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Molecular evidence of *Babesia caballi* and *Theileria equi* in equines and ticks in Nigeria: prevalence and risk factors analysis

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Research Article

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Abstract

Babesia caballi and *Theileria equi* are biological agents responsible for equine piroplasmosis (EP). We conducted a robust and extensive epidemiological study in Nigeria on the prevalence and risk factors of EP. Blood (468, both horses and donkeys) and ticks (201 pools) were screened using polymerase chain reaction (PCR). DNA of equine piroplasms was observed in tick pools with *B. caballi* amplified in *Rhipicephalus evertsi evertsi* only [minimum infection rate (MIR) of 7.6%] while *T. equi* was observed in *R. e. evertsi* (MIR, 61.6%), *Hyalomma dromedarii* (MIR, 23.7%) and *H. truncatum* (MIR, 50.0%). Overall results showed that 196/468 (41.9%) animals were positive for equine piroplasms (both *B. caballi* and *T. equi*). The prevalence for *T. equi* was 189/468 (40.4%) compared to 7/468 (1.5%) for *B. caballi*. In the horses and donkeys, respectively, the prevalence for *T. equi* was (39.9%; 112/281) and (41.2%; 77/187) compared with (1.4%; 4/281) and (1.6%; 3/187) due to *B. caballi*. Our analysis showed that location (Jigawa state), Talon breed, horses used for work and reproduction, unsatisfactory husbandry practices, contact with other mammals are risk factors that associated positivity to *T. equi* infection in horses, whilst horses kept on intensive management appeared to be less prone to infection. On the other hand, Jangora breed of donkeys and location (Jigawa state) are risk factors to infection with *T. equi* in donkeys. Findings suggest the persistence of EP in equids and ticks in Nigeria.

Introduction

Ticks and associated tick-borne parasitic diseases are a major constraint to the health and productivity of livestock and indirectly affecting the livelihood of their owners particularly in developing countries of the world (Dahiya *et al.*, 2018). Equine piroplasmosis (EP) is an important tick-borne (haemoprotozoan) disease of equids (including horses, donkeys and zebras) caused by *Babesia caballi* and *Theileria equi*. This disease is widespread in most subtropical and tropical countries of the world where competent tick vectors are present. Ixodid ticks belonging to the genera *Rhipicephalus*, *Amblyomma*, *Dermacentor*, *Hyalomma* and *Haemaphysalis* are known vectors for these pathogens (Scoles and Ueti, 2015). However, the above-mentioned pathogens can also be transmitted *via* blood transfusion, contaminated syringes and clinical instruments (Short *et al.*, 2012), although the latter is of minor epidemiological significance (Onyiche *et al.*, 2019).

EP is associated with morbidity and mortality in acute cases with impact on the equine industry. Clinical signs of EP in acute form include fever, inappetence, pale mucous membrane, malaise and increased heart and respiratory rates, while in chronic form of infection, the clinical signs include transient fever, mild anaemia, enlarged spleen, weight loss and reduced work efficiency (Zobba *et al.*, 2008). *Theileria equi* infection is responsible for severe clinical disease compared with *B. caballi*. Nevertheless, clinical signs and disease severity vary considerably between regions (Wise *et al.*, 2013). Recovered equines with *T. equi* infection remain long life carriers, whereas *B. caballi* infection is self-limiting and infected animals clear the infection within 4 years (Rothschild, 2013).

EP has been categorized by the OIE as a notifiable disease due to the socioeconomic impact of the disease as it leads to restrictions in the international movement of horses (OIE, 2014). This has led many countries which are EP free to put in place stringent measures to prevent introduction into their territories. Knowledge of the presence of a disease agent and the epidemiological factors that are responsible for transmission or perpetuation within a particular geographical space is vital in the design of effective control measures. Epidemiological factors/variables associated with the occurrence of EP can vary across different geographical areas, altitude, management system and areas of origin of the equids (Santos *et al.*, 2011).

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Nigeria, a country in West Africa, has huge livestock population, especially in its northern region due to abundance of savannah type vegetation and absence of tsetse flies. Horses are kept in small flocks ranging from 5 to 30 in numbers and are used for polo sport and during traditional ceremonies. On the other hand, donkeys are kept in small flocks ranging from 2 to 5 in numbers and are used as working animals in the transportation of goods as they are relatively cheaper (Hassan *et al*., 2013). However, limited information is available on the status of EP in equids in Nigeria. Only recently, the first molecular evidence of equine piroplasms was reported in Nigeria equids (Idoko *et al*., 2020). Prior to this recent report, all the studies conducted so far on the prevalence of EP in Nigeria have been largely based on microscopic examination of blood smears and serological tests (Sanusi *et al*., 2014; Turaki *et al*., 2014; Oladipo *et al*., 2015; Mshelia *et al*., 2016).

Serological tests have been widely adopted in recent times in epidemiological surveys of apicomplexan protozoan parasites. However, cross-reactivity of closely related species has been widely reported due to lesser specificity (Mans *et al*., 2015). Therefore, further optimization for precision is required. On the other hand, molecular and bioinformatics techniques including gene sequencing have improved our knowledge and understanding of the genetic relatedness between different organisms (Chan and Ragan, 2013). Therefore, the aim of this study was to (i) investigate the prevalence of *B. caballi* and *T. equi* in equines (horses and donkeys) in North-Western Nigeria using a combination of microscopy and molecular techniques; (ii) to identify the possible risk factors associated with the occurrence of these pathogens in horses and donkeys; (iii) to determine the tick species which are potentially involved in the transmission of these piroplasms to equids in the study areas by detecting *B. caballi* and *T. equi* DNA in the ticks.

Materials and methods

Study area

The North-West region is a semi-arid zone and is the largest region in Nigeria with a human population of 35 786 944 (NPC, 2006). The current estimates put the population heads of horses and donkeys in the country as 206 212 and 936 832, respectively (FAO, 2016). This region has a savannah type of vegetation. The temperature ranges from 18 to 45°C with a mean of 27°C. The raining season usually begins in May through to October with a mean annual rainfall of 508–1016 mm and dry season is from October to April. Samples (blood and ticks) from horses were collected in four states (Kano, Kaduna, Jigawa and Katsina) while donkeys were sampled in three states (Kano, Jigawa and Katsina). Sampling points are presented on the map of Nigeria shown as Fig. 1.

