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Full Length Research Paper

Molecular detection of concurrent infections of *Anaplasma* sp *Omatjenne*, *Theileria mutans*, *Babesia bigemina* and *Anaplasma marginale* in calves and yearlings in a tick endemic Guinea savannah ecosystem in Cameroon

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Ticks play a major role in limiting profitable livestock production in sub-Sahara Africa and the region is beleaguered by a paucity of data on diseases implicated in high young stock morbidity and mortality. In a tick endemic high Guinea savannah ecosystem 20 calves were raised in a traditional grazing system and blood collected from them during their first eighteen months of life. PCR, Restriction Fragment Length Polymorphism (RFLP) and sequencing were applied on DNA of tick-borne pathogens in animal blood buffy coat to amplify and characterize the 16S rRNA genes of *Anaplasma*, *Ehrlichia* and 18S rRNA gene for *Babesia* spp. All animals had different combinations of mixed infections of these haemoparasites. *Anaplasma* sp. *Omatjenne* (reported for the first time in this region) and *T. mutans* infected all and four of these animals, respectively. *Babesia bigemina*, and *Anaplasma* sp. *Omatjenne* concurrently occurred in all 20 experimental animals; *A marginale* in 15 while no *Ehrlichia ruminantium* was detected. The presence of *Anaplasma marginale* and *Anaplasma* sp. *Omatjenne* in the blood significantly reduced haematocrit ($p<0.0001$) while *Babesia bigemina* and *Theileria mutans* had no such effect ($P>0.05$). The mean first-time contact periods (in weeks) for *B. bigemina*, *T. mutans*, *Anaplasma* sp. *Omatjenne* and *A. marginale* were 15 (3-37), 30(9-43), 21(5-55) and 25(7-55) respectively; and they were not significantly different ($P>0.05$). The sequences for new pathogens we found in the region: *Theileria mutans* and *Ehrlichia* Bom Pastor or (*Anaplasma sp. Omatjenne*) have been deposited in the GenBank database with accession numbers MN719893.1 and MN719091.1. The absence of disease states during the study demonstrates an endemically stable situation in the region for these infections. With no clinical data on *A.* sp. *Omatjenne* and *T. mutans* infections in this area, further insights into their epizootiology should be of interest.

Keywords: Endemic stability, *Anaplasma marginale*, *Babesia bigemina*, *Anaplasma* sp. *Omatjenne, Theileria mutans*, Zebu cattle, Guinea savannah.

INTRODUCTION

In Sub-Saharan Africa, ticks and tick-borne diseases are to a large extent responsible for the stagnation in the livestock industry and their inefficient management constitutes an important limiting factor to beef and dairy livestock production, particularly in smallholder farms with low input options (LSR, 1989).The sub-Saharan African region is beleaguered by a paucity of data with respect to what extent tick-borne diseases are implicated in the high morbidity and mortality recorded in most livestock production systems. In Wakwa, located in the high Guinea Savannah zone of Cameroon, tick burdens are influenced by season and infestation varies greatly between breeds as well as amongst individuals of the same breed (Tawah, 1992; Stachurski et al. 1993; Achukwi et al. 2001). About 63% of cattle mortalities at IRAD Wakwa, have been associated with ticks and tick-borne diseases (Mbah, 1982). The situation is thought to be similar in livestock production systems where deficient tick control programmes persist (LSR, 1989). Exotic taurines and cross-bred animals between local cattle and exotic ones, usually with the hope of improving their productivity, are unable to adapt in the local production systems plagued with inefficient tick control options [unpublished data]. The resulting heavy losses through high morbidity and mortality rates (Mbah, 1982) contribute to poverty among local breeders with low input systems, frustrates and hinders them from developing the beef and milk industries.

Apart from causing disease, tick infestation also has a negative impact on zebu cattle live weight gain (Stachurski et al.,1993). Factors favouring tick-borne diseases in low input small-scale livestock extensive grazing systems include the high cost of acaricides, irregularity in supply and lack of knowledge concerning the risks from various tick species and management of acaricides (Awa et al., 2009). Hand tick removal from animals is still very popular as a means of tick control in the High Guinea Savannah of Cameroon (LSR, 1989). The abundance of different genera of ixodid ticks in most grazing pastures of Cameroon and particularly in our study site (Awa et al.2015; Stachurski et al.1993) suggest that tick-borne pathogens transmitted by these ticks, are important factors limiting livestock productivity, yet studies on their relative importance with respect to early calf morbidity and mortality in this area are almost inexistent.

