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Original Article

First report of *Theileria buffeli/Theileria orientalis* group and identification of piroplasms via Nested PCR-based RLB Hybridization assay in zebu cattle in the Western Highlands of Cameroon

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Summary
Piroplasms infections are tick-borne diseases caused by haemoparasite of the genus *Theileria* or *Babesia*. They have a great impact on livestock production, especially cattle in sub-Saharan countries. However, data on the prevalence of bovine piroplasms and their genetic diversity are scanty in Cameroon. This study was aimed at highlight the species composition and determine the prevalence of piroplasms infecting cattle in the Western Highlands of Cameroon. To achieve this aim, blood samples from a total of 162 cattle were collected and examined using Reverse Line Blot hybridization (RLB) assay. The amplified hypervariable V4 region of the 18S rRNA gene of bovine piroplasms species, including *Theileria parva, T. annulata, T mutans*, *T. velifera*, *T. buffeli/T. orientalis*, *T. taurotragi*, *Theileria* sp (buffalo), *Babesia bovis*, *B. bigemina*, *B. divergens*, *B. major* and *B. occultans* was hybridized against species-specific probes. RLB hybridization assay revealed the presence of four piroplasms species with the overall prevalence of infection of 82.1%. *Theileria velifera* (71.6%) was the most prevalent species followed by *Theileria mutans* (43.21%), *Theileria buffeli/T. orientalis* (5.55%) and *Babesia bigemina* (3.7%). However, the study provided the first molecular evidence for the presence of *T. buffeli/T. orientalis* group species in cattle in Cameroon. Higher overall prevalence of infection of tick-borne pathogens was observed in this study area as well as the increase in prevalence and widespread of *T. velifera* and the observance of a new species of piroplasms. These results are an indication that special attention should be given to epizootiological investigations alongside well-adopted control programs.
Keywords: Piroplasms,*Theileria buffeli*, Babesia, RLB, Prevalence, Cameroon

Introduction

Piroplasmosis, caused by intra-erythrocytic protozoan parasites of the genus *Babesia* and *Theileria* is among the most relevant diseases of domestic and wild animals. It is a tick-transmitted disease and is distributed worldwide (Zanet et al., 2014) but mainly on cattle in tropical and sub-tropical regions (Noaman, 2012). It is a cause of tick-borne zoonosis worldwide, where free-living animals are reservoir hosts of pathogens (Lemma et al., 2016). Among principal pathogens in central and east Africa with the most devastating economic impact on livestock are *Babesia*, *Theileria*, *Anaplasma* and *Rickettsia* species (Mukhebi et al., 1999; Jongejan and Uilenberg, 2004), where their presence is tightly linked to the presence and

distribution of potential tick vectors (Morel, 2000). Piroplasms cause heavy economic losses to the livestock industry and affect the health of wild animals in parasite endemic areas (Li et al., 2014). It was previously estimated that 200 million cattle were exposed to theileriosis, and 200 million to anaplasmosis and babesiosis throughout the tropics (Mukhebi et al., 1992). Likewise, more than one million cattle were estimated to have died from tick-borne diseases in 11 countries of central, eastern, and southern Africa in 1989. Therefore, the economic losses in livestock industry and funding for prevention, control, and research programs were estimated to US$168 million that year (Mukhebi et al., 1992). In eight Latin American countries, anaplasmosis and babesiosis cause an annual economic losses estimated at US$1.5 billion. It was estimated that 2-20% of Southeast Asia's 337 million cattle are affected either by anaplasmosis or babesiosis (ILRAD, 1991). According to Silatsa et al. (2019), more than thirteen species of ticks were found widespread in Cameroon while, Abanda et al. (2019) identified more than eleven tick-borne haemoparasites in cattle from the North Region of the country with highest prevalence assessed to about 89.1%.

