

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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Keywords: Endemic stability, *Anaplasma marginale*, *Babesia bigemina*, *Anaplasma* sp. *Omatjenne*, *Theileria mutans*, Zebu, PCR-RFLP, Ixodid ticks, Guinea savannah, Cameroon

Posted Date: April 20th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-22365/v1>

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Abstract

Background

Ticks infesting animals constitute one of the most important factors limiting profitable livestock production in sub-Saharan Africa and the region is beleaguered by a paucity of data on diseases implicated in high morbidity and mortality in most of its livestock production systems.

Methods

In a tick endemic Guinea savannah ecosystem, ticks infesting calves and yearlings during their first eighteen months of life were collected weekly and morphologically identified. 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.

Results

Of 31 364 adult ticks, nymphs and larvae collected from the 20 experimental animals, 6525 (20.1%), 7399 (23.6%), 990 (3.2%), 16450 (52.4%) were *Amblyomma variegatum*, *Rhipicephalus* spp., other genera, and tick larvae respectively. Tick infestation rates during the rainy season for *A.variegatum*, *Rhipicephalus* spp. and larvae were 50.3, 12.4 and 28.3, respectively, while in the dry season the proportions were one, 26.2 and 72.8 respectively. *A. variegatum* had no effect on haematocrit ($P > 0.05$) but high *Rhipicephalus* spp. (*Boophilus*) infestation rates significantly reduced haematocrit ($P < 0.01$). All animals had mixed infections of haemoparasites. The effect of season on haematocrit was not significant ($P > 0.05$). 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 effect ($P > 0.05$). No *E. ruminantium* was detected. We report for the first time in Cameroon, the detection of *Anaplasma* sp. *Omatjenne* and *T.mutans* infecting all and four of the animals, respectively. *Babesia bigemina*, and *Anaplasma* sp. *Omatjenne* concurrently occurred in all 20 experimental animals, *A.marginale* in 15 and *T.mutans* in four. 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 ($P > 0.05$).

Conclusion

The absence of clinical cases of the four pathogens detected 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.

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 [1]. The sub-Saharan African region is beleaguered by a paucity of data with respect to what extent ticks and tick-borne diseases are implicated in the high morbidity and mortalities 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 [2, 3, 4]. About 63% of cattle mortalities at IRAD Wakwa, have been associated with ticks and tick-borne diseases [5]. The situation is thought to be similar in livestock production systems where deficient tick control programmes persist [1]. Exotic taurines and cross-bred animals between local cattle and exotic ones, 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 [5] 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 [2]. Factors favouring tick-borne diseases in low input traditional extensive production 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 [6]. Hand tick removal from animals is still very popular as a means of tick control in the High Guinea Savannah of Cameroon [1]. The abundance of different genera of ixodid ticks in most grazing pastures of Cameroon suggest that tick-borne pathogens (babesiosis, ehrlichiosis, anaplasmosis and theileriosis), transmitted by these ticks, are important factors limiting livestock productivity, yet studies on their relative importance with respect to early calf morbidity and mortalities 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 [1]. Adult *A. variegatum* are the main vectors of *E. ruminantium* in cattle [7, 8] although other tick species in the region are possible vectors of the disease. Taken together, the role of these four tick-borne pathogens in constraining ruminant production (usually occurring in definitive hosts as high morbidity and mortality) in the pastoral regions in sub-Saharan Africa is only faintly understood by pastoralists and the available information is obsolete because most of the available results are based on assumptions [5], serology and microscopy with obvious limitations [9, 10, 11, 12].

In this longitudinal study we used molecular tools (PCR-RFLP analysis and sequencing) to identify, monitor and confirm natural transmission by ixodid ticks of four tick-borne pathogens: *Babesia*, *Theileria*, *Ehrlichia* and *Anaplasma* in zebu Gudali (*Bos indicus*) calves and yearlings during their first eighteen months of life. The study determined animal age at first contact with the different pathogens, the effect

of tick infestation burden and season on pathogen presence in blood and packed cell volume of the animals in the tick-endemic ecosystem.

Materials And Methods

Animals and production system

Twenty naive zebu Gudali female and male calves born on the experimental animal farm of IRAD, Wakwa, 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 animals were raised in a sedentary low input traditional cattle production system with grazing in fenced paddocks. 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 in both pre- and post-weaning management. The calves were weaned at the age of nine months.

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 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[13]. 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 savanna grasses such as *Hyparrhenia*, *Panicum* and *Sporobolus*.

The Gudali breed

The Gudali is a short-horned and short-legged zebu cattle (*Bos indicus*) mainly occurring in Nigeria and Cameroon. A small population of the Gudali breed have 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 [14]. Only the Ngaoundere Gudali were used without spraying with any acaricide during the entire study period.