The general perception amongst local livestock owners is that *Ehrlichia ruminantium* causes huge economic losses to the livestock industry in the region (LSR, 1989). Adult *A. variegatum* are the main vectors of *E.ruminantium* in cattle (Gueye et al. 1993; Mattioli et al.2000) although other tick species in the region are possible vectors of the disease. Taken together, the role of tick-borne pathogens in constraining ruminant production (usually occurring in definitive hosts as high morbidity and mortality) in the pastoral regions in sub-Sahara Africa is only faintly understood by pastoralists and the available scanty information is obsolete because most of the available results are based on assumptions (Mbah, 1989), serology and microscopy with obvious limitations [Awa, 1997; Bose et al. 1995; Chollet, 1995; Mamoudou, 2017).

In this longitudinal study that was undertaken in the high Guinea Savannah of Cameroon, a tick-endemic ecosystem, we used molecular tools (PCR-RFLP analysis and sequencing) to identify, monitor and confirm natural occurrence of four tick-borne pathogens: *Babesia, Theileria, Ehrlichia* and *Anaplasma* in zebu Gudali (*Bos indicus*) calves and yearlings during their first 18 months of life. The study also determined animal age at first contact with the different pathogens and the effect of pathogen presence in the host blood on packed cell volume of the animals.

MATERIALS AND METHODS

Animals, breed and production system

Twenty naive zebu Gudali female and male calves born on the experimental animal farm of the Institute of Agricultural Research for Development (IRAD) Wakwa Centre, located in the high Guinea Savannah (Adamawa plateau) of Cameroon were successively recruited into the study soon after birth (between October 2005 and May 2006) and were identified by ear tags. The Gudali cows (mothers to our experimental animals) were raised in a low input small-scale livestock extensive grazing system here described. Sparing salt supplementation was all year round and cotton seed cake was fed only during the dry season. From birth, the experimental calves were left to graze with their mothers in the same paddocks during pre- and post-weaning management. All the calves were dewormed with oral administration of 400 mg albendazole tablets (Cipla, India) when they were one month old. The calves were weaned at the age of nine months. The Gudali is a short-horned and short-legged zebu cattle (*Bos indicus*) mainly

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Table 1: Primers used in PCR for detection of *Babesia/Theileria* and *Anaplasma/Ehrlichia* groups.

Pathogen group	Name	Sequence
Babesia* and *Theileria		
	BabF3	ATGTCTAAGTACAAGCTTTTTACGGT
	BabR2	TTGATTTCTCTCAAGGTGCTGAAGGAGTCG
	BabR3	AAAGGCGACGACCTCCAATCCCTAGT
Anaplasma* and *Ehrlichia		
	EHR 16SD	GGTACCYACAGAAGAAGTCC*
	EBR3	TTGTAGTCGCCATTGTAGCAC
	EBR2	**TGCTGAC**TTGACATCATCCC

Primer F: forward, R: reverse. *The primers are based on the amplification of the16S rRNA and 18S rRNA genes for the simultaneous detection of *Anaplasma/Ehrlichia* and *Babesia/Theileria,* respectively. That for the 16S rRNA were developed at ITM (Antwerp, Belgium).

occurring in Nigeria and Cameroon. A small population of the Gudali breed has also been seen in Burkina Faso (personal communication). They have a high potential for beef and consist of two major sub-types: the Sokoto and Adamawa Gudali. The latter comprises three regional variants, namely the Ngaoundere, Banyo and Yola Gudali (Tawah and Rege, 1994). Only the Ngaoundere Gudali were used without spraying with any acaricide during the entire study period. Typically as practised in the low input small-scale livestock extensive grazing system the animals were restrained once a week and all ticks were removed using a forceps.

Local conditions including altitude, seasons, temperature and hygrometry

The experimental farm is located at E07", N013"35.895' and at an altitude of 1217 metres. The high altitude of this region (ranging between 1000 and 1300 meters) provides a relatively cool climate but since about two decades ago the effect of climate change saw increasing temperatures ranging between 22–25°C and the average annual rainfall decreasing to between 900 mm to 1500 mm (Mbahe, 1998). However, the farm's weather station recorded 1600 mm of rainfall during the year of the study. The climate is of the Sudanese tropical type with two seasons: the dry season that occurs from November to March, followed by the wet season (April to October). The area is covered by discontinuous wooded vegetation consisting of savannah grasses such as *Hyparrheenia*, *Panicum* and *Sporobolus*.

Collection of blood and packed cell volume measurement

Blood was collected from the jugular vein of each animal fortnightly for the first year and thereafter once a month until the animal attained the age of 18 months when it was removed from the study. The blood was used for measurement of packed cell volume (PCV) (Figure 1) and DNA extracted from its buffy coat. Each buffy coat sample was stored at +4°C until it was used for DNA isolation.