The importance of tick-borne diseases is increasing all over the world, including Cameroon and especially in its third Agro-Ecological Zone (AEZ), the Western Highlands where incidentally is the third major cattle producing area in the country (IRAD, 2008; MINEPIA, 2012). It must not be forgotten that human activities continue to change the landscape vastly, altering faunal associations and thereby contact with arthropod vectors, producing circumstances that serve as a basis for the emergence of tick-borne infections.

Piroplasms, a pathogenic protozoa responsible of piroplasmosis are considered as a public and veterinary health concern as the prevalence of diseases they cause increases, coupled with its increase in geographical spread or expansion where they are found. Since there is paucity of information available on tick-borne diseases in cattle in the Western Highlands of Cameroon, there is a need for updating. Based on the foregoing, Reverse Line Blot hybridization (RLB) assay was performed to better identify *Theileria* and *Babesia* species in cattle from the Western highlands of Cameroon.

Material 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 3000 m above sea level. The climate of this region is the humid tropical 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 mid-November to mid-March. The temperatures vary between 20 and 32°C. The dominant vegetation is residual savannah and the region is designated grassland because the greater proportion of the area is covered by grassland than forest. This AEZ 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 (Nchinda and Mendi, 2008; Jiotsa et al., 2016).

Study population

The zebu cattle (*Bos indicus*), mainly the local breed namely Aku, Gudali and M'bororo cattle which is commonly found in the Western Highlands of Cameroon was the target population for the study. The majority of livestock is in extensive management system with a usual practice of transhumance (Boukar et al., 2015). This region is known to be the third major cattle producing area of the country with the livestock population estimated to 500,000 (about 17% of the total cattle) head (IRAD, 2008; MINEPIA, 2012) and no or adequate tick control program is implemented.

Collection of the samples

A total number of one hundred and sixty-two cattle were sampled between March 2019 to January 2021, according to their sex (66 males and 96 female cattle) and age (152 adults and 10 yearling cattle) for blood sampling. Five ml of blood samples were collected from jugular or coccygeal vein of each cattle into EDTA tubes, preferably potassium–ethylenediamine tetra-acetic acid (EDTA/K3) with a concentration of 1.27 mg EDTA/K3 per ml of blood and into Dried Blood Spot (DBS) specimen collection cards prepared for the purpose. A combination of nested-PCR and RLB hybridization assay was performed to simultaneously detect piroplasms from blood samples collected on DBS cards. The use of DBS cards facilitated the collection, storage and shipment of the blood samples at the Laboratory of Molecular Parasitology, Department of Parasitology, Faculty of Veterinary Medicine, University of Firät, Elazig, Turkey.

DNA extraction and PCR

Genomic DNA was extracted by a commercial DNA isolation kit (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. For amplification of *Theileria* and *Babesia* species, a nested PCR was performed using two sets of primers. In the first round PCR, the universal primers Nbab-1F and Nbab-1R were used to amplify a 1,600-bp fragment of the 18S rRNA gene of *Theileria* and *Babesia* species (Table 1). The amplification mixture contained 2.5 µl PCR buffer, 2.5 µl $MgCl_2$, 2 µl dNTPs mix (1.25 mM dNTP), 0.1 µl Taq DNA polymerase (1.25 U), 1.25 µl each primer (10 pmol/µl), 2.5 µl template DNA, and nuclease-free water to a total volume of 25 µl. PCR amplification was performed in PCR Sprint (Thermo Electron Corporation, Sprint, USA). The initial denaturing was performed at 94°C for 2 min followed by 40 cycles of 94°C for 30s, 55°C for 1 min, and 72°C for 1 min. A final extension was at 72°C for 7 min, after which the products were stored at 4°C. In the second round PCR, genus-specific primers, RLBF2/RLBR2, were used to amplify a fragment of 460–540 bp of the 18S rRNA gene of the V4 region of *Theileria* and *Babesia* species (Table 1). For the second amplification, 1 µl of first round PCR products were used as a template DNA in the second round PCR. To reduce non-specific amplification, a touchdown program was performed. The touchdown PCR was performed in a total volume of 25 µl, and positive and negative control DNA samples were used in each reaction.

