Research Article
Volume 13:7, 2022

Journal of Veterinary Science and Technology

ISSN: 2157-7579 Open Access

Prevalence, associated risk factors, morphological and molecular characterization of piroplasms in the blood of infected donkeys from Gombe and Yobe States, Nigeria

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Abstract

Four hundred and twenty-six (426) donkeys were sampled using the convenience sampling technique in markets, loading areas, and watering points to determine the prevalence, associated risk factors, morphological and molecular characterization of piroplasms present in the blood of infected donkeys from Gombe and Yobe States, Nigeria. Fifty-three ticks and 426 blood samples were collected from donkeys for the identification of piroplasms using microscopy and molecular techniques. The prevalence of piroplasms observed in the blood samples of donkeys via microscopic examination was 12 (2.81 %; CI = 1.62%, 4.86%) for B. caballi and none for T. equi while multiplex PCR (MPCR) showed a prevalence of114 (26.76%; CI = 22.78%, 31.16%). Out of which 33 (7.75 %; CI = 5.75, 10.68) represent T. equi and 78 (18.31%; CI = 14.93%, 22.26%) represent B. Caballi and 3 (0.07%; CI = 0.24, 2.04) represents a mixed infection of B. caballi and T. equi. The prevalence of piroplasms in the internal organ of ticks was 21 (77.8%; CI =59.25%, 89.39%) in the primary screening of ticks by PCR out of the 27 DNA extracted from the 53 ticks sampled. Out of 53 ticks sampled Riphicephalus had a prevalence of 52 (98.11%; CI = 90.05%, 99.67%) and Amblyomma varigatum had a prevalence of 1 (1.89%; CI = 0.33%, 9.95%) and are the species of ticks found on the donkeys in the studied areas. Phylogenetic analysis was performed after the 18SrRNA gene from 20 positive samples (10 each from blood and ticks) were sequenced. The sequencing analysis suggested a 99-100% similarity of T. equi with the other T. equi in the gene bank and after blasting alignment and analysis of the genes, accession numbers from the gene bank were assigned. The accession numbers were MH355571, MH355572, MH355573, MH355574 and MH355575. It was also found that the group D isolates of T. equi were closely related to the T. equi reported in Nigerian waterbucks. This is the first report of equine piroplasms sequencing from the studied areas to the best of our knowledge.

Keywords

Prevalence• Risk factors• Morphological• Molecular• Piroplasms• Donkeys• Gombe• Yobe• Nigeria

Introduction

The donkey or ass (Equus africanus asinus) is a descendant of African wild ass and was domesticated 6000 years ago. Nigeria is one of the countries where donkeys contribute to the daily socio-economic activities of the citizens and the primary role of donkey in Nigeria has been traditionally for draught purposes. In addition to this,

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Date of Submission: 01 July, 2022, Manuscript No. jvst-22-44669; **Editor assigned:** 04 July, 2022, PreQC No. P-44669; **Reviewed:** 15 July, 2022, QC No. Q-44669; **Revised:** 22 July, 2022, Manuscript No. R-44669; **Published:** 29 July, 2022, DOI: 10.37421/2157-7579.2022.13.137

donkeys are used in many regions across the globe and in particular their application in providing mobility for the conveyance of people and goods from one location to another, ploughing, threshing, milling, conveyance of firewood, loads, including water and household structures. Donkeys provide food in terms of meat and milk. Donkeys can live on a very poor diet without clinical symptoms of anaemia or metabolic disorders and are tolerant of a variety of infectious pathogens and pests that cause clinical diseases in ruminants such as tick and fly-borne infections prevalent in the tropics [1]. In Australia, Equus asinus, is used as packaging animals and as transport teams. Donkeys played a very important role in the growth of long-distance trade in Egypt due to their weight-bearing ability and adaptation to desert travel [2].

The population of donkey in Africa, particularly Nigeria and Niger Republic are reducing sharply This reduction trend is due to very high demand for donkey products in China. According to Baker, there has been a large-scale global trade in donkey hides over the last two years, with figures of at least 1.8 million donkey hides traded annually [3]. Global demand, on the other hand, has been estimated approximately at up to four million, with some reports suggesting higher demand limits in China at 10 million donkey hides per year. Due to enormous value of donkeys in North Eastern Nigeria, including Gombe and Yobe states, equine piroplasmosis, especially in drought donkeys, have serious socio-economic implications. Conversely, notwithstanding a prominent role in the rural farming system, donkeys are being vulnerable to poor management, lack of health care and a negative attitude of owners towards donkeys and knowledge of diseases of donkeys is limited and is often referenced from the knowledge of diseases of horses [4].