Study design and sampling locations

In this study, a cross-sectional study design was used. Non-probability sampling, combining both convenient and snowball sampling techniques, was employed. Samples were collected from May to September 2017. The population of equines (horses and donkeys) is not evenly distributed within these study areas, and therefore, representative samples were collected in these areas. A total of 468 samples were collected from 281 horses (Kano = 113, Jigawa = 67, Katsina = 54 and Kaduna = 47) and 187 donkeys (Kano = 57; Jigawa = 70 and Katsina = 60). Within each study area, samples were collected from different sampling points. We visited stables and individual owned horses for sampling after obtaining owner's consent. Samples were also collected from working donkeys at known points where their owners congregate with their animals and clients come around to hire them for their services.

Blood and tick sample collection

About 5 mL of whole blood was collected from the jugular vein of horses and donkeys after which the collected whole blood was transferred into labelled EDTA coated tubes. All animals were apparently healthy during sampling. All collected blood samples were transported to the laboratory on ice pack within 4 h after collection. In the laboratory, 125 μL of blood was dispensed on the marked spot within the Classic FTA card (Whatman® GE Healthcare, UK). All cards were labelled, air dried and stored at room temperature for further analysis.

The skin on both sides in horses and donkeys covering known predilection sites for ticks (the perineum region, abdomen, thigh, ear, and neck) was carefully examined for the presence of ticks. Ticks were collected using forceps into labelled tubes plugged with cotton wool for each animal. The labelled tubes contained information of the identity of the animals including their location.

Epidemiological questionnaire

To assess the possible risk factors associated with exposure to tick-borne apicomplexan parasites (*B. caballi* and *T. equi*) in equids, a closed-ended, semi-structured questionnaire was used to collect information of each horse and donkey on individual and ecological factors that might influence positivity to EP. Some of the factors investigated by the questionnaire were organized as follows: satisfactory husbandry practices which include (i) appropriate husbandry practices for the horses, (ii) farm with adequate infrastructure and (iii) presence of sanitary control and constant veterinary assistance; unsatisfactory husbandry practices include (i) farm with inadequate infrastructure and (ii) farm lacking systematic sanitary control, and when veterinary assistance was present, it was sporadic or used only in cases of emergency; presence or absence of ticks on animals; horse bred in close contact with other mammals (if cattle and horse shared the same pasture or were located close in proximity) or without contact with cattle; breeding systems: intensive (confined system: animals had no access to outside areas), semi-confined/extensive system (limited access or total access to pasture areas) or extensive (complete access to the environment) (Santos *et al*., 2011); activities of the horses (sport exhibition; sports and ceremony only, ceremony and recreation only, work/walk and reproduction); acaricide used (carbamate, cypermethrin, diazinon, abomec, kerosine); frequency of acaricide use (monthly, quarterly and occasionally); gender (male or female); age (<6; 6–12; over 12 years); breed definition and location. Due to the differences in the management systems between horses and donkeys in the study areas, all the variables listed above were collected for horses, while for the donkeys, we collected age, gender, breed, location, presence of ticks and altitude only. Data were collected using Open Data Kit (ODK), a digital application system for Android devices. We uploaded the questionnaire on the online platform (https://odk.ona.io/). Subsequently, we downloaded the offline version on a mobile phone and available offline for usage without mobile network. The captured data were first locally stored on the mobile phone. The collected data were later transferred to the cloud at the end of the day's using Internet service for safe retrieval. On completion of the study, the data were downloaded from the server as a Microsoft Excel worksheet file.

Microscopy for the detection of piroplasms

Thin blood smear was prepared using a drop of blood on a grease-free glass slide. After air drying and fixing with absolute

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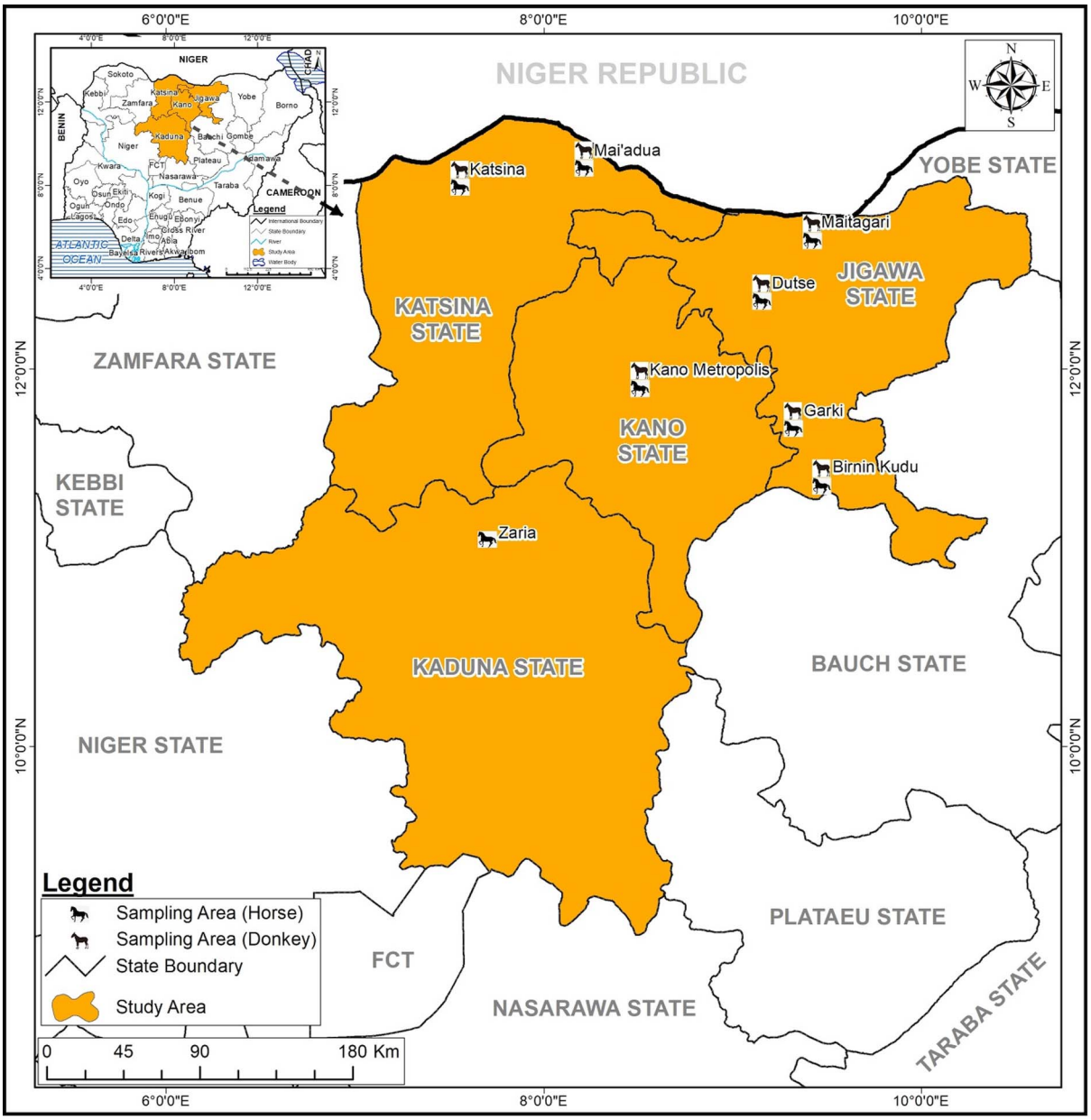