Probes of catchall, genus, and species-specific for *Theileria*/*Babesia* were used with a range of 200–800 pmol/150µl concentration, and containing N-terminal N-(trifluoracetamidohexyl-cyanoethyl,N,N-diisopropyl phosphoramidite [TFA])-C6 amino linker. The oligonucleotide probes were synthesized by The Midland Certified Reagent (Midland, Texas, USA). The oligonucleotide probes used in this study are listed in Table 1.

Preparation, hybridization and stripping of the RLB membrane were performed as previously described with minor modifications (Gubbels et al., 1999; Georges et al., 2001; Altay et al., 2008).

Statistical analysis

Data were uploaded into Microsoft Excel 2016 computer program. Statistical calculations were performed using SPSS V.23 software, and Chi-square tests were used to compare prevalence of infection of different parasites detected via RLB hybridization assay according to age and sex.

Results

Out of the 162 cattle blood sample screened, 133 were found positive with the presence of at least one haemoparasite, essentially piroplasms belonging to two genera which were *Theileria* sp. and *Babesia* sp. (Figure 1).

Table 1. Oligonucleotide primers and probes used in this study

Primer	**Sequence (5'-3')**	**References**
Nbab1F	AAGCCATGCATGTCTAAGTATAAGCTTTT	Oosthuizen et al., 2008
Nbab1R	CCTCTCCTTCCTTTAAGTGATAAGGTTCAC	Oosthuizen et al., 2008
RLB-F2	GACACAGGGAGGTAGTGACAAG	Georges et al., 2001
RLB-R2	biotin-CTAAGAATTTCACCTCTGACAGT	Georges et al., 2001
Probe	**Modification (5'-3')**	**References**
Theileria/*Babesia* Catchall	C6 amino-TAATGGTTAATAGGARCRGTTG	Gubbels et al., 1999
Theileria spp. (*Theileria* genus-specific)	C6 amino-TGATGGGAATTTAAACCYCTTCCA	Gubbels et al., 1999
T. annulata	C6 amino-CCTCTGGGGTCTGTGCA	Georges et al., 2001
T. parva	C6 amino-GGA CGG AGT TCG CTT TG	Gubbels et al., 1999
T. buffeli/orientalis	C6 amino-GGCTTATTTCGGWTTGATTTT	Gubbels et al., 1999
T. mutans	C6 amino-CTTGCGTCTCCGAATGTT	Gubbels et al., 1999
T. velifera	C6 amino-CCTATTCTCCTTTACGAGT	Gubbels et al., 1999
T. taurotragi	C6 amino-TCTTGGCACGTGGCTTTT	Gubbels et al., 1999
Theileria sp. (buffalo)	C6 amino-CAG ACG GAG TTT ACT TTG T	Oura et al., 2004
Babesia Catchall 1	C6 amino-ATTAGAGTGTTTCAAGCAGAC	Adamu et al., 2014
Babesia Catchall 2	C6 amino-ACTAGAGTGTTTCAAACAGGC	Adamu et al., 2014
B. bovis	C6 amino-CAGGTTTCGCCTGTATAATTGAG	Georges et al., 2001
B. bigemina	C6 amino-CGTTTTTTCCCTTTTGTTGG	Gubbels et al., 1999
B. divergens	C6 amino-GTTAATATTGACTAATGTCGAG	Gubbels et al., 1999
B. major	C6 amino-TCCGACTTTGGTTGGTGT	Georges et al., 2001
B. occultans	C6 amino-CCT CTT TGG CCC ATC TCG	Ros-García et al., 2011
Babesia sp. (sable)	C6 amino-GCG TTG ACT TTG TGT CTT TAG C	Oosthuizen et al., 2008