DNA extraction, amplification and analysis

DNA extraction from the buffy coat was undertaken using the Pure Gene Genomic DNA Isolation kit (Biozym, Belgium) following the instructions of the manufacturer. The DNA samples were amplified by semi-nested polymerase chain reaction (PCR) and gel electrophoresis was used to visualise the amplicons. The analysis was based on the amplification of the 18S rRNA gene for the detection of all species of *Babesia* and was similar to that described by Devos & Geysen (Devos and Geysen, 2004). To amplify the 16S rRNA gene for the detection of *Anaplasma* spp. and *Ehrlichia* spp. the primers used were developed at IMT (Antwerp, Belgium). The isolated DNA was used for species-specific detection of the pathogens by restriction fragment length polymorphism (RFLP).

Polymerase Chain Reactions

In each reaction microtube, 5 µl of template and 20 µl of the master mix were added. The latter contained 1µl of Yellow Sub™ (Geneo BioTech Hamburg, Germany), 11.2 µl distilled water, 5µl of buffer (20 mMTris-HCl, pH 8.4; 100mM KCl), 1.6µl of MgCl (25 mM), 0.2µl of dNTP (100mM each), 0.4 µl each of *Babesia, Theileria, Anaplasma* and *Ehrlichia* primers (Table 1) (25µmol/µl) and 0.2µl of Taq polymerase (5U/µl). A thin layer (2 drops) of mineral oil was placed over the content of all microtubes. The whole tube was put in a preheated (84°C) thermocycler (PTC- 100 TM or T3 Biometra®, Westburg, Germany) programmed as follows: denaturing at 92°C for 30 seconds, annealing at 62°C for 45 seconds, extension at 72°C for 1 minute and this whole cycle was undertaken 39 times. A last extension of 8 minutes was added before

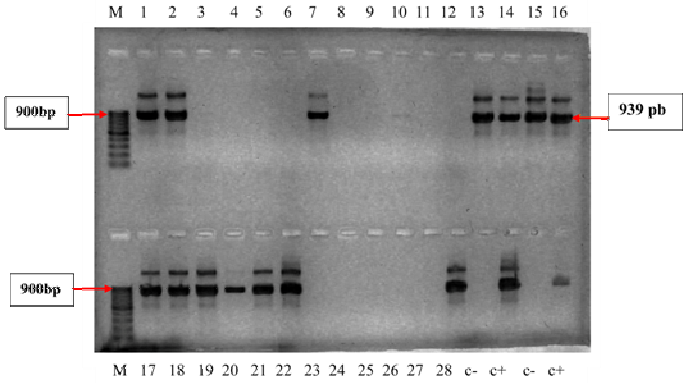

Figure 2 Samples of PCR positive parasites (*Babesia* spp. and *Theileria* spp.) resolved in 2% agarose gel.

Positive lanes: 1, 2, 7, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 and 28. Negative lanes: 3, 4, 5, 6, 8, 9, 10, 11, 12, 23, 24, 25, 26 and 27. M: molecular marker; C^-: Negative control; C^+: Positive control. Gels were coloured with ethidium bromide and photographed under UV light

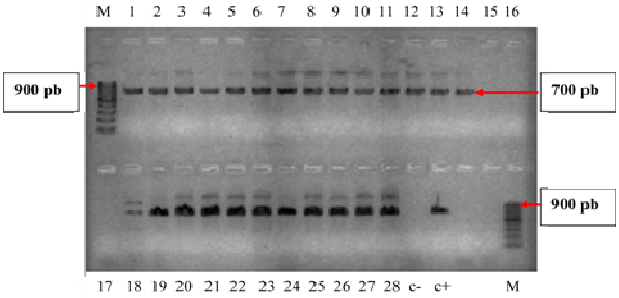

Figure 3 Samples of PCR positive bacteria (*Anaplasma* Spp. and *Ehrlichia* Spp.) resolved in 2% agarose gel.

Positive lanes: 1-14 and 18-28; Negative lines: 15,16,and 17; M: molecular marker; C^-: Negative control C^+: Positive control. Gels were coloured with ethidium bromide and photographed under UV light.

further analysis. Double distilled water was used as a negative control and a known *Babesia bigemina, T.mutans,* Anaplasma or Ehrlichia sample was used as a positive control in each case.

