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Serosurvey for equine piroplasms in horses and donkeys from North-Western Nigeria using IFAT and ELISA

ThankGod E. Onyiche[a,b,c], Thillaiampalam Sivakumar[a], Bumduuren Tuvshintulga[a], Arifin Budiman Nugraha[a,d], Believe Ahedor[a,e], Lehlohonolo Mofokeng[b], Joshua Luka[c], Ali Mohammed[c], Albert W. Mbaya[c], Abdullahi A. Biu[c], Naoaki Yokoyama[a], and Oriel Thekisoe[b]

[a]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan; [b]Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, Potchefstroom, South Africa; [c]Department of Veterinary Parasitology and Entomology, University of Maiduguri, Maiduguri, Nigeria; [d]Faculty of Veterinary Medicine, IPB University, Indonesia; [e]Department of Animal Experimentation, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra, Ghana

ABSTRACT

Equine piroplasmosis is caused by apicomplexan parasites, namely, *Babesia caballi* and *Theileria equi*, which are transmitted to equids principally through ticks. To ascertain the exposure of equines to agents of equine piroplasms, we tested serum samples collected from horses (n = 272) and donkeys (n = 170) in North-Western Nigeria for the presence of antibodies against *B. caballi* and *T. equi* using IFAT and ELISA. The seroprevalence of *T. equi* in the horses determined using IFAT and ELISA was 48.89% and 45.96%, respectively, while for *B. caballi*, it was 6.3% and 0.4%, respectively. For *T. equi*, the seroprevalence based on IFAT and ELISA results in donkeys was 14.1% and 2.9%, respectively, while for *B. caballi*, the seroprevalence was 2.4% and 0.6%, respectively, for ELISA and IFAT. Mixed infection detected in the horses using IFAT and ELISA was 5.5% and 0.4%, respectively, while no mixed infection was observed in the donkeys. The seroprevalence of *T. equi* was significantly ($P < .0001$) higher than that of *B. caballi* in both horses and donkeys. Comparatively, the IFAT detected a greater number of piroplasm seropositive animals than ELISA, indicating a difference in their diagnostic accuracy. Findings from this study confirm the existence of equine piroplasms in both horses and donkeys in North-Western Nigeria and highlights the need for robust and effective control measures against the disease.

KEYWORDS

Horses; donkeys; *Babesia caballi*; *Theileria equi*; Nigeria; IFAT; ELISA

Introduction

Babesia caballi and *Theileria equi* are two important hemoprotozoan parasites, which are causative agents of tick-borne disease of equids commonly referred to as equine piroplasmosis [EP].[1] The EP is characterized by anemia, fever,

CONTACT ThankGod E. Onyiche et.onyiche@unimaid.edu.ng National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

My permanent address is Department of Veterinary Parasitology and Entomology, University of Maiduguri, P. M. B. 1069, Maiduguri, 600230, Nigeria.

edema, icterus, hemoglobinuria, and in some cases, death.[2,3] In the acute phase, infection with *T. equi* is considered more virulent than *B. caballi*.[4] Whilst infection with *B. caballi* is usually self-limiting, *T. equi* infection may persist for life if untreated. The transmission of *T. equi* and *B. caballi* is mainly through the bites of tick vectors broadly belonging to four (4) genera, namely, *Hyalomma, Rhipicephalus, Haemaphysalis* and *Dermacentor*, which are widely distributed worldwide.[5] Other methods of transmission, such as contaminated clinical instruments, syringes, and blood transfusion, are possible, but they are of lesser epidemiological significance.[6]

EP is endemic in tropical and subtropical regions of the world and it is a notifiable disease according to the World Organization for Animal Health (OIE).[7] Control of EP is an important priority in the equine industry, because it is an important constraint in the international movement of horses for equitation sport and trade.[5] Therefore, serological testing for EP is mandatory before equines are transported out of an endemic country to prevent the spread of infection. In addition, epidemiological surveys to identify the carrier horses are often conducted using serodiagnostic tests, among which indirect fluorescent antibody tests (IFAT) and enzyme-linked immunosorbent assays (ELISA) are the most commonly employed.[8]

In West Africa, Nigeria is an important player in the livestock industry and is a crossroad in animal transportation within the sub-region. There is a paucity of surveys on the prevalence of EP in Nigeria. Prior to the last 5 years, all the studies conducted have been based on microscopy.[9–12] Only recently, molecular techniques were employed for the detection and genetic characterization of agents of EP in Nigeria.[13,14] Previously, we reported the use of PCR for the detection of DNA of equine piroplasms in Nigerian equids.[14] Compared with the molecular detection, the advantage of serodiagnosis is that it easily enables large-scale epidemiological studies, resulting in generation of information on exposure of animals to infection. However, serological tests have been used only sparingly for epidemiological surveys of equine piroplasm parasites in Nigeria.[13,15] In this study, our objective was to determine the serological prevalence of two important protozoal organisms in equids in four states of North-West Nigeria. Therefore, we used IFAT and ELISA to detect anti-*T. equi* and anti-*B. caballi* antibodies in serum samples from equids (horses and donkeys) to ascertain the rate of exposure to these piroplasms in North-Western Nigeria.