Collection of blood and identification of ticks

Once a week, the animals were restrained and all ticks were removed using a forceps. The ticks were stored in labelled vials containing 70% alcohol and as soon as possible they were counted and identified morphologically using a low-power stereomicroscope with the aid of standard identification keys [15]. All

16450 *Amblyomma* spp. collected, 1101 nymphs and larvae and 573 ticks of the *Boophilus* (*Rhipicephalus*) genera were examined for spp. identification (Fig. 1).

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) 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 PureGene 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 [16]. 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 1 µl of Yellow Sub™ (Geneo BioTech Hamburg, Germany), 11.2 µl distilled water, 5 µl of buffer (20 mM Tris-HCl, pH 8.4; 100 mM KCl), 1.6 µl of MgCl (25 mM), 0.2 µl of dNTP (100 mM each), 0.4 µl each of *Babesia*, *Theileria*, *Anaplasma* and *Ehrlichia* primers (Table 1) (25 µmol/µl) and 0.2 µl 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^R, 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 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.

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

In the second round reactions, microtubes containing 24.5 µl of the master mix consisting of 1 µl of Yellow Sub™ (Geneo BioTech Hamburg, Germany), 15.7 µl of distilled water, 5 µl of buffer solution, 1.7 µl of MgCl₂ (25 mM), 0.4 µl of each primer, 0.13 µl of Taq polymerase and 0.5 µl 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 (1 µg/ml) for 30 minutes to permit visualisation of the DNA using UV light. A photograph of each gel was made (Figs. 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 (*Hind*61 incubation temperature at 37 °C and *Bse*DI 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 6 µl of enzyme-digested product was mixed with 2 µl 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 2hours 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 our 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 [17].

Analysis of collected data

Data was collected from each animal only during its first 18 months of life. Adjustments were made to permit data collected from animals with the same age and during the same season to be grouped together for analysis. Analysis of covariance to look for the effect of season and tick infestation rate on PCV was undertaken. A logistic regression was undertaken to determine the effect of the different tick genera infestation rates on animal blood parasite/bacteria incidence. The logit model employed the maximum likelihood function using the Newton-Raphson algorithm. Threshold significance value was 5%.

Results

Of 31 364 adult ticks, nymphs and larvae collected from the 20 experimental animals during the 18 months study period, 6525 (20.1%), 7399 (23.6%), 990 (3.2%), 16450 (52.4%) were *A.variegatum*, *Rhipicephalus* spp., other genera and tick nymphs and larvae respectively. All 6525 adult *Amblyomma* ticks collected from the animals were screened and confirmed to be *A.variegatum*. Also all 1101 nymphs and larvae examined from the 16450 collected were *A. variegatum*. 573 ticks of the *Rhipicephalus* (*Boophilus*) genera examined for species identification, were made up mainly of *R.decoloratus* (72%), *R.annulatus* (6%) and the other species which included *B.geigy*, *R.lunulatus*, *H.rufipes* and *H.truncatum* occurred only sparingly.

In total during the rainy season tick burdens were twice that of the dry season. However, tick infestation rate in the rainy season was made up of 12.4, 50.3, 9, and 28.3 for *Rhipicephalus* spp., *A. variegatum*, others and larvae respectively while in the dry season the proportions were 26.2, 1, 0.4 and 72.8 for *Rhipicephalus* spp., *A. variegatum*, others and nymphs and larvae, respectively (Fig. 1). Put together, tick species abundance and seasonality were similar to those earlier reported by Stachurski et al.[2] and so could not be the object of more detailed analyses.

Age at first contact

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 ($P > 0.05$). Animals born between October and April (mostly dry season) were younger at their first contact with *B.bigemina* or *T. mutans* while those born between May and September (mainly rainy season) were older at first contact with *B.bigemina* or *T. mutans*.

Mortalities

Two of the experimental animals died of intestinal blockage due to *Toxocara vitulorum* infestation: one at 9 and the other at 11 months age. 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.

Effect of tick infestation rate on PCV and the incidence of blood parasites

Mean PCV were 33.3 and 33.5 in the dry and rainy season respectively. Season had no significant effect ($P > 0.05$) on haematocrit (normal value range 32–45%). From a logistic regression that was run to determine the effect of tick infestation rate on the incidence of blood parasites (Table 2) it was found that *A. variegatum* had no effect on haematocrit ($P > 0.05$) but high *Rhipicephalus (Boophilus)* spp. infestation rate significantly reduced haematocrit ($P < 0.01$). The effect of season on haematocrit was not significant ($P > 0.05$). Furthermore, interaction between season and *Rhipicephalus* spp. infestation rate significantly ($p < 0.05$) reduced haematocrit. All animals had mixed infections of haemoparasites. Infection of the animals by the different haemoparasites was affected to different levels by combinations of either *A. variegatum* or *Rhipicephalus (Boophilus)* spp. (Table 2). 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 effect. Infestation with *A. variegatum* correlated highly with the detection of *A. marginale*, *Anaplasma* sp. *Omatjenne* and *T. mutans* ($p < 0.001$) while infestation of *Rhipicephalus (Boophilus)* spp. correlated highly only with the detection of *A. marginale* and *A. sp. Omatjenne* (Table 2).