In the second round reactions, microtubes containing 24.5µl of the master mix consisting of 1µl of Yellow SubTM (Geneo Bio Tech Hamburg, Germany), 15.7µl of distilled water, 5µl of buffer solution, 1.7 µl of MgCl2 (25 mM), 0.4 µl of each primer, 0.13µl of Taq polymerase and 0.5µl of the first round PCR product (template) were each covered with 2 drops of mineral oil. The negative and positive control samples were similarly prepared. The reaction tubes were then preheated and maintained at 84°C and the thermocycler was programmed as follows: denaturation at 92°C for 30 seconds, annealing at 62°C for 45 seconds, extension at 72°C for 1 minute and this whole cycle was undertaken 24 times. A last extension of 8 minutes was added before further analysis. A total of 548 samples were examined using this nested PCR technique.

Five microliters of each PCR product was mixed with 2 µl of loading buffer and loaded into a 2% agarose gel wells and allowed to migrate in the agarose gel in TAE (0.5%) buffer at 100 Volts for 20 minutes. Thereafter, the gel was dipped in an ethidium bromide solution (1µg/ml) for 30 minutes to permit visualisation of the DNA using UV light. A photograph of each gel was made (Figure 2 and 3).

Restriction Fragment Length Polymorphism (RFLP) and Sybr Green digestion by enzymes

Positive PCR samples were further analysed by RFLP to determine the pathogen species implicated. Four to six microliters of each positive PCR product was digested by restriction enzymes (*Hind6*1 incubation temperature at 37°C and *BseD*I incubation temperature at 55°C overnight) in a mixture of RO-DI water, the enzyme and

corresponding buffer in a final volume of 15 µl consisting of 11 µl of reaction mix and 4-6 µl of PCR product.

Four to 6µl of enzyme-digested product was mixed with 2µl of loading buffer and loaded into marked wells of a polyacrilamide (PAGE) gel that was totally immersed in TBE buffer. The power unit was set at 100Volts and allowed to run for 2 hours 30 minutes. After migration, the gel was transferred into Sybr Green and maintained therein for 40 minutes. All positive PCR samples were submitted to RFLP where enzymatic digestion and their different fragment profiles were used to identify most of the parasite species**.**

Sequencing and Blast

On RFLP we could not distinguish the fragment profile of *Theileria* spp. from *Ehrlichia* spp. For that reason, we had to sequence those PCR products to confirm the diagnosis of infection by *Theileria* spp. and/or *Ehrlichia* spp. In each case one animal with a respective positive RFLP profile was randomly selected for sequencing. Purification of the PCR products was done using QIAquick® purification kit (QIAGEN®) according to the manufacturer's protocol. Cloning was then carried out using the TOPO TA Cloning® kits 2006 (Invitrogen™, California USA) according to the manufacturer's protocol. Positive colonies were selected for sequencing and further analysed by comparing the sequences to the NCBI nucleotide database using Blast and Jalview 2.8.2 (Waterhouse et al., 2009).

Analysis of collected data

Data were collected from each animal only during its first 18 months of life. Adjustments were made to permit PCV data collected from animals with the same age and during the same season to be grouped together for analysis. The time (in weeks) of each animal's first contact with any of the four haemoparasites was calculated. A logistic regression was undertaken to determine the effect of the different animal blood parasite/bacteria incidence on PCV. The logit model employed the maximum likelihood function using the Newton-Raphson algorithm. Threshold significance value was 5%.

RESULTS

Age at first contact with different pathogens

The mean age at first contact for the different pathogens was 15 weeks (range 3-37 weeks) for *B. bigemina*, 30 weeks (range 9-43 weeks) for *T. mutans*, 21 weeks (range 5-55 weeks) for *A.* sp. *Omatjenne* and 25 weeks (range 7-55) for *A. marginale* with no significant difference (P>0.05).

B. bigemina

All 20 first *B. bigemina* infections occurred during the first 12 months of life, 16 of which occurred during the first six of life while four were after 7th month of life. 11 of the *B. bigemina* infections occurring during the first six months of life came in the rainy season while the five remainder occurred in the dry season. Four other first time infections occurred when the calves were more than seven months (two of which were in the rainy season while the two others occurred during the dry season.).

A.* sp. *Omatjenne

13 first infections with *A.* sp. *Omatjenne* occurred during the first six months of life (10 of which were in the rainy season and 3 in the dry season). After the 7th month of life six other *A.* sp. *Omatjenne* infections occurred (3 in the rainy season and 3 in the dry season. One *A.* sp. *Omatjenne* infection occurred in the dry season when the animal was older than13 months.

A. marginale

Of the 15 first infections of *A. marginale* recorded during the first 18 months of life of the animals, eight occurred during the first six months of life (six in the rainy season and two in the dry season). When the animals were between seven and 12 months old, five other first time infections with *A. marginale* were recorded (four of which occurred in the rainy season while one was in the dry season). The two other first time infections with *A. marginale* occurred in the rainy season when the animals were above 13 months of life.

