy seasons. Rainfall varies between 1300-3000 mm with peaks occurring between mid-July and mid-September. The rainy season extends from mid-March to mid-November while the dry season runs from end of mid-November to mid-March. The maximum temperatures vary between 20 and 32°C. The dominant vegetation is residual savannah and the region is designated grassland because a greater proportion of the area is covered by grassland than forest. This Region is characterized by a rapid population growth (128.5 inhabitants per km^2), most of whom live in rural areas (67.8%) and depend on crop

and livestock activities. It is the third major cattle producing area, with 500,000 Zebu cattle, and one of the most important agricultural production zones of the country **(**IRAD, 2008; Nchinda and Mendi, 2008; Jiotsa *et al.,* 2016).

Collection of the samples

Between March 2019 and January 2021, one hundred and sixty-two (162) zebu cattle (*Bos indicus*), mainly the local breed (Aku, Gudali and M'bororo) commonly found in the Western Highlands of Cameroon were sampled according to their age and sex for blood sampling. This target population was in extensive management with no or adequate tick control program implemented. Five ml of blood samples were collected from jugular or coccygeal vein of cattle into EDTA tubes, preferably potassium–ethylenediamine tetra-acetic acid (EDTA/K3) with a concentration of 1.27mg EDTA/K3 per ml of blood and into Dried Blood Spot (DBS) specimen collection cards prepared for the purpose. Simultaneous detection of rickettsial bacteria in the blood samples was done using nested-PCR based RLB hybridization assay in the Laboratory of Molecular Parasitology, Department of Parasitology, Faculty of Veterinary Medicine, University of Firät, Elazig, Turkey.

DNA extraction and PCR

DNA was extracted by a commercial DNA isolation kit (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. Then, for the amplification of *Anaplasma/Ehrlichia* spp., a nested PCR was performed using two universal primer pairs. The primers EC9/EC12A were used for the first round PCR amplification of 1462 bp fragments of the 16S rRNA gene of *Anaplasma*/*Ehrlichia* spp. The nested amplification, using the primers 16S8FE/BGA1B, produced a 492–498 bp fragment in the hypervariable V1 region of the 16S rRNA gene of the *Anaplasma*/*Ehrlichia* species. For the second amplification, one µl of first round PCR products was used as a DNA template. To reduce non-specific amplification, a touchdown program was performed. Touchdown PCR involves the use of an annealing temperature that is higher than the target optimum in early PCR cycles.

RLB hybridization

Probes of catchall, genus and species-specific for *Anaplasma/Ehrlichia* were used with a range of 200–800 pmol/150µl concentration and contain N-terminal N-(trifluoracetamidohexyl-cyanoethyl,N,N-diisopropyl phosphoramidite [TFA])-C6 amino linker in the study. The oligonucleotide probes were synthesised by The Midland Certified Reagent (Midland, Texas, USA). Preparation, hybridisation and stripping of the RLB membrane were performed as previously described with minor modifications (Georges *et al.,* 2001).

Table 1. Oligonucleotide primers and probes used in this study

Primer	Sequence (5'-3')	Reference
EC9	TACCTTGTTACGACTT	Kawahara *et al.,* 2006
EC12A	TGATCCTGGCTCAGAACGAACG	Kawahara *et al.,* 2006
16S8FE	GGAATTCAGAGTTGGATCM*TGGYTCAG	Schouls *et al.,* 1999
B-GA1B	biotin-CGGGATCCCGAGTTTGCCGGGACTTCTTCT	Schouls *et al.,* 1999
Probe	Modification (5'-3')	Reference