Table 2

Effect of tick infestation rate on the incidence of blood parasites

Haemoparasites	<i>Amblyomma variegatum</i>	<i>Boophilus</i> (<i>Rhipicephalus</i>)
Anaplasma spp.	+++	+++
Babesia spp.	+	+
Ehrlichia spp.	+++	+++
Theileria spp.	+++	+
+: Significant ($p \leq 0.05$); +++: Highly significant ($p < 0.001$)		

Molecular analysis of blood samples

Animals positive for *Babesia* and *Theileria* gave a PCR product at 939 bp (Fig. 2) while animals positive for *Anaplasma* and *Ehrlichia* gave a product at 700 bp (Fig. 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 [18] 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 [19]. This pathogen had been renamed *Anaplasma* sp. *Omatjenne*. The two sequences were submitted to GenBank (MN719893.1 for SN404_ and MN719091.1 for SN119). Although more than 20% of adult ticks and all nymphs and larvae infesting the animals were *A. variegatum* only *A. sp. Omatjenne* but no *E. ruminantium* infection was detected in this group of animals.

During the study period the following number of animals indicated in brackets were found positive for any of the following pathogens: *B. bigemina* (20), *Theileria mutans* (4), *A. sp. Omatjenne* (20) and *Anaplasma marginale* (15).

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) (Figs. 4 and 5).

Discussion

The practice of tick control by hand removal of ticks from their predilection sites on animals has remained the tick control method of choice for most local farmers even though it is labour-intensive, cannot be 100% efficient and is considered in the local context to permit disease transmission [1]. This implies that the livestock owners allow ticks to infest their animals before they follow up to remove them. Thus, the time lapse between tick fixation and its removal by the herdsman is enough for the ticks to inoculate pathogen. However, this depends on two factors: the periodicity of tick removal and the

pathogen species that eventually get inoculated. For example, if it is a *Babesia* or a *Theileria*, one needs three days approximately to get the pathogen transmitted. 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 small-scale livestock breeders mainly because of their high cost and irregularity in supply [6]. Other constraints include the supply of fake and dangerous products by charlatans and the lack of technical knowhow in the appropriate use of the products [6].

The 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 (3-7weeks) 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 less attractiveness of some calves to tick vectors in their early life and/or inherited maternal resistance to ticks that may be associated with breed difference to tick infestation and 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 [20]. The established immunity may wane in old animals and disease state resurgence occurring in stressed animals could cause mortalities.

In the present study, the detection rate of *A.marginale* pathogen (in 15 out of 20 animals) was lower than that of *B.bigemina* and *Ehrlichia* spp. (*A. sp. Omatjenne*) both of occurred in 20 out of 20 animals exposed. This finding corroborates with that of Chollet [11] who reported a seroprevalence of antibodies to *Anaplasma* of between 40 and 76% in a cross-sectional study in the same region in which our study was conducted. In these highly endemic areas it would be logical to expect the development of a “pre-immune state” that is due to constant exposure of the animals to the pathogen causing clinical cases to 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. The detection of *T. mutans* pathogens in four out of 20 animals in the present longitudinal study had not been previously reported in the area. Given our small sample size, the presence of other pathogenic strains in the region cannot be excluded and ought to be the subject of future investigations.