Materials and methods

Study area

The North-West region of Nigeria is a semi-arid zone with a savannah type of vegetation. There is a single rainy season (May to October) with a mean annual

rainfall of 508–1016 mm and dry season (October to April). Within these study areas, samples were collected from several sampling points across these states. These sampling points include stables and farms with respect to samples from horses, while in the case of donkeys, samples were collected from working donkeys at known points where the owners congregate with their animals and clients come around to hire them for their services. These donkeys are primarily used as working animals to carry goods. Most of the animals were kept under semi-intensive system. Details of their age, sex, breed and management have previously been reported.[14]

Sample collection

Blood samples were collected from apparently healthy horses and donkeys between May and September 2017 from four states in North-West Nigeria, comprising Kano, Jigawa, Kaduna and Katsina (Figure 1). The blood samples from which sera were prepared and used in this study were used in a previous study for the molecular detection of equine piroplasms by PCR.[14] Details of the sampling technique are available elsewhere.[14] Serum samples were harvested from blood collected in vacutainer tubes without anticoagulants from the jugular veins. We initially collected a total of 468 blood samples from both horses (n = 281) and donkeys (n = 187).[14] However, 26 serum samples were unsuitable for further analysis due to hemolysis of the red blood cells. Therefore, we used a total of 442 serum samples in this study (Horses, n = 272; Donkeys, n = 170). All serum samples were stored at −20°C until tested. Collected samples were shipped chilled to the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan, after due approval for shipment. The study was approved by the ethics committee, North-West University with an approval number NWU-01242-19-A9.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted for detecting antibodies to *T. equi* and *B. caballi* from serum samples using equine truncated merozoite antigen 2 (EMA-2 t) and 48 kDa merozoite rhoptry protein (*BC*48), respectively.[16,17] Serum samples from equids were used at 1:200 dilution and the absorbance (OD415 nm) was measured. All serum samples were tested in duplicates. Both negative and positive controls were included in each plate in triplicates. The positive controls were previously prepared from horses that were experimentally infected with *B. caballi* or *T. equi*.[17] The sum of the mean optical density (OD) value of sera from 10 negative horses and the threefold standard deviation was used to arrive at the cutoff value, as described previously.[17,18] A sample is considered positive if the mean OD value was greater than the cutoff. The

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

relative percent positivity (RPP) for each tested sample was computed using the formula described previously.[19] The cutoff RPP value for a sample to be considered positive for both *EMA*-2 t and *BC*48 ELISAs was set at 20%.

Immunofluorescence antibody test assay (IFAT)

IFAT was used for serological screening to detect the antibodies to *B. caballi* and *T. equi* in horses and donkeys. Antigen for the IFAT slides was prepared from in-house *in vitro* cultures of *B. caballi* and *T. equi* as described previously.[20] All prepared slides were stored at −80°C until used. Briefly, the antigen-coated IFAT slides were removed from−80°C freezer and kept in a drying oven at 50°C for 2 min. Thereafter, non-antigen coated spots were blocked with 20 µL of freshly prepared 5% fetal bovine serum (FBS) in

phosphate buffered saline (PBS) for 30 min at room temperature in a humid chamber. Incubated slides were washed three times using PBS for 3 min (slides were not allowed to dry after washing before the addition of serum samples into the wells). The donkey and horse serum samples were diluted with 5% FBS at 1:80 dilution, and 10 µL of the freshly prepared serum samples were added to each antigen-containing well. After incubation at 37°C for 30 min in a humid chamber, slides were washed with PBS for 3 min with three repetitions. Then, 10 µL of species specific secondary antibodies, that is, Rabbit anti-donkey IgG (H + L) (Invitrogen, Massachusetts, USA) for donkeys and Goat anti-horse IgG-fluorescein isothiocyanate (FITC)-conjugated (Bethyl Laboratories, Inc., USA) for horses, which were prepared with 5% FBS at a dilution of 1:250, were added to the wells on the slides and incubated at 37°C for 30 min in the dark. The slides were then washed thrice with PBS for 3 min. The slides were overlaid with 50% glycerol-PBS (1:1), covered with a 24 × 60 mm cover slip and examined under a fluorescence microscope. Every run had positive and negative controls.