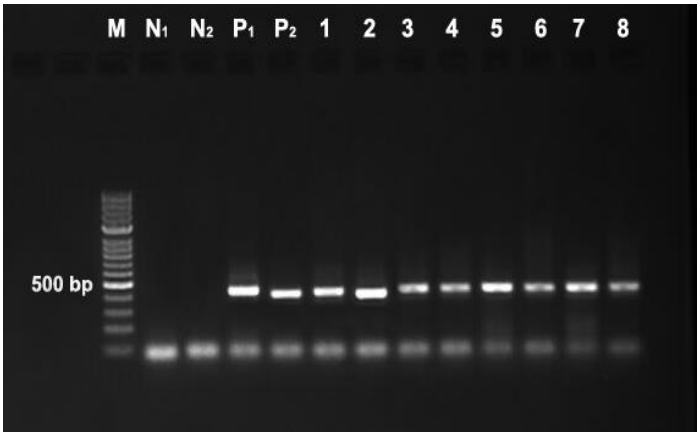

Fig. 1: Gel electrophoresis of PCR product of *Theileria* and *Babesia* species
M: 500 bp ladder, **N1-N2**: negative controls (**N1**: DNA from uninfected cattle blood; **N2**: Sterile deionized water), **P1-P2**: positive controls (**P1**: *T. annulata*; **P2**: *B. bigemina*). Lanes **1-8**: positive field samples signaling *Theileria/Babesia* catchall probe in the RLB.

After confirming the presence of 18S rRNA gene of *Theileria* and *Babesia* in the blood samples, RLB was performed to specifically identify these parasites and, 4 of them were detected namely, *T. mutans*, *T. velifera*, *T. buffeli/T. orientalis*, and *B. bigemina* (Figure. 2).