Equine Piroplasmosis (EP) is a tick-borne infection transmitted by intraerythrocytic apicomplexan protozoan parasites, the infection also known as tick fever. Ticks are very significant donkey ectoparasites that transmit numerous diseases, including equine piroplasmosis. The disease poise great liability to the equine industry because of severe economic losses. The disease has an impact primarily on donkeys, mules, horses, and zebra, but the parasite DNA has often been identified in camels and dogs that raise doubts regarding the host specificity [5]. The infection is widespread in temperate and tropical regions of the world where competent tick vectors are ubiquitous. Usually infections with T. equi infections are more prevalent than B. caballi and the infections predominate in areas and habitats where tick-infested donkeys are common. In particular, a typical example includes the Dermacentor, Hyalomma, Rhipicephalus and ticks of the Ixodes family belonging to the Amblyomma group. The main component of transmission has been through the saliva of infected Ixodide ticks during a blood feeding; numerous different means of infection usually involve iatrogenic and transplacental transmission through infected needles and/or blood transfusion Infected equids remain life carriers of T. equi infection, while infection with B. caballi are cleared in couple of years [6].Balkaya reported the prevalence of T. equi and B. caballi in Donkeys from Erzurum in Eastern Turkey using microscopic examination, which divulged no parasite detected. However, Tefera indicated a prevalence of 3.13% in donkeys in and around Debre Zeit, Central Ethiopia using microscopy technique. The prevalence of EP was reported from Northern Nigeria by Sunday after examining 57 blood samples of donkeys using PCR, where 25 (43.8%) and 5 (8.82%) were recorded as positive for T. equi and B. caballi respectively. One hundred and thirty eight mixed breeds of donkeys were studied in central Italy, out of which the PCR results showed that 17.4% of the animals tested positive for T. equi and 3.6% for B. caballi. Age, sex and body condition scores are profound risk factors for disease dissemination in donkeys Therefore, the present study is designed to determine the prevalence, associated risk factors, morphological and molecular characterization of piroplasms present in the blood of infected donkeys from Gombe and Yobe States, Nigeria [7].

Materials and Methods

Study Area: The two states (Gombe and Yobe) are located in the northeastern part of Nigeria at latitude 10.38 36oN and 11. 190321E, and 12. 29o031N and 11. 43o031E, the states have a combined land mass of 54,270 km2 and the communities are largely farmers. Donkeys of all ages and of both sexes selected from markets, loading and water points within the geographical locations of Gombe and Yobe states. The climate, ecology and vegetation vary within the zone ranging from Sahelian to savannah with semi-arid and flooded pastures towards Lake Chad and mountain regions in the southeast. The relative humidity is generally as low as 13 % in the driest month of February and March, around Yobe State and up to 80 % between the rainy months of July and August [8]. Mean humidity is generally high in Gombe State. Temperatures may be as high as 50℃ around the study areas. Temperature and relative humidity were recorded with portable thermohygrometer (H1936440N, Hanna instrument Romania) [9].

Donkeys were assigned a Body Condition Score (BCS) using a standard scale of 1-9. A convenient sampling technique (Non-Probability Sampling) was used to obtain a sample size of 426 donkeys within the markets, watering and loading points in villages and towns of Gombe and Yobe States. In Gombe State, 202 samples were collected from 10 locations while in Yobe State, 224 samples were collected from 4 locations Blood was obtained from each animal through the jugular vein and neck collar was used for restrain to make sure the donkey(s) were at rest, undisturbed or under least excitement, to allow for smooth collection. The jugular surface was disinfected before blood samples collections in commercial sample bottles containing Ethyl Diamine Tetraaceticacid (EDTA). The blood samples were placed into a flask containing ice, and then transported to the Parasitology Division, National Veterinary Research Institute Vom, Plateau State for analysis [10].

Figure 1. Map Showing Gombe (Orange Star) and Youb (Blue Star) States Nigeria Source Google Map.

Morphological Identification of piroplasms

Piroplasms infected donkeys were determined by demonstrating the parasites in stained blood smears (thick and thin) films using Giemsa stain. The method involved making smear on a clean slide, fixing the smear with absolute alcohol, staining it with Giemsa stain for 45 minutes, washing the stained slide with water and air dried, and viewed under 100 magnifications with oil of immersion, parasites were identified using a guide as described by Soulsby [11].

Molecular detection of piroplasms

Genomic DNA was extracted from blood samples according to manufacturer's protocol using Quick-DNATM miniprep kit as reported by Alhassan, with some modifications. Briefly, 50 µL of each donkey blood samples was washed 3 times with cold phosphate buffered saline by centrifuging at 1000g for 5 minutes at room temperature and re-suspended in 100 µL of DNA extraction buffer (0.mM Tris-HCl [pH 8.0], 0.1 % sodium dodecyl sulfate, 100 mM NaCl, 10 mM EDTA, and 100µg mL-1 proteinase K) (TBE) and incubated at 55oc for 2 hrs. The parasite DNA was extracted with phenol-chloroform and precipitated with ethanol [12]. The purified DNA pellets were dissolved in 20 µL of double-distilled water for subsequent PCR reactions.