Anaplasma/Ehrlichia catch-all	C6 amino-GGG GGA AAG ATT TAT CGC TA	Bekker *et al.,* 2002
A. marginale	C6 amino-GAC CGT ATA CGC AGC TTG	Bekker *et al.,* 2002
A. centrale	C6 amino TCG AAC GGA CCA TAC GC	Bekker *et al.,* 2002
A. bovis	C6 amino-GTA GCT TGC TAT GRG AAC A	Bekker *et al.,* 2002
E. ruminantium	C6 amino-AGT ATC TGT TAG TGG CAG	Bekker *et al.,* 2002
Ehrlichia sp. 'Omatjenne'	C6 amino-CGG GTT TTT ATC ATA GCT TGC	Bekker *et al.,* 2002
A. phagocytophilum group	C6 aminoTTG CTA TRR AGA ATA RTT AGT GG	Bekker *et al.,* 2002
A. phagocytophilum 1	C6 amino-TTGCTATAAAGAATAATTAGTGG	Schouls *et al.,* 1999
A. phagocytophilum 3	C6 amino-TTGCTATGAAGAATAATTAGTGG	Schouls *et al.,* 1999
A. phagocytophilum 5	C6 amino-TTGCTATAAAGAATAGTTAGTGG	Schouls *et al.,* 1999
A. phagocytophilum 7	C6 amino-TTGCTATAGAGAATAGTTAGTGG	Schouls *et al.,* 1999
A. phagocytophilum A-HE	C6 amino-GCTATAAAGAATAGTTAGTGG	Schouls *et al.,* 1999
A. phagocytophilum A-D- HE	C6 amino-GCTATGAAGAATAGTTAGTG	Schouls *et al.,* 1999

Statistical analysis

Statistical calculations were performed using SPSS V. 23 software and Chi-square tests was used to statistically compare different prevalence of infection.

Result

A total number of 162 cattle blood samples were screened for detection of rickettsial organisms. Seventy-two (72) were found positive for the presence of 16S rRNA gene of *Anaplasma* and *Ehrlichia* species. We then identified after examination these blood samples of two genera of rickettsial bacteria such as *Anaplasma* sp. and *Ehrlichia* sp. (Figure 1).

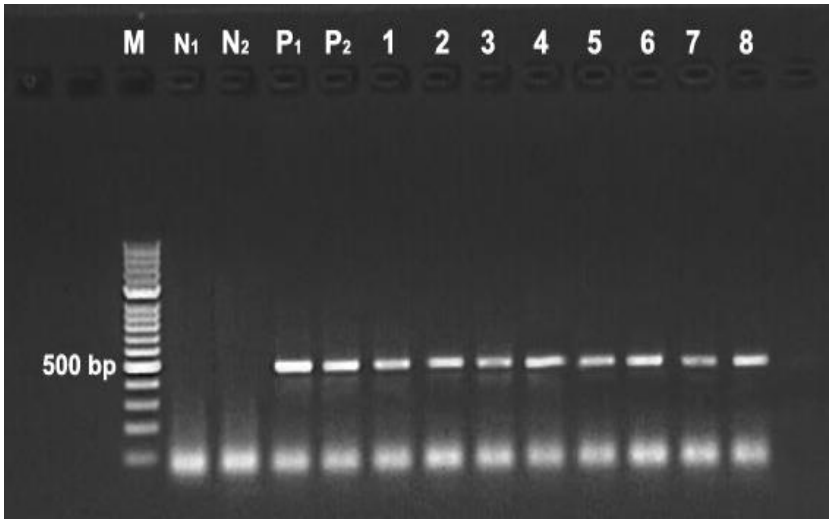

Figure 1. Gel electrophoresis of PCR product of *Anaplasma* and *Ehrlichia* species

The detection of 16S rRNA gene of *Anaplasma* and *Ehrlichia* species is done by nested PCR using genus-specific primers 16S8FE/B-GA1B. **M:** 500 bp ladder; **N1-N2:** standard negative controls (N1, DNA isolated from uninfected cow blood; N2, Sterile deionized water); **P1-P2:** positive controls (P1, *Anaplasma marginale*; P2, *Anaplasma phagocytophilum*). Lanes **1-8:** positive field samples signalling *Anaplasma/Ehrlichia* catchall probe in the RLB.

After confirmation of the presence of 18S rRNA gene of *Anaplasma* and *Ehrlichia* species in the blood samples, RLB was performed to identify these parasites at the level of species and so, the following four of them were incriminated: *Anaplasma marginale*, *Anaplasma centrale, Anaplasma* sp. 'Omatjenne' and *Ehrlichia ruminantium* (Figure 2).