Fig. 1. Map of Nigeria showing the study areas where samples were collected from horses and donkeys. (Map was created using ArcGIS version 10.6 by ESRI, Redlands, CA, USA.)

methanol, glass slides were stained with 10% Giemsa stain in the laboratory. All slides were observed using immersion oil at 100 × objective for the presence of *B. caballi* and *T. equi* using a light microscope (Olympus®, Japan) (Bose *et al.*, 1995).

Morphological identification of ticks

All tick samples collected from infested animals were identified to species level based on standard keys using a stereomicroscope (Olympus®) (Walker *et al.*, 2003; Apanaskevich and Horak, 2005). Species identification and gender of the ticks were recorded. All tick specimens were preserved in 70% ethanol and kept at 4°C.

DNA extraction from blood dried on FTA cards

Genomic DNA was extracted from Whatman FTA™ cards using 5% Chelex 100 resin following the manufacturer's instructions. Briefly, about 6 mm punch of the dried blood spot was removed from the card. The disc was macerated using 20 G needle in a 2 mL microcentrifuge tube. Thereafter, 1000 μL of double distilled water (DDW) was added and incubated at room temperature for 10 min. After incubation, the supernatant was removed, and the step was repeated. The tubes were centrifuged for 3 min at 20 000 ***g***. The supernatant was removed after which 200 μL of the freshly prepared 5% Chelex was added using a large-bore pipette tip. The samples were incubated at 56°C for 20 min after which they were briefly vortexed for 15 s and further incubated for 8 min at 100°C. Samples were again centrifuged for 3 min at 20 000 ***g***. The supernatants containing DNA were transferred to a new 1.5 mL microcentrifuge tubes and stored at −20°C until used for downstream analysis.

Washing and homogenization of ticks

Prior to washing and homogenization, ticks were pooled. Pooling was carried out for ticks of the same species and on the same animal with a maximum of five ticks per pool. Ticks were washed twice using DDW after the removal of ethanol in microcentrifuge

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tubes as described by Silaghi *et al.* (2011). A sterile scissor was used to cut the ticks into small pieces and 200 μL of sterile PBS was added. Ticks were homogenated using Tissue Lyser LT (Qiagen, Hilden, Germany) for 10 min at an oscillation frequency of 30 Hz. The supernatant was removed after centrifugation at 3000 ***g*** for 3 min.

Extraction of genomic DNA from tick homogenate

A maximum of 80 μL of the tick homogenate (supernatant) was used for DNA extraction. DNA was extracted using QIAamp DNA mini Kit (Qiagen) following the manufacturer's instruction.

Amplification of B. caballi *and* T. equi *DNA in tick and blood*

Species-specific polymerase chain reaction (PCR) was carried out for detecting *B. caballi* and *T. equi* DNA in both blood and ticks from equines. For *T. equi*, the reaction was carried out using primer pairs Bec-UF2 (forward): 5′-TCGAAGACGATCAGATACCGTCG-3′ and Equi-R (reverse): 5′-TGCCTTAAACTTCCTTGCGAT-3′ (Alhassan *et al.*, 2005, fragment size: 392 bp), while for *B. caballi*, the primer pairs used are Bec-UF2 (forward): 5′-TCGAAGA CGATCAGATACCGTCG-3′ and Cab-R (reverse): 5′-CTCGTT CATGATTTAGAATTGCT-3′ (Alhassan *et al.*, 2005, fragment size: 540 bp). The reactions were performed in a total volume of 25 μL consisting of 12.5 μL of Amplitaq Gold® 360 master mix, 1 μL of each primer (10 μM each of both forward and reverse primer), 5.5 μL Nuclease-Free Water and 5 μL template DNA using a proFlex thermocycler (Applied Biosystem, California, USA, Supplied by Thermo Fisher Scientific) with the following cycling conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 60 s and a final extension at 72°C for 7 min. Every reaction set had a positive and negative control (molecular grade water). DNA samples prepared from *in vitro* cultures of *B. caballi* and *T. equi* were used as positive controls.

Gel electrophoresis and sequencing

All PCR products were separated on a 1% agarose gel stained with ethidium bromide and visualized under UV trans-illuminator (Gene Genius Imaging System, Syngene, UK). Amplicons were sent for sequencing in the forward direction [Inqaba Biotechnical (Pty) Ltd., Pretoria, South Africa]. Nucleotide searches were carried out to identify highly similar sequences on the NCBI database using BLAST(n).