PCR Reactions

A single and multiplex PCR method were applied for simultaneous detection of B. caballi and T. equi based on the 18S ribosomal RNA genes, which are present in multiple copies through the genome, and evaluated in a field blood sample.The nucleotide sequence of the primers used for the present study was obtained from the design of Alhassan, using 18S ribosomal RNA gene sequence of B. caballi and T. equi. The Accession numbers used in the present study are Z15104 for B. caballi and Z15105, AY150062, and AY150063 for T. equi by aligning these sequences using a Mac Vector (Oxford Molecular, Ltd., Oxford, UK), a universal screening primer pair common for B. caballi and T. equi, Bec-UR, was designed to amplify the DNA of both parasites in one reaction [13]. Additionally, a set of F primer combinations including Bec-UF2 as a universal forward primer and Cab-R and Equi-R as reverse primers specific for B. caballi and T. equi, respectively, was also designed for the species detection [14]. Furthermore, species-specific primer pairs were designed based on the genes of T. Equi Merozoites Antigen 1 (EMA-1) and used to confirm the accuracy of the result obtained by the multiplex PCR. The EMA-1 is encoded by a single copy gene of T. equi. The primer pairs designed from these genes have so far been used for the single detection of these parasites in donkey blood and in ticks [15].

Primers	Sequence
Bec-UF1	5'-GTTGATCCTGGCCAGTAGTCA-3'
Bec UR	5'-CGGTATCTGATCGTCTTCGA-3'
Bec-UF2	5'TCGAAGACGATCAGATACCGTCG 3'
Cab-R	5'-CTCGTTCATGATTTAGAATTGCT3'
Equi-R	5'-TGCCTTAAACTTCCTTGCGAT-3'
BC48-F	5'-GGCTCCCAGCGACTTGATGG-3'
BC48-R	5'-TTAAGTGCCCTCTTGATGC-3'
EMA-1F	5'-GATCCATTGCCATTTCGAG-3'
EMA-1R	5'-TGCGCCATAGACGGAGAAGC-3'

Table 1. List of PCR primers used in the present study.

Bec-UF1 and Bec-UF2; Universal forward primers; Bec-Ur: universal reverse primers; Cab R: B. caballi-specific reverse primer, Equi-R: B. Equi-specific reverse primer. BC48-F: BC48-specific forward primer, BC48-R: BC48-specific reverse primer, EMA-1 F: EMA-1 specific forward primer, EMA-1 specific reverse primer [16]. The nucleotide sequences of the primers used in this study are shown in table 1. PCR was performed in 50 µl reaction mixture (10mM Tris-HC1 [pH8.3], 50mM KC1, and 1.5 mM mgC12) containing 3 µl of the template DNA, 2.5 pmol of each of the primers, 0.2 mM dNTP mixture was heated for 10min at 96°C to activate the Ampli Tag Gold DNA polymerase, and 40 cycles of the following conditions were repeated: denaturation for 1 min at 96°C, annealing for 1 min at 60.5 °C, extension for 10 min at 72°C. The amplified DNA samples were electrophoresed on 1.5 % agarose gel and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light [17].

Sequencing: Sequencing was done at Macrogen incorporation South-Korea for gene analysis to relate differences, similarities and association between isolates from different geographical regions of sampling [18].

Stages in sequencing

The collected blood was subjected to DNA extraction using the manufacturer's protocol where Total Nucleic Acid (TNA) was obtained. The PCR (MPCR) assay was performed under stated conditions using specific primers as described by Alhassan, with some modifications. This was followed by purification of the amplicons and direct sequencing of 18SrRNA gene (hypervariable region) of PCR amplicons (products) with Babesia and Theileria genus specific probes (540 bp and 392 bp respectively). The obtained sequence was edited and purified using MEGA 7.0 software. A nucleotide query for similarity between related organisms was conducted in NCBI using BLASTn. The phylogenetic tree was constructed by the neighbor-joining method [19]. Distance and maximum likelihood were applied using 500 bootstrap value or replicates per tree for each method. Molecular evolutionary analysis was done using MEGA 7.0.

Statistical Analysis

Data was analyzed using chi-square to test for the association between infection status and the risk factors. Confidence interval level of 95% was used for the evaluation of the prevalence of equine piroplasmosis. In all cases, the JMP version 11 software (SAS Institute Inc, Cary, NC) was used and the results were considered significant at $P < 0.05$.

Results

Prevalence of piroplasms in donkeys

A total of one hundred and fourteen (n=114) 26.76%; CI=22.78%, 31.16% samples were positive of piroplasms by PCR. Out of which 33 (7.75 %; CI=5.75, 10.68) represent T. equi and 78 (18.31%; CI =14.93%, 22.26%) represent B. Caballi and 3 (0.07%; CI=0.24, 2.04) represents a mixed infection of B. caballi and T. equi (Table 2). Of the PCR positive samples, 20 representatives (amplicons) 10 each from blood and ticks samples were sequenced. The prevalence of piroplasms observed in the blood samples of donkeys via microscopic examination was 12 (2.81 %; CI=1.62%, 4.86%) for B. caballi and none for T. equi as shown in table 3 [20].