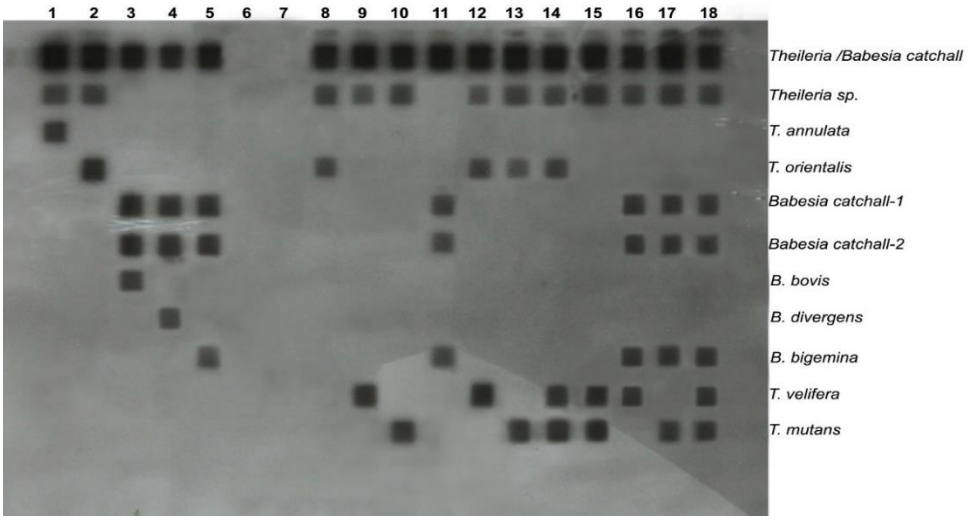

Fig. 2: Detection of *Theileria* and *Babesia* species by RLB

Oligonucleotide probes were applied in columns and PCR products in rows. Lanes 1-5: positive controls (**1**, *T. annulata*; **2**, *T. orientalis*; **3**, *B. bovis*; **4**, *B. divergens*; **5**, *B. bigemina*. **Lanes 6-7**: negative controls (**6**, DNA isolated from uninfected cow blood; **7**, sterile deionized water). **Lanes 8-18**: field samples (single and mixed infection) (**8**, *T. orientalis*; **9**, *T. velifera*; **10**, *T. mutans*; **11**, *B. bigemina*; **12**, *T. orientalis* + *T. velifera*; **13**, *T. orientalis* + *T. mutans*; **14**, *T. orientalis* + *T. velifera* + *T. mutans*; **15**, *T. velifera* + T. *mutans*; **16**, *B. bigemina* + *T. velifera*; **17**, *B. bigemina* + *T. mutans*; **18**, *B. bigemina* + *T. velifera* + *T. mutans*).

Table 2. Prevalence of piroplasms identified by RLB in the study area

	Number of cattle				
	Examined	Infected	Prevalence (%)	95% CI	*P* value
Piroplasms					
T. velifera	162	116	71.6	59.17 – 85.88	*< 0.0001*
T. mutans		70	43.21	33.68 – 54.59	
T. buffeli/T. orientalis		9	5.55	2.54 – 10.54	
B. bigemina		6	3.7	1.35 – 8.06	
Overall	162	133	82.1	68.74 – 97.3	

Of the 162 cattle blood sample screened, 133 were found infected by one or more piroplasms made up of four species. The overall prevalence of infection was assessed to 82.1% in the study area with *Theileria velifera* (71.6%) being the most prevalent species, followed by *Theileria mutans* (43.21%), *Theileria buffeli/Theileria orientalis* (5.55%), and *Babesia bigemina* (3.7%). The prevalence of these tick-borne pathogens was significantly different (Table 2).

A co-infection analysis showed that a total of 38.88% of cattle were found to be simultaneously co-infected with two or more piroplasms (Table 3). Overall, seven different species combinations were observed. The level of co-infection ranged from double to triple. The majority of the mixed infection occurred as double infection (35.8%), while triple infection (3.08%) was the least and significantly different (*P < 0.0001*). The most frequent co-occurrences (parasites association) included *T. mutans* + *T. velifera* (33.33%). It is interesting to note that no significant difference (*P = 0.5439*) was observed between the prevalence of single (43.21%) and mixed infection (38.88%).

Table 3. Prevalence of mixed infection and parasites association

	Frequency	Prevalence (%)	Number of species combination
Level of co-infection			
Double	58	35.8	5
Triple	5	3.08	2
Overall	63	38.88	7
Parasites association			
T. mutans + T. velifera	54	33.33	
T. mutans + T. buffeli/T. orientalis	1	0.62	
T. mutans + B. bigemina	1	0.62	
T. velifera + T. buffeli/T. orientalis	1	0.62	
T. velifera + B. bigemina	1	0.62	
T. mutans + T. velifera + T. buffeli/T. orientalis	2	1.23	
T. mutans + T. velifera + B. bigemina	3	1.85	

Table 4. Prevalence of infection of cattle in the study area

	Number of cattle				
Sex	Examined	Infected	Prevalence (%)	95% CI	*P* value
Male	66	55	83.33	62.78 – 108.47	0.8857
Female	96	78	81.25	64.22 – 101.4	
Age					
Yearling	10	7	70	28.14 – 144.23	0.6629
Adult	152	126	82.89	69.05 – 98.7	

The prevalence of infection of cattle was assessed according to sex and age. It appeared that male cattle (83.33%) were more infected than female (81.25%), while adult (82.89%) were found more infected than yearling cattle (70%). However, these results revealed that piroplasm infection was not significantly associated to sex and age (Table 4).