Statistical analysis

Data obtained from the prevalence study from horses and donkeys were analysed using SPSS version 20.0. We used the PCR results and a linear model to identify variables associated with *B caballi* and *T. equi* infections. Univariable regression model was built to estimate the risk factors associated with positivity to *T. equi* and *B. cabalii* in both horses and donkeys to a variety of independent variables. The output from the univariable analysis was used in the multivariable model with *P* value set at <0.25. The multivariable model was constructed only for infection with *T. equi* and not for *B. caballi* due to few numbers of positives. Stepwise approach was used in the building of the multivariable model creating a main effects model with a significance level set at $\alpha = 0.10$. Changes in the coefficient (cofounding) were examined in the model for the remaining variables that were significant after expulsion of the cofounding variable. The fitness of the model was assessed following completion of the multivariable model using the Hosmer–Lemeshow test (Bursac *et al.*, 2008). Confidence intervals (CI: lower and upper) at 95% were calculated for infection rates with piroplasms in ticks. The minimum infection rate (MIR) was accepted and expressed in simple percentages (only one sample was considered as positive, in a pool of adult tick). Statistical significance of differential infection rates in the ticks was analysed using GraphPad Prism version 5.0. Significant level ($P < 0.05$) was used throughout the analysis.

Results

Microscopic detection of equine piroplasms in blood

Microscopic examination of Giemsa stained blood films from 468 equid samples revealed the presence of *B. caballi* and *T. equi*. In total, 43 equids were positive with an overall prevalence of 9.2%. The prevalence was higher in the donkeys (25/187; 13.4%) as compared to horses (18/281; 6.4%). Based on parasite species and host, the prevalence of *T. equi* was higher in both horses and donkeys (4.9 and 11.8%) compared with *B. caballi* (1.4 and 1.6%), respectively.

Molecular detection of T. equi *in the blood of equines*

Theileria equi infections were demonstrated in horses and donkeys by amplifying 392 bp fragment of 18S rRNA. The overall prevalence for *T. equi* in horses in all sampled areas of Nigeria was 39.9% (112/281) while that in the donkeys was 41.2% (77/187). BLASTn query of the sequences obtained from selected PCR amplicons showed 96.9–99.8% homology with *T. equi 18S rRNA* sequence from horses in Israel (GenBank accession no: MK392054.1 and MK392059.1). The *T. equi* sequences generated in the present study have been registered with the NCBI GenBank with the following accession numbers: MN620483, MN620483, MN620484, MN620485, MN620486 and MN620487.

Risk factors analysis in relation to T. equi

Horses

A total of 281 horses were tested of which 112 were positive with an overall prevalence of 39.9% (Table 1). The multivariable logistic regression analysis showed statistically significant ($P < 0.05$) influence on risk factors such as location, breed, activities of horses, husbandry practices, contact with other mammals and management system on the prevalence of *T. equi* in horses (Table 2). Based on location, horses in Jigawa state were 3.89 times (OR = 3.890, 95% CI 1.849–8.219, $P = 0.001$) more likely to be infected while those in Kaduna state are 0.234 less prone to infection (OR = 0.234, 95% CI 0.108–0.506) (Table 2). The odds of a positive result were almost 4-fold (OR = 3.872, 95% CI 1.752–8.558) greater in horses involved in reproduction and work (Table 2). Concerning management system, horses on extensive system were found to be more positive to infection compared to those on intensive management (51.4 *vs* 29.6%) in the univariable analysis (Table 1). The multivariable analysis also confirmed that horses on intensive management were 2.5-fold less prone to infection (OR = 2.513, 95% CI 1.201–5.261, $P = 0.019$) (Table 2). According to breed, Talon are 0.307 times less prone to infection as compared to other breeds comprising of Argentine and Thoroughbred. Furthermore, horses with satisfactory husbandry practices were 0.489 times less prone (OR = 0.489, 95% CI 0.301–0.796, $P = 0.005$) to get infection. Additionally, horses with no contact with other mammals are 0.278 times less prone to infection (OR = 0.278, 95% CI 0.119–0.649, $P = 0.004$). Other risk factors which were not statistically significant in the multivariable regression analysis include gender, age and frequency of acaricide use (Table 2).

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Table 1. Results of the univariable analysis associated with *Theileria equi* and *Babesia caballi* PCR positivity in horses in North-Western, Nigeria

			Theileria equi			*Babesia caballi*		
Variables	Category	Number examined	Number positive (%)	χ^2	*P* value	Number positive (%)	χ^2	*P* value
Locations	Kano	113	49 (43.4)	44.80	$P < 0.001$	4 (3.5)	6.033	0.110
	Kaduna	47	36 (76.6)			0 (0)		
	Katsina	54	16 (29.6)			0 (0)		
	Jigawa	67	11 (16.4)			0 (0)		
Breeds	Arewa	182	64 (35.2)	10.36	0.016	2 (1.1)	5.43	0.143
	Sudanese	55	22 (40.0)			0 (0)		
	Talon	36	23 (63.9)			2 (5.6)		
	Others	8	3 (37.5)			0 (0)		
Gender	Male	198	74 (37.4)	1.75	0.189	2 (1.0)	0.82	0.366
	Female	83	38 (45.8)			2 (2.4)		
Age (years)	Under 6	77	37 (48.1)	3.06	0.216	1 (1.3)	0.75	0.687
	Between 6 and 12	172	64 (37.2)			2 (1.2)		
	Over 12	32	11 (34.4)			1 (3.1)		
Altitude (meters)	Btw 0 and 399	212	86 (40.6)	0.18	0.671	3 (1.4)	0.19	0.978
	Above 400	69	26 (37.7)			1 (1.5)		
Activities of horses	Sports (Polo)	107	54 (50.5)	14.14	0.012	0 (0)	2.14	0.829
	Sports and ceremony	26	7 (26.9)			2 (7.7)		
	Ceremony and recreation	100	41 (41.0)			2 (2)		
	Reproduction and work	48	10 (20.8)			0 (0)		
Husbandry practices	Satisfactory	160	52 (32.5)	8.39	0.004	1 (0.6)	1.69	0.194
	Unsatisfactory	121	60 (49.6)			3 (2.5)		
Presence of ticks	Yes	105	41 (39.1)	0.05	0.830	1 (0.9)	0.27	0.607
	No	176	71 (40.34)			3 (1.7)		
Acaricide used	Carbamate	4	0 (0)	4.67	0.457	0 (0)	23.36	$P < 0.001$
	Cypermethrin	40	17 (42.5)			0 (0)		
	Diazinon	54	23 (42.6)			0 (0)		
	Abomec	5	1 (20.0)			0 (0)		
	Kerosene (lamp oil or paraffin)	3	2 (66.7)			1 (33.3)		
	Not sure	175	69 (39.4)			3 (1.7)		
Frequency of acaricide use	Monthly	5	0 (0)	6.04	0.109	0 (0)	0.36	0.947
	Quarterly	3	0 (0)			0 (0)		
	Occasionally	100	44 (44.0)			1 (1)		
	Not sure	173	68 (39.3)			3 (1.7)		
Contact with other mammals	Yes	51	13 (25.5)	9.14	0.010	1 (1.9)	0.88	0.643
	No	49	27 (55.1)			0 (0)		
	Not sure	181	72 (39.8)			3 (1.7)		
Mgt. system	Intensive	142	42 (29.6)	25.84	$P < 0.001$	1 (0.70)	1.17	0.559
	Extensive	37	19 (51.4)			1 (2.70)		
	Semi-intensive	102	51 (50.0)			2 (1.9)		
Total		281	112 (39.8)			4 (1.4)		