Total tested	piroplass	Number positive	Prevalene(%)	95%CI
426	B. caballi	12	2.81	1.62,46
426	T. equi	0	0	0.0,0.9

Table 3. Prevalence of equine piroplasmosis in Donkeys in Gombe and Yobe States Nigeria.

CI=Confidence interval (lower and upper).

Prevalence of Ticks on Donkeys: A total of 53 ticks were collected on 11 donkeys that were selected randomly from the different locations and five ticks were collected on each donkey and three ticks was collected on the 11th donkey out of 426 donkeys sampled in different locations in the study areas 53(12.44 %; CI=9.64%, 15.91%). Of the Fifty Three ticks collected, n=30 (56.60 %; CI=43.26%, 69.05%) were identified as males and n=23 (43.4%; CI=30.95%, 56.74%) were identified as females. Amblyomma and Riphicephalus were the genera found, with 52 (98.11%; CI=90.05%, 99.67%) identified as genus Rhipicephalus comprising of different species, and 1 (1.89%; CI=0.33%, 9.95%) as an Amblyoma varigatum specie (Table 4).

		No of Ticks	Sex	Prev. %	95% CI
426		53		12.44	9.64, 15.91
Ticks	Genera of Ticks	No of Ticks	Sex	Prev. %	95% CI
53	Rhipicephalus	30	Male	56.6	43.26, 69.05
53	Rhipicephalus	23	Female	43.4	30.95, 56.74
53	Rhipicephalus	52		98.11	90.05, 99.67
53	Amblyomma	1		1.89	0.33, 9.95

Prev. % = Prevalence %

Table 4. Prevalence of Ticks infestation on Donkeys in Gombe and Yobe States, Nigeria.

	Location	Number (n =202)	Percentage (%)	95% CI
	Gombe	13	6.44	3.8, 10.7
	Liji	14	6.93	4.17, 11.3
	Kundulum	35	17.33	12.73, 23.15
Gombe	Barunde	35	17.33	12.73, 23.15
	Byepass	5	2.43	1.06, 5.67
	Bogo	12	5.94	3.43, 10.09
	Galdimari	24	11.88	8.11, 17.07
	Yankari	20	9.9	6.50, 14.80
	Zagaina	17	8.42	5.32, 13.07
	Mal. Sidi	18	8.91	5.71, 13.64
	Bajoga	9	4.46	2.36, 8.26
State	**Distribution of Donkeys sampled in Yobe state**			
	Location	Number (n = 224)	Percentage (%)	95%CI
	Ngalda	95	42.41	36.12, 48.96
Yobe	Potiskum	12	5.36	3.09, 9.13
	Babbangida	40	17.86	13.40, 23.40
	Gashua	77	34.38	28.47, 40.82

Table 5. Distribution of Donkeys sampled in Gombe state.

Association between Body Condition Score (BCS) and Natural piroplasms infection in Donkeys in Gombe and Yobe States Nigeria: The study observed that there is an association between Body Condition Score (BCS) and natural piroplasms infection in the study areas whereby the examined donkeys were classified into nine scales based on fats and protein mass on the following positions (neck, crest down back, behind the shoulder, ribs, head of tail and pelvic) and the scales were; very thin=1, thin=2, poor=3, below moderate=4, moderate=5, above moderate=6, fleshy=7, fat=8 and obese =9 (Table 11). The association suggested that piroplasms infection is more in donkeys with poor, very thin and thin of body condition scores while comparatively the fleshy, fat and obese classes were not affected ($P < 0.05$).

BCS	No examined	No infected	% infected	P-value
1	50	2	4	
2	60	2	3	
3	60	2	3	
4	40	1	2.5	
5	60	1	1.6	
6	65	1	1.5	0.031
7	41	1	2.4	
8	30	1	0.03	
9	20	1	5	

P< 0.05 is significant

The Risk Factors Associated with Natural piroplasms Infection of Donkey in Gombe State, Nigeria:

The risk factors include sex of the animal studied, presence or absence of ticks, age and Body Condition Score (BCS). The analysis of the risk factors indicated that of the total number of donkeys examined (426), three hundred and thirty seven (337) are males out of which nine (9) donkeys are positive (P=0.726) for piroplasms whereas of the eighty nine (89) female donkeys examined, three (3) were positive for piroplasms. Thirty-nine donkeys have ticks on them, out of which 3 donkeys were positive (P=0.068) for piroplasms while, 387donkeys don't have ticks on them and 9 out of them were positive for piroplasms. Also, among the 352 adult donkeys sampled, 7 were positive (P=0.031) for piroplasms while among the 74 young donkeys sampled only 5 were positive for piroplasms.