Statistical analyses

Data generated were analyzed using descriptive statistics for both IFAT and ELISA and event outcome was expressed in total (absolute) and percentage (relative) frequencies. All analyses were carried out using SPSS® version 20. All *P* values are considered statistically significant at <0.05 unless otherwise stated. Odds ratio and chi-square tests were used to determine the association between seropositivity of *T. equi* and *B. caballi* using Graph Pad Prism version 5.0. We used MedCalc statistical software version 13.0.2 for Windows (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org) to compare and compute the diagnostic accuracy between the two assays (IFAT and ELISA) using four likelihood ratios, namely, PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; PPV: Positive predictive value; NPV: Negative predictive value. The level of agreement between the two serological techniques (IFAT and ELISA) was determined using kappa coefficient.[21]

Results

Serological detection of equine piroplasms using ELISA

The serum samples from horses and donkeys were immune-reactive to the *T. equi*- and *B. caballi*-specific ELISAs. The ELISA results demonstrated that 45.9% (125/272) and 2.9% (5/170) of horses and donkeys, respectively, were *T. equi*-positive, while 0.4% (1/272) and 2.4% (4/170) of horses and donkeys, respectively, were seropositive to *B. caballi* (Table 1). The overall seropositive

rate was significantly higher for *T. equi* compared to *B. caballi* in horses ($P < .0001$), but not in donkeys ($P = 1.000$). Similarly, in the horses, the odds for seropositivity to *T. equi* determined by ELISA was 230 times more likely (OR = 230.4; 95%CI: 31.86–1667) than *B. caballi* positivity. Mixed infection was observed only in horses at 0.4% (1/272) using ELISA (Table 1).

Serological detection of equine piroplasms using IFAT

The IFAT slides were validated using known positive sera before the Nigerian samples were screened for anti-*B. caballi* and anti-*T. equi* antibodies. No cross-reactions were observed between *T. equi* and *B. caballi* when known positive sera samples were tested. The IFAT detected antibodies to both parasite species with strong fluorescence signals in serum samples (1/80 dilution) from horses and donkeys.

Based on the IFAT results, 48.9% (133/272) and 14.1% (24/170) of horses and donkeys were positive to *T. equi* respectively, while only 6.3% (17/272) and 0.6% (1/170) were positive to *B. caballi* respectively (Table 2). The overall seropositive rate was significantly higher for *T. equi* as compared to *B. caballi* in both horses and donkeys ($P < .0001$). The odds for seropositivity to *T. equi* was 14-times likely (OR = 14.35; 95%CI: 8.32–24.77) in the horses than that of *B. caballi*. Mixed infection with both piroplasms was observed at

Table 1. ELISA results for equine piroplasms in equids in North-Western Nigeria.

Variables	*Babesia caballi*	*Theileria equi*	Mixed
Horses			
Positive	1 (0.4%)	125 (45.9%)	1 (0.4%)
Negative	271 (99.6%)	147 (54.0%)	271 (99.6%)
Total	272 (100.0%)	272 (100.0%)	272 (100.0%)
Donkeys			
Positive	4 (2.4%)	5 (2.9%)	0 (0%)
Negative	166 (97.6%)	165 (97.1%)	170 (100.0%)
Total	170 (100.0%)	170 (100.0%)	170 (100.0%)

Table 2. IFAT results for equine piroplasms in equids in North-Western Nigeria.

Variables	*Babesia caballi*	*Theileria equi*	Mixed
Horses			
Positive	17 (6.3%)	133 (48.9%)	15 (5.5%)
Negative	255 (93.8%)	139 (51.1%)	167 (61.4%)
Total	272 (100.0%)	272 (100.0%)	272 (100.0%)
Donkeys			
Positive	1 (0.6%)	24 (14.1%)	0 (0%)
Negative	169 (99.4%)	146 (85.9%)	170 (100.0%)
Total	170 (100.0%)	170 (100.0%)	170 (100.0%)

Table 3. Comparison of the ELISA and IFAT for detecting *T. equi* in equids in North-Western Nigeria.

	IFAT *Theileria equi*		
Variables	Positive	Negative	Total
ELISA *Theileria equi*			
Positive	76 (17.2%)	54 (12.2%)	130 (29.4%)
Negative	81 (18.3%)	231 (52.3%)	312 (70.6%)
Total	157 (35.5%)	285 (64.5%)	442 (100.0%)

Table 4. Comparison of ELISA and IFAT for detecting *Babesia caballi* in equids in North-Western Nigeria.