T. mutans

Four infections with *T. mutans* were recorded during the entire study period (one occurred in the rainy season during the first six months of life and the three others (one in the rainy season while two in the dry season) were recorded when the animals were between seven and 12 months of age.

Mortalities

Two of the experimental animals died of intestinal blockage due to *Toxocara vitulorum* infestation: one at 9 and the other at 11 months of age because these animals must have thrown out the tablets of albendazole administered to them orally at the end of their first one month of life. Of these, one tested positive for *Babesia bigemina* and *Ehrlichia* Spp. while the other was positive for the four pathogens. Both of them presented neither clinical nor post-mortem signs for these infections.

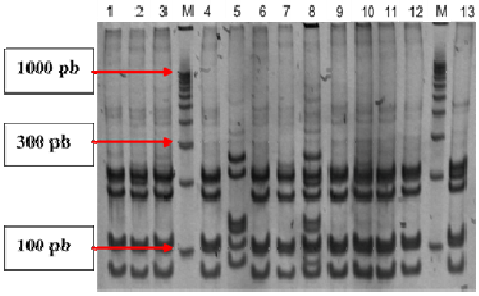

Figure 4 Parasite (*Babesia bigemina* and *Theileria* spp.): RFLP profiles of samples positive post-PCR, digested with BseDI enzyme, resolved and visualized in PAGE gels.

Gels were coloured with Sybr Green and UV photographed. Positive lines for *B. bigemina*: 1, 2, 3, 4, 6, 7, 9, 10, 11,12 and 13; Positive lane for *Theileria* Spp.: *5*. Co-infection *B. bigemina*/*Theileria* Spp.: lane 8; M: RFLP Marker.

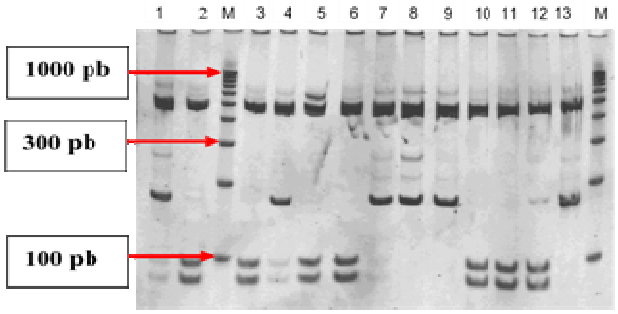

Figure 5 RFLP profiles of samples positive post-PCR, digested with Hind6I enzyme, resolved and visualized in PAGE gels.

A. marginale: lanes 3, 5, 6 and 11; *Ehrlichia* Spp.: lanes 8, 9 and 13; *Co-infection A. marginale*/*Ehrlichia* Spp.: lanes 1,2,4,10 and 12; M: RFLP marker. Gels were coloured with Sybr Green and UV photographed.

Effect of the incidence of blood parasites on PCV

Mean PCV were 33.3 and 33.5 in the dry and rainy season respectively for animals of the same age. Season had no significant effect (P>0.05) on haematocrit (normal value range 32-45%). The presence of *A. marginale* and *Ehrlichia* spp. had highly significant effects on PCV (p<0.0001) while *B. bigemina* and *T. mutans* had no such effect.

Molecular analysis of blood samples

Animals positive for *Babesia* and *Theileria* gave a PCR product at 939bp (Figure 2) while animals positive for *Anaplasma* and *Ehrlichia* gave a product at 700bp (Figure 3). The PCR product SN404 of calf G144/9 was further processed for sequencing and gave on Blast alignment 99,68% similarity with a *T. mutans* isolate (Muhanguzi et al. 2010) from Uganda (GenBank: KU206320.1). The sequence alignment of calf G92/67 (PCR nr SN119) with the RFLP profile typical of *Ehrlichia* spp. was identified to be *Ehrlichia* sp. Bom Pastor having almost 100% similarity with one that was previously reported in goats in Mozambique (Bekker et al., 2001). This pathogen had been renamed *Anaplasma* Spp. *Omatjenne*. No *E. ruminantium* infection was detected in any of the animals. The two sequences from our study were deposited in the GenBank (MN719893.1for SN404_ and MN719091.1for SN119).

All 20 animals had mixed infections with different combinations of haemoparasites. The numbers in brackets represent the number of animals with indicated pathogen combinations: *A.* sp. *Omatjenne* and *B. bigemina* (3), *Theileria mutans, A.* sp. *Omatjenne* and *B. bigemina* (2), *A.* sp. *Omatjenne, B. bigemina* and *A. marginale* (13), *A.* sp. *Omatjenne, B. bigemina, A. marginale* and *Theileria mutans* (2) (Fig 4 and 5).