Despite the high density of *A. variegatum* ticks and their larvae that infested the animals, no clinical case of cowdriosis (*E.ruminantium*-also commonly called heartwater disease) infection was recorded during the first 18 months of life of the animals. Heartwater disease has been previously diagnosed between 1988 and 1994 (unpublished data) using crushed brain smears stained with Giemsa in adult Gudali cattle and in Gudali X Holstein cross yearlings in the same experimental farm. The observation on *Ehrlichia* sp. detection rate is in line with the findings of Merlin et al. [21] 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. That all animals (20) under two years of age were infected with *Ehrlichia* sp. (*A. sp. Omatjenne*) indicates the endemicity of this pathogen in the region and one is poised to believe that such early infections without clinical disease occurrence may confer cross protecting immunity in the local animals against the other *Ehrlichia* spp. including *E. ruminantium*. Interestingly, a recent molecular screening of over 1500 adult cattle [22] in the three northern regions of Cameroon (including Adamawa where our study was undertaken) detected only one case of *E.ruminantium* in Faro et Deo division of the Adamawa region. In other studies in Cameroon *E. ruminantium* DNA was detected in 142 (28.4%) of 500 un-engorged *A. variegatum* ticks collected from 182 cattle at, 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 from cattle at UFR [23]. A serological study 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 [10]. In Benin, a study undertaken in 4 localities [24] 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% [25]. 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. [26] 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-tRNA^{fMet}*) and detected *Rickettsia africae* in *A. variegatum* ticks, *R. aeschlimannii* in *H. truncatum* and *H. rufipes* ticks, *R. massiliae* in *R. lunulatus* ticks, *R. sibirica* in *H. truncatum* ticks and Candidatus *R. barbariae* in *R. lunulatus* ticks. That *E. ruminantium* was not found in our samples and those of Vanegas et al.[26] and only one case reported recently in the same ecosystem [22], should be a matter of great concern for epizootiologists. Put together, this data disagrees with the general perception of the local animal breeders that heartwater caused by *E. ruminantium* is one of the greatest killer of indigenous young and adult cattle in the High Guinea Savannah zone of Cameroon [1]. This perception of the local cattle breeders may be unfounded. Observed mortalities may be due to other pathogens. 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 [27]. In the present study we found for first time in central Africa that the *Ehrlichia* sp. infecting calves and yearling cattle was *A.sp. Omatjenne* previously reported in goats in Mozambique as *Ehrlichia* sp. Bom Pastor [19] and later renamed *E. sp. (Omatjenne)*. *Ehrlichia* sp. (*Omatjenne*), which is apparently apathogenic has also been detected in several ruminants in South Africa including Boer goats [28], in Uganda [18] and in Ethiopia [29, 30]. 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 [18]. Also, such immune

cross-reactivity under natural conditions hinder, through cross-protection the expression of pathology by those otherwise pathogenic *Ehrlichia* spp. concurrently infecting the animals. Therefore, 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. Although *A. sp. Omatjenne* which we found in our study is apparently apathogenic [28, 31] in ruminants, a strain (*E. sp. (Omatjenne)* 1) of this parasite has been used experimentally to produce disease indistinguishable from cowdriosis in sheep [31] 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 [18]. More so, *Anaplasma* and *Ehrlichia* spp. are reported to cause significant economic losses to the livestock sector in Uganda [18].

Babesia bigemina was detected in all 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 [16] 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 [32, 33]. It has also been reported that in *B. bigemina* infection, calves from non-immune cows are as sensitive as adult animals [34, 35].

In the present study, calves that had their first contact with the pathogen within the first 18 months of life did not reveal any clinical disease condition during their first 18 months of life. This 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 [36].

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 [37], increasing aridity [38] 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 [39, 40]. A situation of endemic stability has been reported more than two decades ago in an *A. variegatum*-infested ecosystem inhabited by indigenous cattle [7]. Maintaining the equilibrium state or balance between host, vector and pathogen is therefore crucial in ensuring endemic stability and thus avoiding economic losses.

Conclusion

This longitudinal study reported on the fate of animals during their first 18 months of life raised in a tick-endemic Guinea savannah ecosystem, in Cameroon. *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 our *Theileria* and *Ehrlichia* samples. Sequencing of these revealed that *T. mutans* occurred only in four of the animals while *Ehrlichia Bom Pastor* (*E. sp. (Omatjenne)*) or (*A. sp. Omatjenne*)

which we report in Cameroon for the first time, infected all the animals during the study. *Anaplasma* sp. *Omatjenne* is apparently an apathogenic bacterium whose presence may protect the animal from infection by a pathogenic species such as *E. ruminantium* which did not occur in any of our experimental animals during the entire study period. It may be that infected animals frequently recover and acquire full immunity to subsequent infections through natural challenge as calves and yearlings, remain carriers of the pathogen without any clinical signs. As such, the tick-endemic Guinea savannah ecosystem of Cameroon is an 'endemically stable' region where an established equilibrium between the host, the vector and the parasite is maintained in the interest of the animals. Disease control strategies in the study area should therefore aim at reducing the tick vector population to low levels resulting in low disease transmission while avoiding complete vector eradication. More pathogen isolations and extensive field surveys should provide further insights on the epizootiological picture of *E. sp. (Omatjenne)* and *T. mutans* in the local livestock production systems.

Declarations

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.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

Funds for this research were provided by the first author and some reagents were bought with funds from a DGCD grant to Professor Dirk Geysen of the Institute of Tropical Medicine (ITM) Prince Léopold (Antwerpen, Belgium).