Discussion

We carried out a cross-sectional survey in order to assess the prevalence of *Theileria/Babesia* species in blood samples collected from cattle in the Western Highlands of Cameroon via reverse line blot hybridization assay. The overall prevalence of infection of cattle was highest and assessed to 82.1%. This result was comparable to the finding of Abanda et al. (2019) who reported a prevalence of 78.8% in Cameroon and, low than the 87.3% reported in Nigeria by Famuyide et al. (2020). However, it was highest compared to 18.5% and 20.3% reported in Algeria respectively by Boularias et al. (2020) and Boularias et al. (2021). This finding may reflect a high endemic level of haemoparasites in the Western Highlands of Cameroon and, suggests an early exposure of cattle to these haemoparasites due to early infestations with *Amblyomma* and *Rhipicephalus* ticks (Lorusso et al., 2013). This could also indicate that there is no implementation of an acceptable control strategy program in the study area.

Of the four piroplasms identified in the study area, *T. velifera* (71.6%) was the most prevalent, followed by *T. mutans* (43.2%), *T. buffeli/T. orientalis* (5.55%), and *Babesia bigemina* (3.7%). The result of this study contrast the previous report of Lako et al. (2007), who rather reported *Babesia bigemina* (11.2%) as the most prevalent piroplasm in the Western Highlands of Cameroon. The Western Highlands of Cameroon is the main destination of herds resulting from transhumance and trade, originating from the neighbouring region, where *Theileria velifera* and *Theileria mutans* seem to be endemic (Abanda et al., 2019; Silatsa et al., 2020), favouring the widespread of these piroplasms and could explain the replacement of *Babesia bigemina* in the study area. However, same species with different proportion were identified by Lorusso et al. (2016) in Nigeria, Hailemariam et al. (2017) in Ethiopia, as well as Abanda et al. (2019) and Silatsa et al. (2020) in Cameroon, although they detected rather *Theileria mutans* as species having the higher prevalence assessed respectively to 66.3%, 66.1%, 92.2%, and 41.1%. *Theileria velifera* and *Theileria mutans* are transmitted by *Amblyomma variegatum* ticks (Lorusso et al., 2016; Abanda et al., 2019). Moreover, other main vectors for *Theileria velifera* are hard ticks of the genera *Rhipicephalus*, *Hyalomma*, and *Haemaphysalis* (Gebrekidan et al., 2020). As Silatsa et al. (2019), we also identified *Amblyomma* and *Rhipicephalus* as the first and second most common tick genera in the study area, correlating with the high prevalence of these pathogens, especially *Theileria velifera.*

The cattle sampled in the present study were apparently healthy and presented no clinical signs of oriental theileriosis as described by Watts et al. (2015). However, result revealed the first record of *Theileria buffeli/T. orientalis* species in cattle population in Cameroon with the prevalence of 5.55%. The prevalence of this parasite newly identified in Cameroon was comparable to 7% reported in Turkey by Aktas et al. (2006). However, it was greater than the 2.8% reported in China/Pakistan by Hassan et al. (2018) and, lowest than the 51.8% and 32.8%, respectively, reported in Ethiopia by Hailemariam et al. (2017) and in Kyrgyzstan by Aktas et al. (2019). This notable result proposes that cattle in Cameroon are subclinical carriers of *Theileria buffeli/Theileria orientalis* parasite. The main vector of *Theileria buffeli/Theileria orientalis* is *Haemaphysalis longicornis* (Hammer et al., 2015), which has fortunately not been identified in Cameroon. Nevertheless, other *Haemaphysalis* and *Ixodes* species (Yokoyama et al., 2012; Hammer et al., 2015) and some mosquitoes (Hammer et al., 2015) have recently been implicated in the transmission of this piroplasm. Considering that *Haemaphysalis leachi* was identified in the study area (Ngangnang et al., 2021), we think that it could act as the potential vector of this piroplasm; however, further investigation is required to confirm it. One important area for future research should be to assess whether potential alternative tick vectors are infected with *Theileria buffeli/Theileria orientalis* or a novel tick species evolves the capacity to transmit it. Moreover, iatrogenic transmission through husbandry procedures is another possible mode of transmission occurring when re-using needles between cattle, contaminated castration knives, ear notching procedures, as well as, injury sustained during transport of cattle (Hammer et al., 2016).