	IFAT *Babesia caballi*		
Variables	Positive	Negative	Total
ELISA *Babesia caballi*			
Positive	0 (0%)	2 (0.5%)	2 (0.5%)
Negative	21 (4.8%)	419 (94.8%)	440 (99.6%)
Total	21 (4.8%)	421 (95.3%)	442 (100.0%)

5.5% (15/272) in horses, whereas none were detected in donkeys using IFAT (Table 2).

Comparison between IFAT and ELISA for the detection of equine piroplasms

For *T. equi*, IFAT detected more positive equids (157/442, 35.5%) as compared to ELISA (130/442, 29.4%), but the difference was not significant (P = .0617) (Table 3). Only 17.2% (76/442) of the screened equids were *T. equi*-positive by both IFAT and ELISA. For *B. caballi*, the number of samples detected positive by IFAT (18/442, 4.1%) was significantly higher than that detected by ELISA (5/442, 1.1%) (P = .0097). We found that the serum samples tested for anti-*B. caballi* antibodies using IFAT were 10 times more likely to be positive (OR = 10.97; 95%CI: 2.56–47.1) as compared with ELISA (Table 4). Kappa statistic for both serological tests (IFAT and ELISA) was higher with respect to the detection of *T. equi* infection (k = 0.559) than for *B. caballi* (k = 0.09) being classified as moderate and low strength of association, respectively.

Table 5. Diagnostic accuracy of ELISA and IFAT in the present study.

Diagnostic techniques	Sensitivity	Specificity	PLR	NLR	PPV	NPV	Accuracy
ELISA *T. equi* vs IFAT *T. equi*	48.4 (40.4–56.5)	81.1 (76.0–85.4)	2.6	0.6	58.5	74.0	69.5
IFAT *T. equi* vs ELISA *T. equi*	58.5 (49.5–67.0)	74.0 (68.8–78.8)	2.3	0.6	48.4	81.1	69.5
ELISA *B. caballi* vs IFAT *B. caballi*	0.0 (0.0–16.1)	99.5 (98.3–99.9)	0.0	1.0	0.0	95.2	94.8
IFAT *B. caballi* vs ELISA *B. caballi*	0.0 (0.0–52.2)	96.1 (93.8–97.7)	0.0	1.0	0.0	98.8	95.0
ELISA *B. caballi* vs IFAT *T. equi*	0.6 (0.02–3.5)	98.6 (96.5–99.6)	0.5	1.0	20.0	64.3	63.8
IFAT *T. equi* vs ELISA *B. caballi*	20.0 (0.5–71.6)	64.3 (59.6–68.8)	0.6	1.2	0.6	98.6	63.8
IFAT *B. caballi* vs ELISA *T. equi*	10.0 (5.4–16.49	98.4 (96.3–99.5)	6.2	0.9	72.2	72.4	72.4
ELISA *T. equi* vs IFAT *B. caballi*	72.2 (46.5–90.3)	72.4 (67.9–76.6)	2.6	0.4	10.0	98.4	72.4

PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; PPV: Positive predictive value; NPV: Negative predictive value

The overall results for the diagnostic accuracy measures in this study are presented in Table 5. The diagnostic accuracy between ELISA *T. equi* vs IFAT *T. equi* had a specificity of 81.1% and sensitivity of 48.4% with a PPV of 58.5% and accuracy of 69.5%. An accuracy of 95.0% with a NPV of 98.8% was observed when the results between IFAT *B. caballi* were compared to ELISA *B. caballi* (Table 5).

Discussion

Seroepidemiological surveys of equine piroplasm parasites are essential because they assist in determining the rate of equid exposure to the causative agents.[22] This is because EP is an economically important disease with significant losses in the equine industry, which range from cost of treatment, abortion, loss of activity and death in some cases.[6,23] In the present study, we investigated piroplasm infections in horses and donkeys in North-Western Nigeria using two serodiagnostic tests, IFAT, and ELISA. Both are OIE-recommended tests for screening of horses for EP.[7] The two serodiagnostic assays (IFAT and ELISA) employed in the current study have been utilized previously for screening EP infections in equine samples from different parts of the world and they displayed great accuracy.[16–18]