Acknowledgment

This publication is dedicated to the Dr. Sali Ngam (Veterinarian) who was an important collaborator in the generation of a good part of the data presented in this paper. He passed into eternity too soon, creating a vaccum with a lot of difficulties and pain in us which delayed the writing up of this paper. We are grateful to Dr Gharbi Mohamed of the University of Tunisia for proof reading and providing very useful suggestions to improve this manuscript. We thank the herdsmen of the veterinary section of IRAD Wakwa for proper herding and restraining of the animals during the entire study period.

Authors' contributions

MDA: Designed experiments, collected samples, extracted DNA, obtained ethical clearance, analysed data, wrote first draft, edited and reviewed the manuscript, supervised all field and some lab experiments.

NS: Analysed samples in ITM using molecular tools, worked on first draft of manuscript.

DG: designed experiments and specific primers, edited and reviewed the manuscript, supervised lab experiments.

MM: Lead the team for identification of ticks, edited and reviewed the manuscript.

MY: collected samples, extracted DNA, analysed data, reviewed the manuscript.

MTK: collected samples and reviewed the manuscript.

NCW: collected samples and reviewed the manuscript.

AND: analysed data, reviewed the manuscript

All authors read and approved the final manuscript.

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Figures

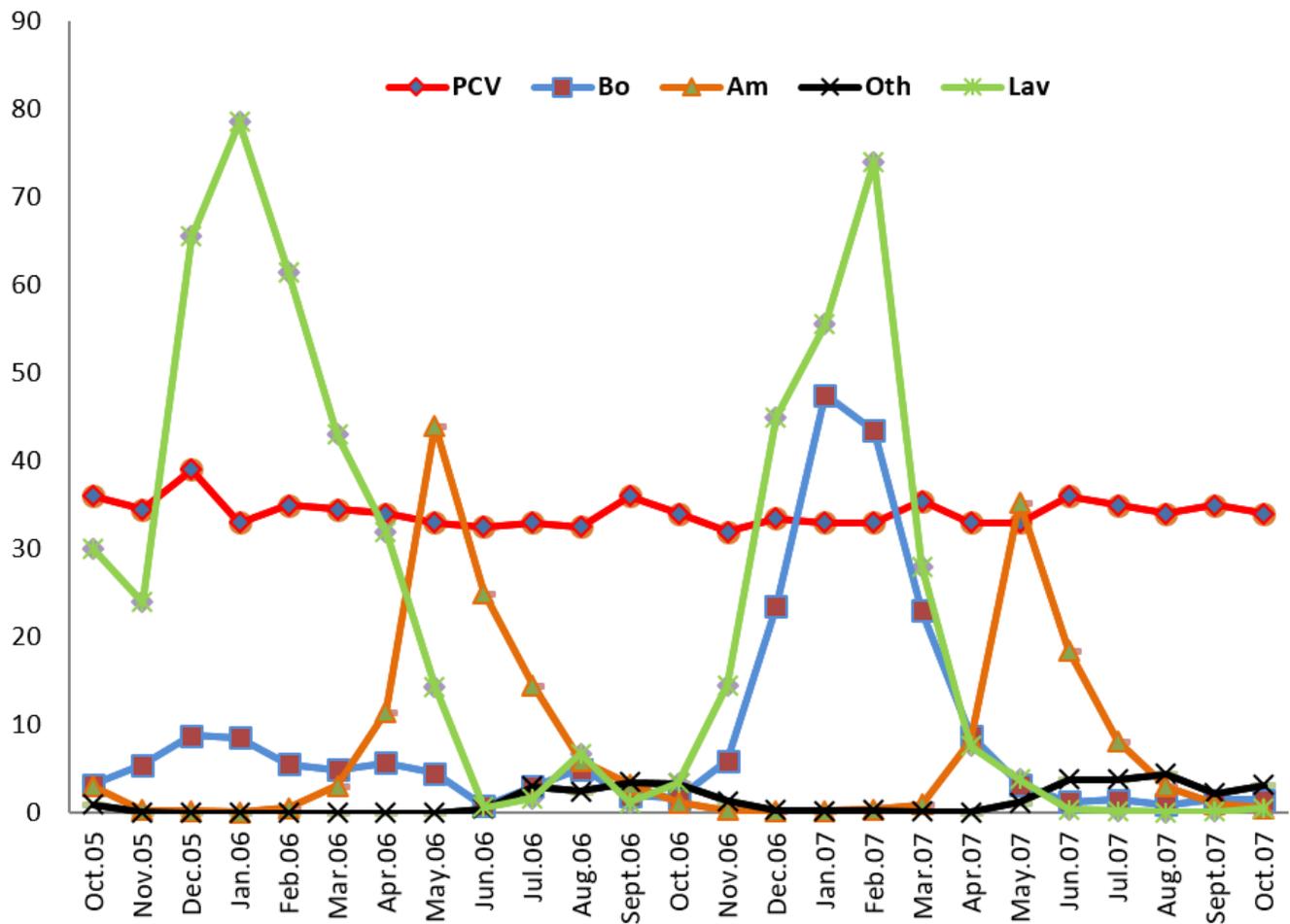


Figure 1

Tick infestation showing seasonal dynamics of various genera and PCV variation during the study period. Dry season: November to March; Rainy season: April to October. 05: 2005; 06: 2006; 07:2007. PCV: Packed Cell Volume; Threshold value for anaemia is <32% PCV. Bo: *Rhipicephalus* spp; Am: *Amblyomma variegatum*; Oth: other tick species including *Hyalomma*; Lav= larvae and nymphs of abundant ticks.