Risk factors		No.examined	No.positive	%positive	Pvalue
Sex	Male	337	9	2.6	0.726
	Female	89	3	3.3	
Ticks	Presence	39	3	7.6	0.068
	Absence	367	9	2.3	
Age	Adult	352	7	6.7	0.03
	Young	74	5	1.9	

The P value is significant at P < 0.05

Table 7. Risk Factors Associated with Natural piroplasms Infection in Donkeys in Gombe State, Nigeria

Blood Smear: Giemsa stained showed bipolar (paired) merozoites of B. caballi as presented in Figure 2. However, T. equi were not seen by microscopy. The parasites seen appeared bluish (cytoplasm of the parasites and a pinkish nucleus) in the background inside the red blood cells with bluish appearance.

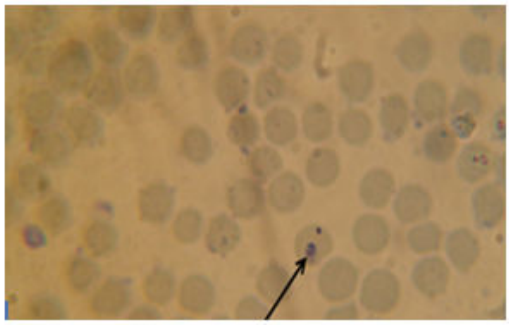

Figure 2. Blood Smear from a Donkey Showing B. caballi (Arrow) Stained with Giemsa stain.

Blood samples from donkeys were subjected to multiplex PCR test for detection of piroplasms (B. caballi and T. equi) with a pair of universal screening primers (Bec-UF1 and Bec-UR) and a set of primer combinations (Bec-UF2, Cab-R and Equi-R), which demonstrated the presence of genetic material of piroplasm of approximately 913 base pairs and 867 base pairs for B. caballi and T. equi respectively in the primary screening, and approximately 540bp and 392 bp for B. caballi and T. equi respectively in secondary or confirmatory tests of the blood of donkeys and ticks on electrophoresis (Figures 2, 3 and 4). Of the PCR positive samples, 20 representatives (amplicons) was 10 each from blood and ticks samples were sequenced.

DNA was extracted from 27 (50.9 %; CI = 37.88%, 63.87%) out of 53 tick samples.

Of the twenty seven (27) DNA amplified only twenty one passes the primary multiplex PCR screening test and 21 (77.8 %; CI =59.25%, 89.39%) were positive for piroplasms in the primary screening of ticks by PCR.

The secondary screening did not yield detection of any amplicon on the ge.

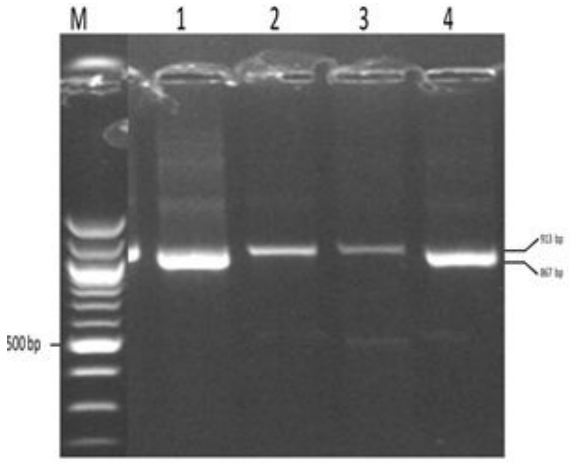

Figure 2. Amplicons from field blood samples showing molecular marker M amplicons 1, 2, 3 and 4 showing molecular size of 913 bp and 867bp in a primary screening.

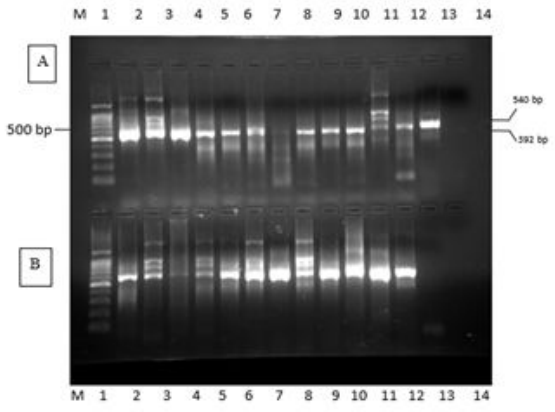

Figure 3. detection of B. caballi and T. equi with a pair of universal screening primers (Bec-UF1 and Bec-UR) (A) and a set of primer combinations (Bec-UF2, Cab-R, and Equi-R) (B). Panel A: M: 100 bp ladder DNA marker. Lane 1: equine whole DNA; lane 2 B. caballi DNA; lane 3: T. equi DNA. Panel B: M: 100 bp ladder DNA maker; lane 1: B. caballi DNA; lane 2: T. equi DNA; Lane 3: a mixture of B. caballi and T. equi DNAs; lane 4: equine whole blood DNA. The band of 500 bp determines from the 100bp ladder DNA marker is indicated on the left. The size of the positive bands is indicated on the right.