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Table 2. Multivariable logistic analysis of risk factors associated with *Theileria equi* PCR positivity in horses in North-Western Nigeria

Variables	Category	OR (95%CI)	*P* value
Location	Kano	a	
	Kaduna	0.234 (0.108–0.506)	$P < 0.001$
	Katsina	1.818 (0.909–3.635)	0.094
	Jigawa	3.890 (1.849–8.219)	0.000
Breed	Arewa	a	
	Sudanese	0.814 (0.438–1.512)	0.526
	Talon	0.307 (0.146–0.646)	$P < 0.001$
	Others	0.904 (0.209–3.907)	1.000
Gender	Male	a	
	Female	0.707 (0.421–1.019)	0.229
Age (years)	Under 6	a	
	Between 6 and 12	1.561 (0.906–2.689)	0.125
	Over 12	1.766 (0.750–4.156)	0.211
Activities of horses	Sports (Polo)	a	
	Sports and ceremony	2.765 (1.074–7.123)	0.047
	Ceremony and recreation	1.466 (0.846–2.541)	0.209
	Reproduction and work	3.872 (1.752–8.558)	$P < 0.001$
Husbandry practices	Unsatisfactory	a	
	Satisfactory	0.489 (0.301–0.796)	0.005
Contact with other mammals	Yes	a	
	No	0.278 (0.119–0.649)	0.004
	Not sure	0.523 (0.261–1.049)	0.072
Frequency of acaricide use	Monthly	a	
	Occasionally	0.115 (0.006–2.145)	0.073
	Not sure	0.140 (0.008–2.575)	0.158
Management system	Extensive	a	
	Intensive	2.513 (1.201–5.261)	0.019
	Semi-tensive	1.056 (0.497–2.241)	0.002

[a]Reference category.

Donkeys

A total of 187 horses were tested of which 77 were positive with an overall prevalence of 41.2% (Table 3). The multivariable logistic regression analysis showed statistically significant ($P < 0.05$) influence on risk factors such as location and breed on the prevalence of *T. equi* in donkeys (Table 3). Based on location, horses in Jigawa state were 3.20 times (OR = 3.200, 95% CI 1.525–6.716, $P = 0.002$) more likely to be infected (Table 4). The odds of positivity were 11-fold greater in Jangora breed (OR = 11.330, 95% CI 1.779–72.200, $P = 0.010$). Other risk factors which were not statistically significant in the multivariable risk factors are age, presence of ticks and altitude.

Molecular detection of B. caballi *in the blood of equines*

Babesia caballi-infected animals were detected by amplifying a 450 bp fragment of rhoptry associated protein-1 (*rap-1*) gene from DNA samples. The overall prevalence of *B. caballi* was 1.4% (4/287) in the horses and 1.6% (3/187) in the donkeys. The sequences obtained from the positive PCR amplicons showed 99.0–100.0% homology with *B. caballi* sequences from a horse in Israel (GenBank accession no: MK346860.1). The *B. caballi* sequences generated in the present study have been registered with the GenBank with the following accession numbers: MT126819, MT126820, MT126821 and MT126822.

Risk factors analysis in relation to B. caballi

Horses

Due to low infection rate, the multivariable regression analysis was not carried out. In the univariable analysis, risk factors such as location and acaricide used were statistically significant ($P < 0.001$). Based on location, *B. caballi* was detected only in Kano with a prevalence of 3.5% (4/113) while no positive sample was found from other sampled locations (Kaduna, Katsina and Jigawa) (Table 1). Horses in which Kerosene was used as possible acaricide had higher prevalence (33.3%) to *B. caballi* compared with those placed on other types of acaricide such as carbamate, cypermethrin and diazinon (Table 1).

Donkeys

The multivariable regression analysis was not carried out for donkeys as well due to very low number of positive animals for the different variables. No significant difference ($P > 0.05$) was

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Table 3. Results of the univariable analysis associated with *Theileria equi* and *Babesia caballi* PCR positivity in donkeys in North-Western, Nigeria