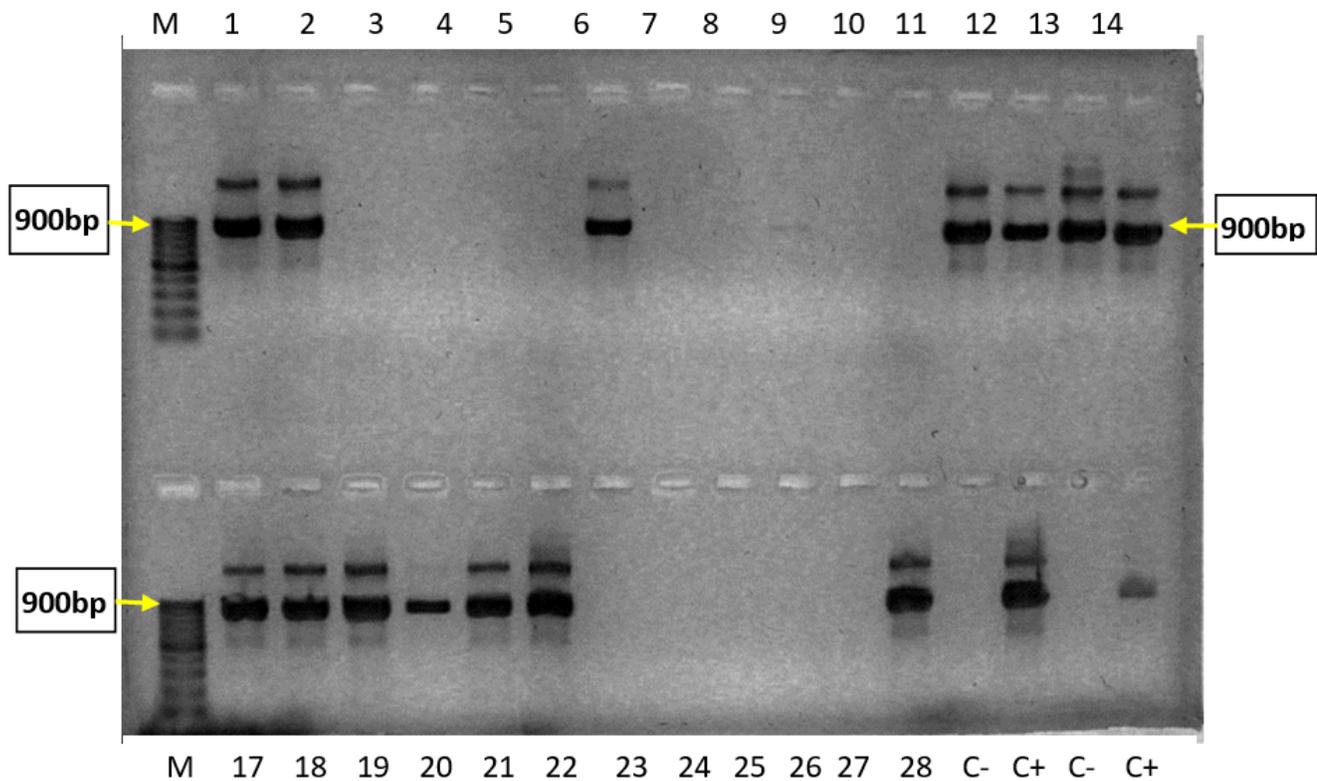


Figure 2

Samples of PCR positive parasites (*Babesia* spp. and *Theileria* spp.) resolved in 2% agarose gel coloured with ethidium bromide and photographed under UV light. 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