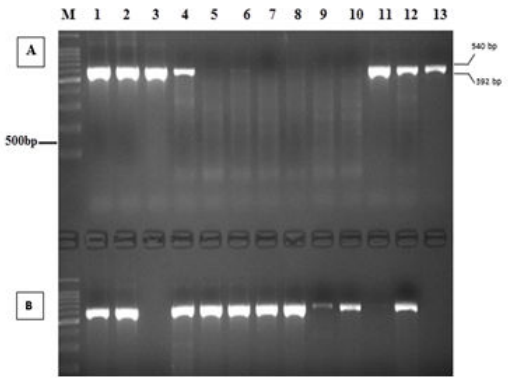

Figure 4. Pictorial presentation of electrophoresis showing amplicons of tick DNA in primary screening. Each level represents 100 base pairs (bp); =showing mix infection of B. caballi and T. equi; 2 and 3 = showing positive amplicons; 4= differentiating B. caballi at 540 base pairs and T. equi at 392 base pairs.

The PCR positive samples, 20 representatives (amplicons) of 10 each from blood and ticks were sequenced. The results of sequencing revealed that there were 99-100% similarities of T. equi with the study sample. The edited sequence was submitted to the Gene Bank under the accession numbers MH355571, MH355572, MH355573, MH355574 and MH355575 for samples with I.D numbers 151, 196, 225, 345 and 350 respectively. The phylogenetic tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic relationship.

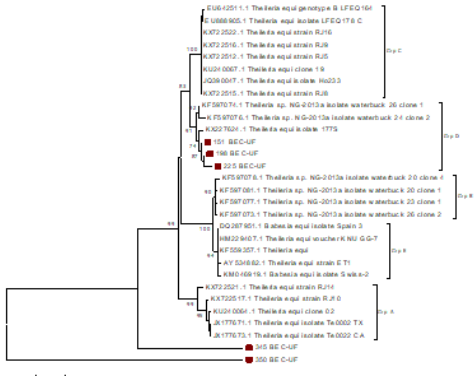

Figure 5. Phylogenetic tree

The evolutionary history was inferred using the Neighbor-Joining method of Saitou and. The optimal tree with the sum of branch length=0.45664542 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) were shown next to the branches as depicted by. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method of Kimura and are in the units of the number of base substitutions per site. The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 711 positions in the final dataset. The colored isolates from the phylogenetic tree showed the samples analyzed. The classified samples fall in group D which is the same group with the T. equi obtained from water buck while the unclassified isolates may probably fall into a new genotype subject to further research.

States	Specimen I.D	Age	Sex	Location	Genotype
Gombe	ND 151	Young	Male	Yankari	D
	ND 198	Adult	Male	Bajoga	D alone in
	ND 225	Young	Male	Ngalda	D
Yobe	ND 345	Adult	Male	Gashua	Unclassifie d
	ND 350	Adult	Male	Gashua	Unclassifie d

Table 2. Characteristics of piroplasms Positive Donkeys and Genotypes.

The genotype of ND151, ND198, and ND225 is Group D while ND345 and ND350 were unclassified groups and may be speculated as novel genotype which need further research to be investigated.

Discussions

The overall prevalence of equine piroplasmosis was found to be 2.81% for B. caballi. Despite the high number of samples taken in Gombe and Yobe states, none of the blood smears were positive for T. equi. These findings were in agreement with previous reports that the blood smear microscopy was not sensitive enough to pick the parasites, especially in those animals with carrier status. However, Khalid, observed the presence of T. equi from blood smear of horses and donkeys but was negative for B. caballi in blood smear prepared from donkeys only. This could be attributed to low sensitivity of the microscopic techniques. In addition, the findings of Abedi who reported a prevalence of 3.77% of T. equi but none of B. caballi was similarly attributable to the same reason expressed by Mahmoud. The prevalence of 2.81% natural piroplasms infection in donkeys was obtained in the present study, and is lower than 4.1% obtained by Khalid and Lokman. Similarly, the findings of Khalid and Lokman, who reported a prevalence of 8.3% and 1.7% respectively for T. equi and B. caballi was higher than the prevalence observed in the present study. The low rate of infection could be as a consequence of the method used which has limited sensitivity and specificity of detection, especially during latent or carrier stage of infection with a low parasitemia level. The finding of the current study is higher than the observation of Mekibib, who obtained 1.75% in working donkeys.