DISCUSSION

Most livestock farmers operating in the low input small-scale livestock extensive grazing system in the region where this study was undertaken use hand picking of ticks as the main line of attack against tick infestation even though it is criticised for being labour-intensive, cannot be 100% efficient and is considered in the local context to permit disease transmission (LSR 1989). Furthermore, at least two previous studies (Stachurski et al. 1993; Awa et al. 2015) carried out in the same experimental farm provided enough data on the tick vector species infesting the animals with seasonal abundance of the tick vectors without any data on which diseases those tick vectors produce in the animals. Those studies indicated generally that peak periods for tick vector occurrence was the rainy season for adult ticks while nymphs and larvae were more abundant in the dry season. The present study however provides enough data to suggest that the tick control by the above local approach may have some overwhelming merits in the sense that it aims at a high reduction of the tick vector population to low levels (and not completely eradicating tick populations) resulting in low disease transmission that apparently tends to provide pre-immunity against a number of tick-borne diseases to yearling animals raised in such a system. On the other hand, various forms of acaricides are available for the effective control of ticks in Cameroon but these drugs are well out of the reach of most low input small-scale livestock breeders mainly because of their high cost and irregularity in supply (Awa et al. 2009). Other constraints include the supply of fake and dangerous products by charlatans and the lack of technical knowhow for the appropriate use of the products (Awa et al., 2009).

Some naive calves in this study quickly acquired three pathogens (*B. bigemina*, *A.* sp*. Omatjenne* and *A. marginale*) at a very early point in life (within the first two months [3-7 weeks]) while earliest contact with *T. mutans* pathogens was much longer (nine weeks). The age at first contact with the pathogens then increased gradually from five months onwards, recording the longest periods as 43-55 weeks in a few animals for *B. bigemina* and *A.* sp. *Omatjenne* and 55 weeks for *A. marginale* and *T. mutans*. This observation probably depicts the similarity in animal contact with vectors of the pathogens, the homogeneity in both the animal production system and ecosystem used for the study. The trends may also be associated with the lower attractiveness of some calves to tick vectors in their early life and/or inherited maternal resistance to ticks or transfer of maternal immunity through colostrum. It has been previously established in the same ecosystem that *A. variegatum* ticks infest lactating animals and older calves to a higher degree than non-lactating and younger calves (Stachurski and Musonge, 1996) but these studies did not generate data on which tick-borne pathogens were found in the animals. The established immunity may wane in old animals and disease state resurgence occurring in stressed animals could cause mortalities as reported earlier (Mbah, 1982).

Despite the high density of *A. variegatum* ticks, their nymphs and larvae observed in this study that have been previously reported (Stachurski et al. 1993; Awa et al. 2015) in the same study site, no clinical case of heart water disease due to *E. ruminantium* infection was recorded during the first 18 months of life of the animals. Heart water disease has been previously diagnosed between 1988 and 1994 using crushed brain smears stained with Giemsa in mostly Gudali X Holstein cross yearlings and a few adult Gudali cattle in the same experimental farm (Achukwi, unpublished data). That zero detection rate of *E. ruminantium* was recorded during the first 18 months of life in the experimental animals is in line with the findings of Merlin et al. (1987) who used microscopic examination of crushed brain tissue stained with Giemsa in the adjacent highland plateau of the north Western Cameroon and concluded that the rate of *E. ruminantium* infection was very low in village herds although *A. variegatum* infestations were very high. We observe that all 20 animals under two years of age were infected with *Ehrlichia* sp. (*A.* sp. *Omatjenne*) indicating the endemicity of this pathogen in the region. Also, from other studies in Cameroon *E. ruminantium* DNA was detected in 142 (28.4%) of 500 un-engorged *A. variegatum* ticks collected in Société de Développement et d'Exploitation des Productions Animales (SODEPA) Dumbo ranch (SDR) in the North West Region and Upper Farms ranch (UFR) in the South West Region. A higher infection rate of *E. ruminantium* (40.9%) was observed in ticks from SDR than in the ticks (24.7%) collected in UFR (Esemu et al., 2012). In Benin, a study undertaken in 4 localities (Farougou et al. 2012) showed that on the overall 10.8% of tested *A. variegatum* were infected by *E. ruminantium* (ranging: 8.9-15.6%), while in Burkina Faso the infection rate of ticks by this pathogen was higher, ranging from 9 to 20% (Adakal, 2009).Taken together, these previous studies confirm the endemicity of *E. ruminantium* in West and Central Africa in the tick vector and indicates that the tick also serves as the reservoir since the pathogen is maintained trans-stadially. On the contrary our study on *Ehrlichia* spp. corroborates with those of Vanegas et al. (2018) who collected ticks, at least five years later after our study from the same High Guinea Savannah zone of Cameroon, from which they sequenced the *ompB* gene and three intergenic spacers (*dksA-xerC, mppA-purC and rpmE-tRNAfMet*) and detected *Rickettsia africae* in *A. variegatum* ticks and many Rickettsia from other tick vectors but not *E. ruminantium.* That *E. ruminantium* was not found in our samples and those of Vanegas et al. (2018) and only one case reported recently in the Faro et Deo division, during a recent molecular screening of over 1500 adult cattle in the same ecosystem