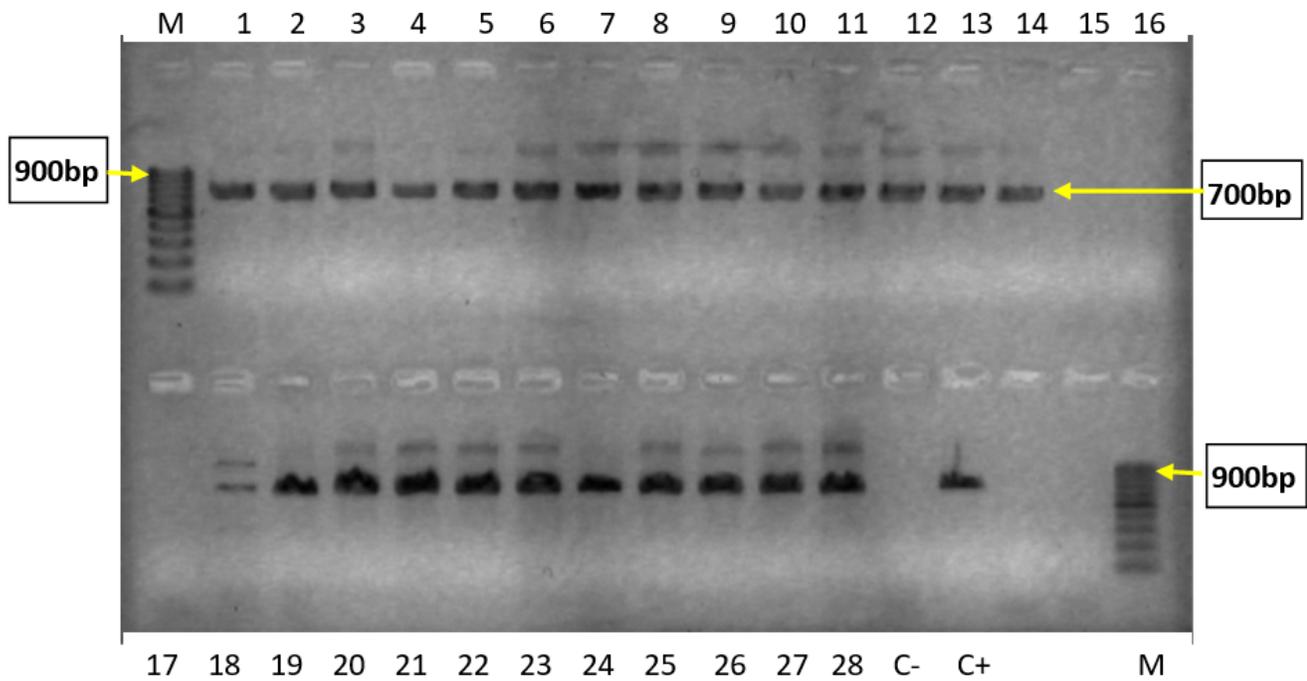


Figure 3

Samples of PCR positive bacteria (*Anaplasma* spp. and *Ehrlichia* spp.) resolved in 2% agarose gel coloured with ethidium bromide and photographed under UV light. Positive lanes: 1-14 and 18-28 Negative lines: 15,16,and 17 M: molecular marker C- : Negative control C+ :: Positive control