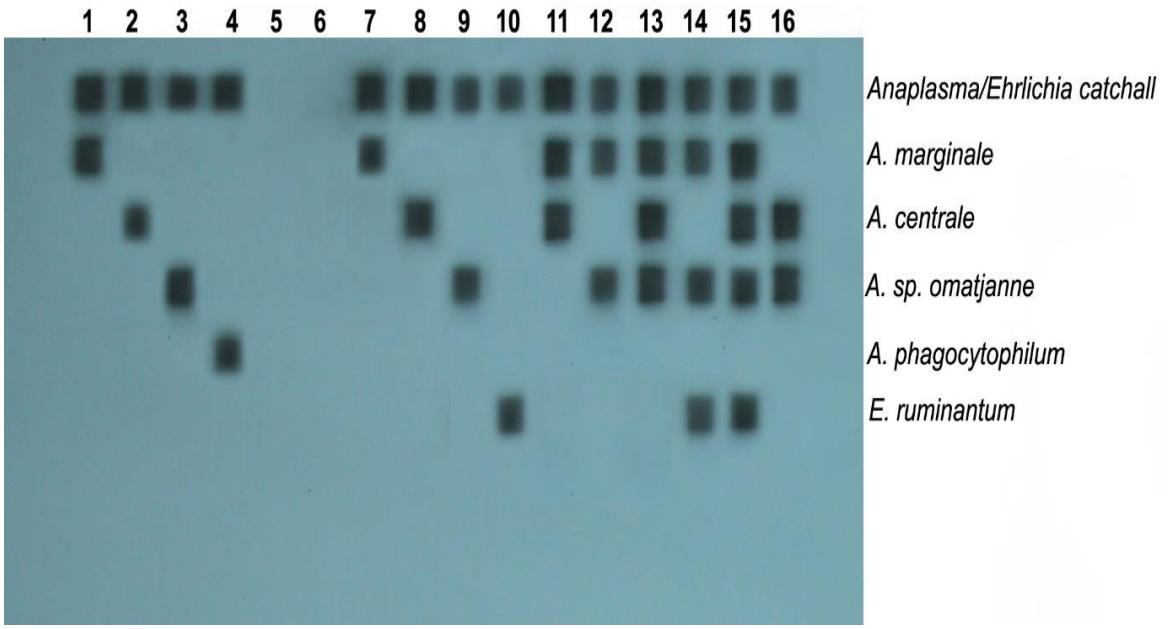

Figure 2. Detection of *Anaplasma* and *Ehrlichia* species by RLB

Oligonucleotide probes were applied in columns and PCR products in rows. Lanes **1-4:** Positive controls (1, *A. marginale*; 2, *A. centrale*; 3, *Anaplasma* sp. 'Omatjenne'; 4, *A.*

phagocytophilum. Lanes **5-6:** negative controls (5, DNA isolated from uninfected cow blood; 6, sterile deionized water). Lanes **7-16:** field samples (single and mixed infection) (7, *A. marginale*; 8, *A. centrale*; 9, *Anaplasma* sp. 'Omatjenne'; 10, *E. ruminantium*; 11, *A. marginale* + *A. centrale*; 12, *A. marginale* + *Anaplasma* sp. 'Omatjenne'; 13, *A. marginale* + *A. centrale* + *Anaplasma* sp. 'Omatjenne'; 14, *A. marginale* + *Anaplasma* sp. 'Omatjenne' + *E. ruminantium*; 15, *A. marginale* + *A. centrale* + *Anaplasma* sp. 'Omatjenne' + *E. ruminantium*; 16, *A. centrale* + *E. ruminantium.*

The overall prevalence of infection in cattle by these rickettsial bacteria was important and assessed to 44.44%. We also noticed that, female cattle (44.79%) were more infected than males (43.93%) with no significant difference while according to age, prevalence of infection was significantly different between yearling (50%) and adult cattle (44.07%) (Table 2).

Table 2. Overall prevalence of infection of rickettsial bacteria in the study area

	Number of cattle blood		Prevalence (%)
	Examined	Infected	
Age	$\chi^2 = 51.681; df = 1; P < 0.0001$		
Yearling	10	5	50
Adult	152	67	44.07
Sex	$\chi^2 = 2.347; df = 1; P = 0.1255$		
Male	66	29	43.93
Female	96	43	44.79
Total	162	72	44.44

We noted that four species of rickettsial bacteria: *Anaplasma marginale, Anaplasma centrale, Anaplasma* sp. 'Omatjenne' and *Ehrlichia ruminantium* were identified. The most prevalent parasite was *A. marginale* (41.35%), followed by *Anaplasma* sp. 'Omatjenne' (15.43%), *A. centrale* (8.64%) and *E. ruminantium* (3.08%). There was a significant difference between the prevalence of infection between the species identified (Table 3).