			Theileria equi			*Babesia caballi*		
Variables	Category	Number examined	Number positives (%)	χ^2	*P* value	Number positives (%)	χ^2	*P* value
Location	Kano	57	31 (54.4)	10.16	0.006	2 (8.8)	2.45	0.293
	Katsina	60	27 (45.0)			1 (1.7)		
	Jigawa	70	19 (27.1)			0 (0)		
Breed	Akaza	9	6 (66.7)	13.19	0.040	0 (0)	6.01	0.422
	Auraki	55	26 (47.3)			0 (0)		
	Fari	18	6 (33.3)			0 (0)		
	Goho	12	8 (66.7)			1 (8.3)		
	Idabari	67	25 (37.3)			2 (2.9)		
	Jaba	6	3 (50.0)			0 (0)		
	Jangora	20	3 (15.0)			0 (0)		
Gender	Male	117	50 (42.7)	0.31	0.576	1 (0.9)	1.11	0.292
	Female	70	27 (38.6)			2 (2.9)		
Age (years)	Under 6	55	17 (30.9)	4.06	0.131	0 (0)	2.22	0.329
	Between 6 and 12	99	43 (43.4)			3 (3.0)		
	Over 12	33	17 (51.5)			0 (0)		
Presence of ticks	Yes	1	1 (100.0)	1.44	0.230	0 (0)	0.02	0.898
	No	186	76 (40.9)			3 (1.6)		
Altitude (meters)	0–199	42	17 (40.5)	3.49	0.175	0 (0)	2.34	0.310
	200–399	91	43 (47.3)			1 (1.1)		
	Above 400	54	17 (31.5)			2 (3.7)		
Total		187	77 (41.2)			3 (1.6)		

observed across all the variables in the risk factors in the univariable analysis (Table 3). Based on location, the prevalence was highest in Kano with 8.8% compared with 1.7% in Katsina. All 70 donkeys screened in Jigawa state tested negative (Table 3). The Goho breed had the highest prevalence of 8.3% compared with 2.9% recorded for the Idabari. The *B. caballi* prevalence in donkeys increased with altitude, and the highest prevalence was found in altitude above 400 m above sea levels (Table 3).

Molecular detection of equine piroplasms in ticks

A total of 538 ticks infesting equines were collected and morphologically identified to species level. Of these ticks, 535 were from 105 horses and three were from a donkey. Altogether, six species were found including *Rhipicephalus e. evertsi*, *Hyalomma dromedarii*, *H. rufipes*, *H. truncatum*, *R. decolaratus* and *R. sanguineus* (Table 5). These ticks were pooled based on species and host and a total of 201 pools were screened for the detection of *B. caballi* and *T. equi*.

Both *B. caballi* and *T. equi* DNA was successfully detected in tick pools using species-specific PCR primers. For *T. equi*, of the 201 tick pools tested, the overall MIR was 57.2% (108/201) (Table 5). Based on location, Kano state had the highest MIR of 64.3% followed by Katsina (MIR 59.9%), Kaduna (MIR 48.1%) and lastly Jigawa (MIR 38.7%). No significant difference ($P > 0.05$) was observed across study locations (Table 5). *Theileria equi* DNA was detected in three tick species namely *R. e. evertsi*, *H. dromedarii* and *H. truncatum*. Out of 176 tick pools of *R. e. evertsi*, 102 were positive with MIR of 61.6%. Only one out of two tick pools of *H. truncatum* was positive with MIR of 50.0% and lastly *H. dromedarii* had MIR of 23.7% (Table 5). BLASTn query of 392 bp sequences obtained in the present study had 96.9–99.8% identity scores with sequences on the NCBI database (Genbank accession no: MK346860.1 and MG052902.1).

For *B. caballi*, 201 tick pools were tested with an overall MIR of 6.9% (Table 5). Only *R. e. evertsi* tick pools were positive for the presence of *B. caballi* DNA with MIR of 7.6% (12/176) (Table 5). Based on location, the highest infection rate was observed in Kano state with eight positive pools comprising of 26 ticks with MIR of 10.7%. This was followed by Kaduna state (MIR 9.1%) and lastly Katsina state with MIR of 2.8% (Table 5). No significant difference ($P > 0.05$) was observed across the four study locations (Table 5). BLASTn query of the sequences obtained from the PCR amplicons confirmed the identity of the pathogen as they shared 100% identity score with a previously registered *B. caballi* gene sequence (Genbank accession no: MK392060.1). Co-detection of *B. caballi* and *T. equi* was observed only in *R. e. evertsi* ticks from Kano and Katsina states with MIR of 1.4% (Table 5).

Discussion

We used a large sample size ($n = 468$) and molecular tools to carry out a robust epidemiological assessment on the prevalence and distribution of the causative agents of EP in horses and donkeys in

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Table 4. Multivariable logistic analysis of risk factors associated with *Theileria equi* in donkeys in North-Western Nigeria

Variables	Category	OR (95%CI)	*P* value
Locations	Kano	a	
	Katsina	1.457 (0.703–3.019)	0.357
	Jigawa	3.200 (1.525–6.716)	0.002
Breed	Akaza	a	
	Auraki	2.231 (0.506–9.838)	0.474
	Fari	4.00 (0.732–21.850)	0.127
	Goho	1.000 (0.159–60.258)	1.000
	Idabari	3.360 (0.771–14.640)	0.147
	Jaba	2.000 (0.241–16.620)	0.622
	Jangora	11.330 (1.779–72.200)	0.010
Age (years)	Under 6	a	
	Between 6 and 12	0.583 (0.290–1.169)	0.168
	Over 12	0.421 (0.173–1.026)	0.071
Presence of ticks	Yes	a	
	No	4.333 (0.174–107.900)	0.412
Altitude	0–199	a	
	200–399	0.759 (0.362–1.593)	0.570
	Above 400	1.480 (0.637–3.437)	0.395

[a]Reference category.

North-Western Nigeria. EP has been reported previously in other parts of Africa including South Africa (Gummow *et al.*, 1996; Bhoora *et al.*, 2020), Sudan (Salim *et al.*, 2008), Egypt (Mahmoud *et al.*, 2016), Kenya (Oduori *et al.*, 2015) and Nigeria (Turaki *et al.*, 2014).

General prevalence in horses

In the horses, the overall prevalence of equine piroplasms was 41.3% using molecular methods and 6.4% using microscopy. The high prevalence in horses due to *T. equi* reported in this study is comparable to the prevalence of 39–42% reported in Romania (Gallusova *et al.*, 2014), Mongolia (Boldbaatar *et al.*, 2005) and Italy (Del Pino *et al.*, 2016). Previous studies conducted in Africa have reported higher prevalence above 50.0% (Motloang *et al.*, 2008; Salim *et al.*, 2008). In Nigeria, the prevalence of equine piroplasms obtained in this study was higher than that reported recently by Idoko *et al.* (2020). The differences could be due to the variation in the sample sizes and study locations. Nevertheless, our result is comparable to the prevalence of 39.2% reported by Turaki *et al.* (2014) using Giemsa-stained blood smears in North-Eastern Nigeria comprising of Borno, Gombe and Taraba states.