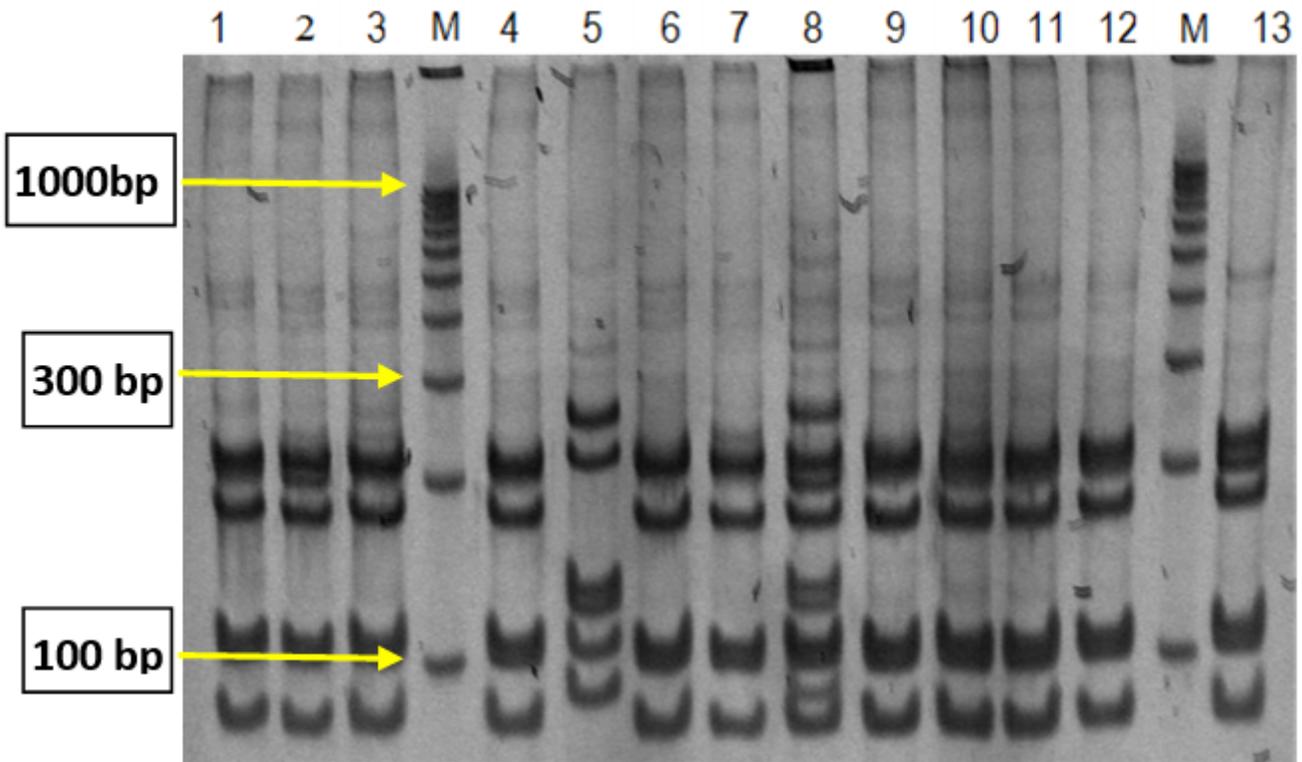


Figure 4

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

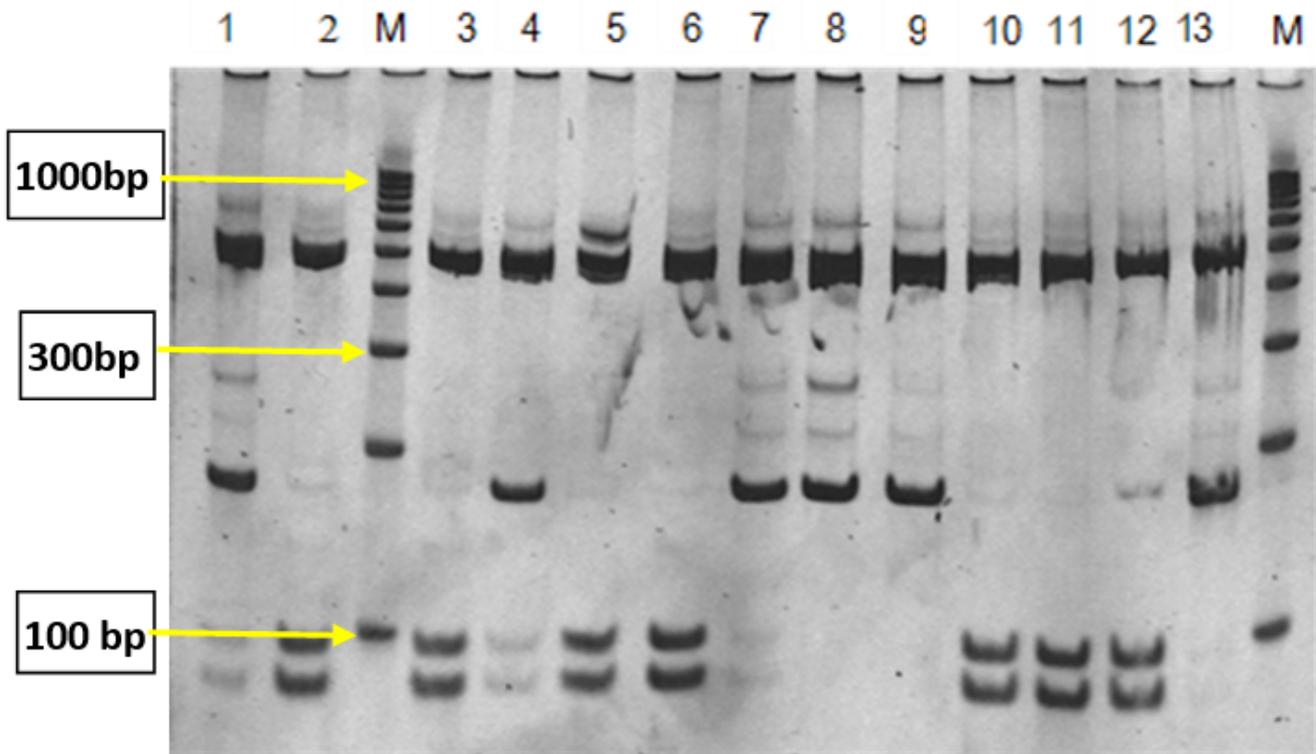


Figure 5

RFLP profiles of samples positive post-PCR, digested with Hind6I enzyme, resolved and visualized in PAGE gels, coloured with Sybr Green and UV photographed. 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

Supplementary Files

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