In Cameroon, some tick-borne pathogens such as *Theileria velifera* and *Theileria mutans* were first recorded by Abanda et al. (2019), as well as *Theileria parva* by Silatsa et al. (2020). To the best of our knowledge, the current study is showing the first recording *Theileria buffeli/Theileria orientalis* in Cameroon. This significant finding suggests that cattle in Cameroon might be subclinical carriers of *Theileria buffeli/Theileria orientalis* parasite and, future research might be carried out on phylogenetic analysis and associated pathogenicity of the parasite. This finding will contribute to a better understanding of the epizootiology of tick-borne infections of cattle in Cameroon. The results generated would ultimately help orientate the designing of control strategies.

The level of co-infection ranged from double to triple with double infection (35.8%) being

significantly most frequent than triple infection (3.08%). The high frequency of co-infections suggests that clinical manifestations of piroplasmosis might be complex in the study area (Hailemariam et al., 2017), influencing the duration of infection and the intensity of the signs, which subsequently impacts the effective control of diseases (Boularias et al., 2020). Single infection (43.21%) was more prevalent than mixed infection (38.88%), but no considerable difference was found. The result disagreed with the finding of Hailemariam et al. (2017) in Ethiopia and Nyabongo et al. (2021) in Uganda, who respectively reported 100% and 82.91% as the prevalence of mixed infection, and this might be the consequence of heavy infection of cattle in the study area and even the dominance of competent vectors. However, the same circumstance as we found was described in China by Abdallah et al. (2017) and in Algeria by Boularias et al. (2020), although they found that there was significant difference between the two prevalence. During this study, we reported that the most frequent parasites association was between *T. mutans* and *T. velifera* (33.33%), and this might be because they were the two most prevalent pathogens identified in cattle. Nevertheless, this result contrast with the previous report of Hailemariam et al. (2017) and Silatsa et al. (2020), who observed that the most co-occurrences included, respectively, *T. velifera* + *T. buffeli*, and *T. mutans* + *T. parva*. The result of this study showed the importance of the two piroplasms and, further research might be carried out to better understand their pathogenicity on cattle of the study area.

It was clear from the result of this study that male (83.33%) were most infected than female cattle (81.25%). This study matched with the finding of Hailemariam et al. (2017) in Ethiopia and Famuyide et al. (2020) in Nigeria but, contrast with the report of Aktas et al. (2019) in Kyrgyzstan and Nyabongo et al. (2021) in Uganda. It is interesting to note that there was no association in piroplasms infection according to sex in this study and, this was because prevalence was found to be equally distributed between male and female cattle. Adult cattle (82.89%) were found more infected than yearling (70%), with no significantly difference. This finding was consistent with those reported by Hailemariam et al. (2017) and Famuyide et al. (2020). Although, the prevalence of piroplasm infection was found to be equally distributed (Aktas et al., 2019), it would seem that the slightly difference which was observed might be due to rapid immune responses of yearling cattle to primary infection through acquired maternal immunity (Lorusso et al., 2016; Boularias et al., 2020). However, the main reason should be the lowest number of yearling cattle sampled for the study, due to the fact that the herdsmen did not accept to take the blood samples from calves and yearling cattle, arguing that they feared wound and even stress and death of animal. So, the lowest number of yearling might have obfuscated the effect of age on the prevalence of transmitted pathogens, as well as it reflects the age composition of cattle in the Western Highlands of Cameroon.

The present study reports the simultaneous detection of multiple piroplasms species and their genetic relationship in the Western Highlands of Cameroon. It also revealed a very high prevalence of piroplasms and showed that the most common parasite known till date as the most prevalent is going to change to a new species. Results could be helpful for providing a basis development of a new efficient fight and control measures of these economically important vector-borne pathogens.

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Conflict of interest

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

Ethical approval

The use of cattle in this study 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).

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