For *B. caballi* infection, the prevalence was low at 1.4% and comparable to that obtained in other regions of Africa (Ros-Garcia *et al.*, 2013; Salim *et al.*, 2013). Absence of *B. caballi* infection in horses was reported recently in Nigeria (Idoko *et al.*, 2020). The use of small sample size in the latter study might be responsible for their observation. Despite the large sample size used in our study, we detected only seven positive samples. Generally, numerous studies have reported higher prevalence of *T. equi* compared to *B. caballi* in epidemiological studies conducted in several parts of the world including Africa (Gummow *et al.*, 1996; Motloang *et al.*, 2008; Kouam *et al.*, 2010). *Babesia caballi*-infected animals usually clear the infection after 1–4 years in the absence of re-infection while *T. equi*-infected animals remain lifelong carriers (Brüning, 1996; Ruegg *et al.*, 2007). These animals serve as a source of infection to ticks which subsequently transmit the infection to naïve and susceptible uninfected animals. Another possible reason for the differences in the prevalence between the two pathogens could be due to the differences in vector distribution (Salim *et al.*, 2008). Mixed infection of the two piroplasms has been reported in different studies and is unconnected with the presence of the tick vectors responsible for the transmission of both pathogens within the same geographical area.

Diagnosis of EP can be achieved by either the use of direct or indirect methods (Abedi *et al.*, 2015). Recently, combination of both methods is increasingly been adopted. The gold standard for piroplasm's diagnosis is microscopy but poor sensitivity during low parasitaemia limits its use (Böse *et al.*, 1995). PCR is a more sensitive test for the diagnosis of infection with piroplasms. Therefore, PCR was able to detect more infections compared with microscopy in our study. In other studies, piroplasms were not detected in blood smears but the use of PCR and serology on same samples proved otherwise (Abutarbush *et al.*, 2012; Munkhjargal *et al.*, 2013). Generally, differences in prevalence to EP could also be due to differences in diagnostic methods employed in the surveys (Onyiche *et al.*, 2019).

We observed that the prevalence was higher in the donkeys compared with the horses. This agrees with previous reports from other studies (Garcia-Bocanegra *et al.*, 2013; Qablan *et al.*, 2013). This observation could be attributed to the differences in the management systems in which these two species of equids are kept and the possible role of donkeys as reservoirs.

General prevalence in donkeys

The overall prevalence of EP in donkeys in Nigeria in this study was 42.8% using PCR and 13.4% using microscopy. The high prevalence of 41.2% in donkeys due to *T. equi* reported in this study is comparable to that reported in Italy (Garcia-Bocanegra *et al.*, 2013), Spain (Piantedosi *et al.*, 2014) and recently in Nigeria (Idoko *et al.*, 2020). Within Africa, higher prevalences above 50% have been reported by other authors (Gizachew *et al.*, 2013; Hawkins *et al.*, 2015; Oduori *et al.*, 2015). For *B. caballi* infection, the prevalence of 1.6% was low. Other studies from Spain, Kenya and Iran reported absence of infection with *B. caballi* (Garcia-Bocanegra *et al.*, 2013; Abedi *et al.*, 2015; Oduori *et al.*, 2015). Mixed infections of both piroplasms as detected by PCR were low with a prevalence of 1.6%. Low rate of mixed infection in this study is due to low *B. caballi* infection rate and all *B. caballi*-infected animals were also infected with *T. equi*. Low prevalence of mixed infection has also been observed in similar studies in Nigeria (Sanusi *et al.*, 2014), Brazil (Heim *et al.*, 2007) and Greece (Kouam *et al.*, 2010).

Risk factors associated with positivity to equine piroplasms

Horses

In our study, we observed statistically significant differences associated with positive detection of *T. equi* between study locations. Corroborating our findings, location has been considered as a risk factor to EP previously in different epidemiological studies (Kouam *et al.*, 2010; Cortés *et al.*, 2017). Differences in prevalence between locations have been observed in other countries and might be related to several factors including the presence of tick vectors, climatic conditions, host and effectiveness of any control

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Table 5. Minimum infection rate for *Babesia caballi* and *Theileria equi* in tick pools from North-Western Nigeria

Study locations	Total No. of pools tested (*n*)	Minimum infection rate (number of positives) [95%CI]		
		Babesia caballi	*Theileria equi*	*B. caballi* and *T. equi*
Kano	92 (244)	10.7 (8) [7.1–15.2]	64.3 (56) [58.0–70.4]	1.6 (1) [0.4–4.1]
Jigawa	30 (75)	0 (0)	38.7 (11) [27.6–50.6]	–
Kaduna	25 (77)	9.1 (3) [3.7–17.8]	48.1 (12) [36.5–59.7]	–
Katsina	54 (142)	2.8 (1) [0.8–7.1]	59.9 (29) [51.3–68.0]	2.8 (1) [0.8–7.1]
Total	201 (538)	6.9 (12) [4.9–9.4]	57.2 (108) [52.9–61.5]	1.4 (8) [0.6–2.9]
Tick species				
Rhipicephalus evertsi evertsi	176 (484)	7.6 (12) [5.4–10.4]	61.6 (102) [57.1–65.9]	1.7 (2) [0.7–3.2]
Hyalomma. dromedarii	17 (38)	–	23.7 (5) [11.4–40.2]	–
H. rufipes	2 (6)	–	–	–
H. truncatum	2 (2)	–	50.0 (1) [1.3–98.7]	–
R. (Boophilus) decolaratus	3 (7)	–	–	–
R. sanguineus	1 (1)	–	–	–
Total	201 (538)	6.9 (12) [4.9–9.4]	57.2 (108) [52.9–61.5]	1.4 (2) [0.6–2.9]

n = Total number of ticks in the entire pool.

measures (Guidi *et al.*, 2015; Heim *et al.*, 2007; Kouam *et al.*, 2010). The climatic conditions within our study areas are similar and characterized by high temperature and humidity prior to the raining season (May–October) and a dry/cold season (December–March). Therefore, it is possible that differences in sample size across the four study areas could be responsible for this observation.