(Abanda et al., 2019), should be a matter of great concern for epizootiologists.

While the *A.marginale* pathogen occurred in 15 out of 20 animals *Ehrlichia* Spp. (*A.* sp. *Omatjenne)* occurred in 20 out of 20 animals exposed. These high infection levels corroborate with that of Chollet (1995) who reported a high seroprevalence of antibodies (between 40 and 76%) to *Anaplasma* in a cross-sectional study in the same region in which our study was conducted. It is also worth noting that a serological study in the same region reported a high (61–67%) seroprevalence of *E. ruminantium* and demonstrated the association of heartwater with the presence of *A. variegatum* in the north region of Cameroon (Awa, 1997). Put together, the abundance of *E. ruminantium* in the tick vectors, the serological observations in the same study area and the neighbouring regions and our present findings in the definitive host, it will be difficult to understand the absence of *E. ruminantium* in the cattle host. Therefore, one is poised to believe that such early infections without clinical disease occurrence observed in our study may confer cross protecting immunity in the local animals against the other *Ehrlichia* Spp. and probably including *E. ruminantium*. Our present findings therefore disagree with the general unfounded perception of the local animal breeders that heart water caused by *E. ruminantium* is one of the greatest killer of indigenous young and adult cattle in the High Guinea Savannah zone of Cameroon (LSR, 1989). The observed mortalities in calves and yearlings may be due to other pathogens in the region.

It has been indicated that *Ehrlichia* Spp. are maintained in nature through subclinical infections of ruminants (carriers) as well as ticks and have evolved mechanisms to persistently infect mammalian hosts by subverting the innate and adaptive immune responses (Harrus, 1998). In the present study we found for first time in central Africa that the *Ehrlichia* sp. infecting all our calves and yearling cattle was *A*.sp. *Omatjenne* previously reported in goats in Mozambique as *Ehrlichia* sp. Bom Pastor (Bekker et al., 2001) which was later renamed *E.* Spp. (*Omatjenne*). *Ehrlichia* Spp. (*Omatjenne*), apparently thought to be apathogenic has also been detected in several ruminants in South Africa including Boer goats (Allsopp, 1997), in Uganda (Muhanguzi et al., 2010) and in Ethiopia (Teshale et al., 2015; Teshale et al., 2018). The observed epizootiological picture with respect to *Ehrlichia* Spp [*E.* sp. *(Omatjenne)*] and *E. ruminantium* from all the cited studies and ours suggests that other rickettsia or pathogens may be causing pathological or clinical signs similar to those of heartwater in the region since both show crossreactivity (Muhanguzi et al., 2010). Also, such immune cross-reactivity under natural conditions could hinder, through cross-protection, the expression of pathology by those otherwise pathogenic *Ehrlichia* Spp. concurrently infecting the animals. Thus there is need for extensive genetic characterization, transmission, clinical and pathogenicity studies of *Ehrlichia* Spp. from the major livestock raising regions of Cameroon and neighbouring countries to generate more data that could clarify the situation of especially *Ehrlichia* sp. (*Omatjenne*). Although *A.* sp. *Omatjenne* which we found in our study is said to be apathogenic (Allsopp et al., 1997; Du Plessis, 1990) in ruminants, a strain (*E.* sp. [*Omatjenne*] 1) of this parasite has been used experimentally to produce disease indistinguishable from cowdriosis in sheep (Du Plessis, 1990) thereby not ruling out its pathogenicity in natural conditions. *Anaplasma* sp. *Omatjenne* may contribute to seropositivity to *E. ruminantium* with which it greatly cross reacts (Muhanguzi et al., 2010). More so, *Anaplasma* and *Ehrlichia* spp. are reported to cause significant economic losses to the livestock sector in Uganda (Muhanguzi et al., 2010)