Table 3. Prevalence of each rickettsial bacteria identified in cattle blood

		Number of cattle blood Examined	Infected	Prevalence (%)
Rickettsial bacteria	$\chi^2 = 81.252; df = 3; P < 0.0001$			
Anaplasma marginale		162	67	41.35
Anaplasma sp. 'Omatjenne'			25	15.43
Anaplasma centrale			14	8.64
Ehrlichia ruminantium			5	3.08

Several types of co-infections were observed following blood examination. We noted four different types of multiple infection and classified as single, double, triple and

quadruple infections with a prevalence statistically different. We found that, the most prevalent was single infection (24.69%) followed by double infection (16.67%) while triple (1.85%) and quadruple infections (1.23%) were less prevalent. Summarily, difference was not significant between the prevalence of single (24.69%) and whole mixed infection (19.75%) (Table 4).

Table 4. Prevalence of co-infection in the study area

	Single infection		Double infection		Triple infection		Quadruple infection		Total	
	No	(%)	No	(%)	No	(%)	No	(%)	No	(%)
Age										
Yearling	3	30	2	20					5	50
Adults	37	24.34	25	16.44	3	1.97	2	1.32	67	44.07
Sex										
Male	18	27.27	9	13.63			2	3.03	29	43.93
Female	22	22.91	18	18.75	3	3.12			43	44.79
$\chi^2 = 58.111; df = 3; P < 0.0001$										
Total	40	24.69	27	16.67	3	1.85	2	1.23	72	44.44

	Co-infection	
	Single infection	Mixed infection
Frequency	40	32
Prevalence (%)	24.69	19.75
$\chi^2 = 0.681; df = 1; P = 0.409$		

Considering the single infection (Table 5) of these rickettsial infections of cattle blood, we found that the most prevalent parasite was *Anaplasma marginale* (22.22%), while the most prevalent mixed infection was the double infection (16.66%) with the association between *A. marginale + A.* sp. 'Omatjenne' (11.11%).

Table 5. Prevalence of rickettsial bacteria association in infected cattle

	Age				Sex				Total	
	Yearling		Adults		Male		Female		Study area	
	No	(%)	No	(%)	No	(%)	No	(%)	No	(%)
Rickettsial bacteria										
Anaplasma marginale	3	30	33	21.71	16	24.24	20	20.83	36	22.22
Anaplasma centrale			2	1.31	1	1.52	1	1.04	2	1.23
Anaplasma sp. 'Omatjenne'			1	0.66			1	1.04	1	0.62
Ehrlichia ruminantium			1	0.66	1	1.52			1	0.62
A. marginale + A. centrale			8	5.27	3	4.54	5	5.21	8	4.94
A. marginale + A. sp. 'Omatjenne'	2	20	16	10.52	6	9.09	12	12.5	18	11.11
A. centrale + A. sp. 'Omatjenne'			1	0.66			1	1.04	1	0.62
A. marginale + A. centrale + A. sp. 'Omatjenne'			1	0.66			1	1.04	1	0.62
A. marginale + A. sp. 'Omatjenne' + *E. ruminantium*			2	1.31			2	2.09	2	1.23
A. marginale+ A. centrale + A. sp. 'Omatjenne' + *E. ruminantium*			2	1.31	2	3.03			2	1.23
Total	5	50	67	44.07	29	43.94	43	44.79	72	44.44