The low prevalence was due to better veterinary services and also as a result of the study design employed using a cross-sectional study to depict only specific period of infection status in animals examined. The diagnostic capability of the parasitological technique used might be another possible reason. The findings of the current study supported an assertion made by who postulated that the parasites and their natural tick's vectors are endemic to most countries with tropical and subtropical climates. The similarity and differences in prevalence in all of these areas mentioned are directly related to the distribution of tick vectors capable of transmission of the equine piroplasmosis pathogen. The distribution of the tick vectors and their ability for the transmission of infections was variable

in the study areas. The distribution of these tick vectors might be due to seasonal activity patterns and the drivers of distribution and abundance, particularly in heavily populated areas in the current study. This assumption has been highlighted by. This is similarly, in accordance with previous observations by Cortés. The present study observed bipolar piroplasms parasites seen in Giemsa stain with a bluish background appearance inside the red blood cells, which is analogous to the findings of

Multiplex polymerase chain reaction was used in the present study and the observation showed that there was an overall prevalence of 25.47%. Of the results, B. caballi accounted for 18.7%, while T. Equi accounted for 7.7% and mixed infection 0.07%. The findings of the present study are in agreement with the result obtained by Qablan, with a prevalence of 18.8% for T. equi and 7.3% for B. caballi. Moreover, Garba, presented a prevalence of 40.6% for piroplasms infection, which was higher than the findings of the current study. The overall prevalence recorded in the current study was lower, than that of Sunday, However, considering the respective infection rates with T. equi and B. caballi, the findings of the present study was higher than that of Sunday, collected blood from 57 donkeys and the infection with EP was detected and characterized by PCR. Twenty-five (43.8%) donkeys were infected with T. Equi, five (8.8%) with B. Caballi, three (5.3%) with dual infection. This findings could be attributable to the sensitivity and specificity of the PCR technique employed. According to Abedi, in a molecular study of donkeys, the prevalence of 50.94% of T. equi was reported which was higher than the findings of the current study. This finding may also be due to dissimilarities in technique approaches. The PCR reaction using 18SrRNA primers on 87 blood spots from Donkey showed a prevalence of 72% with respect to T. equi, and the results indicated that no positive amplicon was observed for B. caballi as reported by Elaine. According to Laus, a prevalence of 17.4% of T. equi and 3.6% for B. caballi was recorded and was lower than the findings in the present study using the molecular techniques.

A prevalence of 22.1% was recorded using molecular methods from Brazil by Quintana, and was lower than the observation in the present study. Moreover, a molecular prevalence of 4.93% T. equi from Southern Marama was observed by Fatih, Hossein reported from Iran a molecular prevalence of 99%, and was higher than the results of the current study. A molecular detection of Theileria species in livestock on five Caribbean Islands resulted in prevalence records of 20% T. equi in donkeys. This finding was higher than the observation made in the current study. According to Claudia, a prevalence of 54.1% B. caballi and 21.6% of T. equi was recorded higher than the findings of the current study. The sensitivity of molecular method applied during these studies and in others was reported to have higher sensitivity and specificity compared with microscopic assays. Based on the number of identified positive samples recorded in microscopy compared to that in multiplex PCR, it suggest that the findings of the present study are in tandem with the previous findings of Geysen, who recorded higher prevalence using PCR and microscopy. The same observation was recorded by Heim, who also reported the prevalence of 59.7% and 12.5% for T. equi and B. caballi respectively using PCR method. According to Rampersad and Bhoora, development using 18SrRNA gene as target sequence include specie specific nested Polymerase Chain Reaction (PCR) assays. Also, Cacciò, pointed out that Polymerase Chain Reaction (PCR) technique proved very useful for the detection of hemoparasites and was justified following the findings of the present study.

Differences in nucleotide sequences were seen among the isolate of each species and between known sequences available from the Gene Bank. The finding of the present study is similar to the one advanced by Heim. The results of sequencing showed that there was 99-100% similarity with the isolates deposited in the Gen Bank, this finding was in tandem with Bhoora, and Abedi. The 18SrRNA gene found in the present study was only recorded in T. equi but none in B. caballi. This observation contradicts the findings of the phylogenetic relation tree established for the current study showed that group D genotype, with isolates number 151Bec-UF, 198 Bec-UF, and 225 Bec-UF have close similarities to Theileria spp isolated in 2013 from waterbuck clone 1 and clone 2 from Nigeria and has close relationship with genotype of group B of Nigeria Theileria spp. of 2013 clone 1, 2 and 4 isolated from waterbuck. This finding is similar to the observation of Zhang. However, the observation of the spp in 345 Bec-UF and 350 Bec-UF belonging to new genotype may indicate a new finding of Theileria spp (fig. 5; Phylogenetic Tree), even though this requires more research. Different risk factors including age, sex, body condition, tick infestation and management system and were considered an important menace to the development of equine piroplasmosis in donkeys. Among these factors body condition score was found to create a significant difference in piroplasms infection. In the present study donkeys that were parasitologically free of piroplasms had a better body condition score compared to those that were positive for piroplasms.