The management system in which the horses are raised was shown to be significantly associated with positivity. It is well known that animals kept on extensive or semi-intensive system are at the greatest risk to ticks and tick-borne infection. In the course of grazing, they get in contact with other animals in the pasture as well as questing ticks. This puts the animals at risk to likely exposure to piroplasms compared with those exclusively raised on intensive management system. Furthermore, horses raised under unsatisfactory technical and sanitary management with inadequate housing/infrastructure, veterinary care, grooming and poor sanitation are more prone to infection with EP. Vaccination, fly control and deworming are pointers of decent herd management with resultant low exposure to ticks (Santos *et al.*, 2011). According to Guidi *et al.* (2015), they observed in southern France that horses regularly dewormed, with fly control regime present and good husbandry were protected against EP. Additionally, it was also observed in India that irregular grooming or non-grooming practices in horses expose them to high tick infestation and hence the greater the likelihood of infection (Sumbria *et al.*, 2016).

The activity in which the horses are engaged in plays a significant role in the positivity to *T. equi* (Kouam *et al.*, 2010). In our study, we observed that horses used for reproduction and work showed the highest risk of infection to EP. This is because most of these working horses are kept under poor condition and are exposed to tick vectors thereby increasing their risk of infection (Shkap *et al.*, 1998).

Furthermore, contact of horses with other mammals was significantly associated with positivity to EP. Tick vectors are limited in their dispersal, and their abundance depends on the presence of appropriate host (Guidi *et al.*, 2015). The close proximity either through working or living of horses together with cattle has been reported to promulgate the spread of EP (Heuchert *et al.*, 1999; Guidi *et al.*, 2015). Concerning breed, we observed that Talon, a non-native breed, was less prone to EP. This is at variance with a similar study in Bauchi state, Nigeria, where the Arewa breed (native/indigenous horse breed in Nigeria) had the lowest prevalence of EP compared with other non-native breeds (Sanusi *et al.*, 2014). The reasons for the difference in our findings remain unknown but may be related to genetic predisposition. Therefore, further studies on the genetic predisposition of breeds to EP may be necessary in the future. Other risk factors not statistically significant in the multivariable model were age and gender. Gender has been considered to be protective to EP with male horses less likely to be infected compared to females (Guidi *et al.*, 2015). Similarly, the prevalence was lower in males but higher in the females. This observation corroborates the findings of others as reported in Trinidad, Turkey, Nigeria and France (Asgarali *et al.*, 2007; Karatepe *et al.*, 2009; Sanusi *et al.*, 2014; Guidi *et al.*, 2015). Hormonal differences between genders have been postulated to be responsible for this observation (Roberts *et al.*, 2001). Furthermore, Alexander and Stimson (1988) were of the opinion that females are more susceptible to protozoan parasites when compared with males. Majority of the horses we sampled were adults above 6 years of age, hence we could not find any association with age. Other studies found no association between infection occurrence and age (Abutarbush *et al.*, 2012; Sanusi *et al.*, 2014).

Donkeys

In the donkeys, positivity to *T. equi* infection differed significantly with regards to location and breed. Since all the sampling locations lied within the same agro-ecological zone, it is very difficult to ascertain the exact reason for this observation. Animals infected with *T. equi* are known carriers of the pathogen for life (Brüning, 1996; Ruegg *et al.*, 2007). Furthermore, breed variation in prevalence was observed with the Jangora breed of donkey having the highest prevalence. Genetic predisposition could probably play a role in this observation.

Piroplasms in ticks

Generally, limited epidemiological surveys have been carried out regarding the detection of piroplasms in feeding ticks. Feeding ticks were collected from surveyed animals. Therefore, the

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detected parasites might have been ingested during blood meal. Thus, the detection of parasites does not identify these ticks as vectors. It is also important to note that all ticks collected from this study were adult ticks.

Rhipicephalus e. evertsi is a known vector responsible for transstadial transmission of *B. caballi* (De Waal and Potgieter, 1987). DNA of *B. caballi* was detected only in *R. e. evertsi* collected from Kano, Kaduna, Katsina and Jigawa. Furthermore, *T. equi* DNA was detected in *R. e. evertsi*, *H. dromedarii* and *H. truncatum* collected from all study locations. Generally, both *H. dromearii* and *R. e. evertsi* are regarded as vectors of *T. equi* (De Waal and Potgieter, 1987; Ueti and Knowles, 2018). The *H. dromedarii* in horses is regarded as an incidental host especially in areas where camels and cattle are present (Walker *et al*., 2003). Coincidentally, all the sampling locations in our study are livestock production areas of Nigeria with substantial population of cattle and camels. In a previous study, *T. equi* DNA was amplified in *R. e. evertsi* ticks collected from domestic and wild animals in South Africa (Berggoetz *et al*., 2014). We did not amplify any of the piroplasms in *R. (B.) decolaratus*, but in South Africa, DNA of *B. caballi* was amplified in *R. (B.) decolaratus* and this vector has been speculated to be a vector of EP (Berggoetz *et al*., 2014).

Conclusion

We observed a high prevalence of *T. equi* and low prevalence with *B. caballi* in both horses and donkeys. The overall prevalence of equine piroplasms was higher in the donkeys compared with horses. Risk factors associated with positivity to *T. equi* in horses include location, breed, unsatisfactory husbandry practices, contact with other mammal's management system and activities of the horses. Breeds and locations were risk factors to the occurrence of *T. equi* in the donkeys. Additionally, we report for the first time in Nigeria, the molecular evidence of *B. caballi* and *T. equi* in ticks from Nigeria. All positive samples of *B. caballi* were observed in *R. e. evertsi* only while that of *T. equi* was observed in *R. e. evertsi*, *H. dromedarii* and *H. truncatum*. We recommend that further studies be undertaken to ascertain the genetic diversity and haplotypes of the circulating piroplasms within the study areas as well as the probable role of donkey as a reservoir.

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Conflict of interest. No conflict of interest exists among the authors.

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