Discussion

The reverse line blot hybridization was performed to specifically identify simultaneously several species of rickettsial bacteria (*Anaplasma* and *Ehrlichia* species). Of the 162 blood samples screened, 77 were found positive for at least one rickettsial bacteria. The overall prevalence of infection was 44.44%. This result was similar to 40.76% and 41% reported by Hailemariam *et al.,* (2017) in Ethiopia and Nguyen *et al.,* (2020) in Thailand respectively. However, it was highest compared to 9%, 5.3% and 7.1% reported respectively by Aktas *et al.,* (2010) in Turkey, Parvizi *et al.,* (2019) in Egypt and Zaid *et al.,* (2019) in Palestine. Furthermore, this prevalence was lower than the 76.1% found in Northern Cameroon by Abanda *et al.,* (2019). The important prevalence of infection of rickettsial bacteria observed might be associated to the presence of its main vectors, the *Amblyomma* and *Rhipicephalus* ticks (Ngangnang *et al.,* 2021). However, *Hyalomma* and *Haemaphysalis* tick species are also considered as potential vectors (Latif and Walker, 2004; Lankester *et al.,* 2007) and were identified in the study area (Ngangnang *et al.,* 2021). According to this finding, we could conclude that pathogens and vector might be widespread and well established in the Western Highlands of Cameroon and need a great attention for medical and veterinary concern. It had also been noticed that, female cattle (44.79%) were most infected than male (43.93%) but the difference was not statistically significant as found by Nguyen *et al.,* (2020) while, the infection was associated to sex as reported Nyabongo *et al.,* (2021) in Uganda. According to Nyabongo *et al.,* (2021), this risk of infection could be explained by the higher number of female cattle sampled compared to male in the study population. Moreover, male cattle are provided with better health care due to their higher value, as they are used by farmers for reproduction and sold for meat, whereas females are kept for dairy. The prevalence of infection was high and significantly different between yearling (50%) and adult cattle (44.07%). Similarly, Nyabongo *et al.,* (2021) report indicated the same observation while it was different from the finding of Lorusso *et al.,* (2016) in Nigeria. This study showed that yearling cattle had a higher chance of being infected compared to adults. Adult cattle that were infected as calves are resistant to re-infection, which could explain the high risk of infection for calves or yearling compared to adult animals.

Of the 162 cattle blood samples tested using nested PCR-based RLB hybridization assay for detection of rickettsial bacteria, *A. marginale, Anaplasma* sp. 'Omatjenne', *A. centrale* and *E. ruminantium* were identified. The most prevalent rickettsial bacteria identified in this study was *A. marginale* (41.35%) followed by *Anaplasma* sp. 'Omatjenne' (15.43%), *A. centrale* (8.64%) and *E. ruminantium* (3.08%). This finding was in agreement with the report of Lorusso *et al.,* (2016) in Nigeria although they found the prevalence in different proportion and might be due to the sample size or the epizootiological situation of disease in each study site. However, the result contrast the previous report of Eygelaar *et al.,* (2015) in Botswana and Teshale *et al.,* (2018) in Ethiopia who reported respectively *Anaplasma centrale* and *Anaplasma sp. '*Omatjenne' as the most prevalent species. This contrast might be difference among the target population (Buffalo and Cattle) during each study site and even the epizootiology of vectors.

Several categories of co-infection were observed and the most prevalent was the single infection (24.69%) followed by double (16.67%), triple (1.85%) and quadruple infection (1.23%). The single infection (24.69%) was most prevalent than the total mixed infection (19.75%) with no significant difference. These results were different from those of

Hailemariam *et al.,* (2017) and Nyabongo *et al.,* (2021) and could indicate the severity of rickettsial infection in cattle in the given study area.

Conclusion

There was a high level of prevalence and species composition of rickettsial bacteria in the study area. This prevalence could be associated to the previous identification of *Amblyomma* and *Rhipicephalus* ticks in the study area. Likewise, the current description of biological transmission of *A. marginale* by *Rhipicephalus microplus* ticks such as biological intrastadial and transstadial transmission could affect the persistence of rickettsial bacteria. Better prevention and control methods of these microorganisms could be development of new vector control strategies.

Authorization

This study including cattle was authorized by the Regional Delegate for Livestock, Fisheries and Animal Industries of the West Region of Cameroon (Authorization N° 02/19/L/DREPIA-O/SRAG).

Author's contribution

Ngangnang Ghislain Roméo conceived the idea of the study, wrote the research proposal, gathered the data, analysed and interpreted the data, prepared the manuscript, searched the literature and finalized the study.

Vincent Khan Payne and Fonteh Anyangwe Florence proposed the study, analysed and interpreted the data, revised and approved the final version of the manuscript.

Aktas Munir and Ulucesme Mehmet Can designed the methodology, extracted the DNA and performed PCR and RLB assay and revised the final version of the manuscript on molecular biology.

Keptcheu Tchankwe Désiré Léonard gathered and analysed the data.

Conflict of interest

The authors declare that they have no competing interest on this study.

Funding

Not applicable

Acknowledgement

The authors acknowledge farmers for agreeing to participate and consent to collect ectoparasites and blood samples on cattle. We are also grateful to the Department of Parasitology, Faculty of Veterinary Medicine, University of Firät, Elazig, Turkey for providing laboratory space and reagents necessary for accomplishment of this work.

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