Seventy five percent of donkeys that were positive for piroplasms by microscopy fall in the latter category and were classified within the 1-6 scale (poor to moderate). This finding was in agreement with the study of Steinman and Afridi However, no significant difference was found in piroplasms infected donkeys due to body condition. Age dependent prevalence conducted showed that donkeys in the age group of 0-4 years (<4years) have a relatively lower infection compared to donkeys with 4years and above. Such variation in susceptibility to piroplasms infection may be associated with acquired immunity protecting the younger ones from piroplasms infection during their early period of life. But such colostral immunity wean gradually due to age as the young donkeys were left indoors while adult donkeys go out for grazing thereby increasing the chances of contact with the ticks carrying pathogens. This finding is contrary to previous ones as reported by. Sex dependent prevalence on the other hand, was found to have no significant variation in occurrence of piroplasms infections. This is in line with the assertion of. Ticks infestation were considered as a risk factor, although there was no significant variation in the occurrence of equine piroplasmosis. Ixodid ticks of the genera Rhipicephalus and Amblyomma have been identified as vectors for the transmission of either B. caballi or T. equi in the natural host. This finding was in tandem with the findings of Mulugeta, However, Boophilus species were the most common tick frequently encountered in the body of donkeys. This is consistent with the previous study by Teglas, in which Rhipicephalus and Boophilus species were reported as the major vectors of equine piroplasmosis in the specific zone. According to OIE. Amblyomma cajennense and possibly Dermocentor variabilis were implicated in the outbreak of equine piroplasmosis. These assertions are similar to the findings of the current study.

As far as the locations of the present study is concern being Gombe and Yobe States, the tick vectors identified in the transmission of equine piroplasmosis were in the genera of Amblyoma and Rhipicephalus. The similarities of geographical climates play a good role in the findings of Rhipicephalus and Amblyomma ticks in the present study agrees with the finding, from Haryana Indian. The similarity of tropical climates between the two study areas favor the growth and multiplication of the tick vectors. The study was conducted in tropical and subtropical climate which make it convenient for detection and the identification of Ixodid ticks such as Amblyomma and Rhipicephalus. Similar statement was made by it was confirmed that Rhipicephalus and Amblyomma were the vectors of EP in the present study, and similar results were obtained by Scoles. Who implicated Amblyomma and Rhiphicephalus as the transmissible agents of equine piroplasms. Riphicephalus species are native to Africa as vectors of caballi, suggesting a strong affirmation of the same vegetation and ecology (climate) that favors the growth of tick vectors. From Ethiopia, Mekibib reported the acknowledgement of the effect of tick on working donkeys. This is because of the similarity of Ethiopian climate and that of the study area that favor the growth and multiplication of the tick vectors. The same record was reported from Texas stud farms in Florida.

Conclusion

In conclusion, the overall prevalence of piroplasms infection in Gombe and Yobe states, Nigeria was 2.81% by microscopy using stained blood smear and the findings indicated that only B. caballi was observed by microscopy, but no T. equi infection was recorded. The prevalence of piroplasms by Multiplex Polymerase Chain Reaction (MPCR) was 26.06%. B. Caballi was 18.3%, while the prevalence of T. equi was 7.7% and mix infection had a prevalence rate of 0.7%. The amplicons sequenced indicated that genes detected proved to be that of T. equi and similarities and differences in the particulars of B. equi in the gene bank conforms to that of the recorded organism. The results of sequencing suggested that there were 99-100% similarities of T. equi detected from the study sample which is similar to the finding of Knowles. The present study proved that none of the tick sample is positive for Theileria genome, but observation revealed records of uncultured bacteria. The edited sequence was submitted to the Gene Bank under the accession numbers MH355571, MH355572, MH355573, MH355574 and MH355575 for samples with I.D numbers 151, 196, 225, 345 and 350 respectively. The phylogenetic tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic relationship. The analyzed and edited partial sequence of 18SrRNA genes from the present study showed that genes with accession numbers MH355574 and MH355575 falls under the unclassified group and therefore we advocate for more studies/research. Similarly, the outcome of the present study is a contribution to scientific development in the study area in particular and the world at large. This is the first molecular study of equine piroplasmosis in the studied areas based on a literature search. The risk factors (age, sex and body condition score) were significantly associated with natural piroplasms infection in donkeys in the studied areas.

Acknowledgement

I wish to acknowledge with thanks and gratitude to members of Parasitology Division National Veterinary Research Institute Vom, who took all the pains to make sure that the practical aspect of this research was successful.

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How to cite this article: Usman Aliyu Turaki,Adamu Lawan,Alhaji Mairiga Ismaila,Kyari Falmata,Modu Bukar Muhammad,Isaac Ndudim Ogo,Yakubu Bitrus,Ismail Shitu. "Prevalence, associated risk factors, morphological and molecular characterization of piroplasms in the blood of infected donkeys from Gombe and Yobe States, Nigeria ." *J Vet Sci Techno* 12 (2021) : 137.