During this longitudinal study we detected *T. mutans* pathogens in four out of 20 animals and there was no previous report of *T. mutans* in the experimental farm before this study. Given our small sample size, the presence of other pathogenic *Theileria* Spp. in the region cannot be excluded and ought to be the subject of future investigations. *Babesia bigemina* was detected in all 20 exposed animals, implying that it is endemic in the study area. Semi-nested polymerase chain reaction (PCR) was undertaken with the same primers described by Devos & Geysen (2004) based on the amplification of the 18S rRNA gene for the detection of *Babesia* spp. Thus *Babesia* species were perfectly differentiated based on their RFLP profiles. This technique has been described as being more sensitive than microscopic and serologic examinations (Birdane et al., 2006; Lew and Jorgensen, 2005). It has also been reported that in *B. bigemina* infection, calves from non-immune cows are as sensitive as adult animals (Allsopp et al.,.2001; Sahibi and Rhalem, 2007). During their first contact with the pathogen, throughout the first 18 months of life, our experimental calves growing up to become yearlings did not reveal any clinical disease condition, suggesting resistance to develop disease states. This closely corroborates with findings from other studies which reported that cattle between 3 and 9 months of age have higher innate resistance to most tick-borne diseases and consequently disease incidence and corresponding mortality are typically lower for this stock class (Goff et al., 2002). In a tick endemic high Guinea savannah ecosystem such as the one in which this study was undertaken, the constant exposure of the animals to low amounts of the pathogen may cause rare clinical cases, and disease states will occur mainly in extremely stressed animals. A common stress that occurs in the area is the dry season, characterized by fodder and water scarcity and frequent high cattle mortalities even for the local zebu (*Bos indicus*) breeds. Although the rainy season which is the peak period for tick vector infestations of our experimental animals

(Awa et al. 2015; Stachurski et al.1993) tended to have more cases of first contact with three of the pathogens we studied, our small sample size does not allow us to hastily draw the conclusion that targeting calving to occur in the dry season (period of less tick burden) could be an attractive tick-borne disease control strategy.

It has been reported that amongst many other farm management practices, the reduction of the tick vector population by use of acaricides or pasture management (Neves, 1999), increasing aridity (De Vos and Potgieter, 1994) and intensive and prolonged tick control may be associated with breakdown in endemic instability to tick-borne diseases, which generally establishes in indigenous cattle following continuous contact with tick-borne pathogens (Du Plessis et al., 1992; Norval et al., 1995). The removal of ticks on a weekly basis in the present study did not stop the animals from acquiring the various pathogens. A situation of endemic stability has been reported more than two decades ago in an *A. variegatum*-infested ecosystem inhabited by indigenous cattle (Gueye et al., 1993). Maintaining the equilibrium state or balance between host, vector and pathogen is therefore crucial in ensuring endemic stability, which we found in the present study to contribute to avoiding economic loses.

CONCLUSIONS

This longitudinal study reported on the fate of cattle during their first 18 months of life raised in a low input small-scale livestock extensive grazing system within a tick-endemic high Guinea savannah ecosystem, in Cameroon. From their blood *Babesia bigemina* and *A. marginale* were perfectly differentiated based on their RFLP profiles. The nested PCR-RFLP procedures used could not characterise to species level the *Theileria* and *Ehrlichia* samples. Sequencing of these revealed that *T. mutans* occurred only in four of the 20 animals while *Ehrlichia Bom Pastor* (*E.* sp. *(Omatjenne)* or *(A.* sp*. Omatjenne)* infecting all the animals is reported for the first time in Cameroon*.* The findings strongly suggest that intermittent hand picking of ticks from their host fixation sites in the described production and ecological systems could have led to the establishment of an equilibrium between the host, the vector and the parasite, for the safety of the calves growing up in the ecological region to become yearlings. Disease control strategies in the study area should therefore aim at reducing the tick vector population to very low levels so as to obtain low disease transmission while avoiding complete vector eradication. More pathogen isolations from animals using extensive field surveys should provide further insights on the epizootiological picture of *E.* sp. *(Omatjenne)* and *T. mutans* in the local livestock production systems.

Availability of data and materials

All data generated or analysed during this study are included in this published article. Sequences for new pathogens found in the area: *Theileria mutans* and *Ehrlichia* Bom Pastor (*E.* sp. (*Omatjenne*) or (*Anaplasma* sp. *Omatjenne*) have been deposited in the GenBank database with accession numbers MN719893.1 and MN719091.1. Supplementary data information files are available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the scientific directorate of the national Institute of Agricultural Research for Development (IRAD) Cameroon. Blood collection from the experimental animals was undertaken by certified veterinarians of IRAD.

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