Horses

The seroprevalence of *T. equi* determined using IFAT and ELISA was 48.9% and 45.9%, respectively. Previously, Mshelia *et al.*[15] screened horses from Nigeria for antibodies against *T. equi* using ELISA (VMRD) and found that the seroprevalence was 10.3%, 77.8%, 60.0% and 31.3%, respectively, for samples collected in 2007–2010, 2011, 2013 and 2014. Within Africa, lower prevalence of 14.8% and 23.9% was reported in Egypt using ELISA and IFAT, respectively,[24] while in Sudan, a prevalence of 50.6% was observed.[25] Furthermore, the prevalence obtained in the present study is similar to the pooled prevalence estimates of *T. equi* in Africa which currently stands at 44.4%.[26] On the other hand, the prevalence of *B. caballi* in our study was 6.3% and 0.4%, respectively, by IFAT and ELISA. In a previous study in Egypt, the *B. caballi* prevalence determined by IFAT was higher (17.0%) than that observed in Nigeria, but all surveyed animals were negative when ELISA was used.[24] Comparatively, the prevalence of *B. caballi* in this study was similar to that previously reported in Nigeria, which ranged from 2.7% to 4.8%, using ELISA.[15] Generally, the prevalence of *T. equi*, as reported in this study, was higher than that of *B. caballi* corroborating previous findings.[15,24] It is well established that equids infected with *T. equi* are lifelong carriers, while *B. caballi*-infected animals usually clear the parasites within 1–4 years in the absence of re-infection.[23] Therefore, diminishing immunity due to clearance

of the parasite could be attributed to the lower seroprevalence of *B. caballi* compared to that of *T. equi*. It has also been postulated that differences in the density of tick vectors might be a reason for the differential seroprevalence of *T. equi* and *B. caballi*.[18]

Donkeys

In this study, the seroprevalence of *T. equi* in donkeys as determined by IFAT and ELISA was 14.1% and 2.9%, respectively. Within Africa, higher prevalence of 81.2% was reported in Kenya using ELISA[27] and of 55.7% in Ethiopia using IFAT[28] and a moderate prevalence of 23.5% and 31.4% using ELISA and IFAT, in Egypt, respectively.[24] The *T. equi* seroprevalence determined in our study is lower compared to 55.8% reported recently in Nigeria.[13] Differences in the study areas might be a reason for this discrepancy, as the animals in different states within the North-Western geopolitical zone were sampled. Other factors, such as sample size as well as climatic changes which affect vector distribution, may also be attributed to this observation. Variations in seroprevalence between horses and donkeys have been observed. Several studies in different countries have reported higher seroprevalence in horses as compared to donkeys in Brazil, Pakistan, Turkey, and the United Arab Emirates.[29–32] Our recent PCR-based study found that the prevalence was higher in the donkeys than in the horses with regard to both piroplasms.[14] By contrast, in the current study, we observed lower seroprevalence in donkeys compared to horses. It is important to note that while PCR detects the parasites, serology indicates the past exposure as well. Previously, hematological findings in the sampled donkeys reported elsewhere indicated that the total white blood cells count was lower in both the PCR-positive and PCR-negative groups compared with the standard value.[33] It is likely that the majority of the sampled donkeys were recently infected and that the samples were collected before the antibodies reached the levels detectable by serological tests. Furthermore, in endemic settings, clinical signs are often rare or absent in the infected animals.[19,34]

Low seropositivity of equids to both piroplasms was observed in this study. This was largely attributed to the low prevalence of *B. caballi* compared to *T. equi*. This corroborates previous observations on EP seroprevalence in equids.[10,35] Comparatively, IFAT detected higher prevalence of mixed infection compared with ELISA. Mixed infection by both piroplasms has been attributed to the presence of competent tick vectors responsible for their transmission within the same geographical area.[14]

Comparison between IFAT and ELISA

As recommended by OIE, the IFAT remains the gold standard for equine piroplasmosis.[7] In this epidemiological study, IFAT comparatively detected a greater number of animals exposed to both piroplasms compared with ELISA. The odds of positivity to *T. equi* using ELISA was higher as compared to IFAT. On the other hand, IFAT appears more likely for the detection of *B. caballi* when compared to ELISA. IFAT is known for its high sensitivity.[35] Nonetheless, it is important to note that IFAT has several shortcomings, which include low throughput and subjective to operator-dependent reading of results.[36,37] To overcome these drawbacks, ELISA has been preferably used due to its advantages of high throughput analysis and better specificity of the antigen.[37] Furthermore, comparison of ELISA and IFAT for both piroplasms indicated that detection specificity was higher for ELISA when compared to IFAT, while the detection sensitivity was higher for IFAT as compared to ELISA. It is interesting to note that some serum samples were ELISA-positive and IFAT-negative and vice versa. This may be due to genetic variation of the *T. equi* isolates found in the study area. So far, four genotypes (A, B, C, and D) out of the five described (A, B, C, D and E) *T. equi 18S* rRNA genotypes have been identified in Nigeria equids.[13] This may be further compounded by the recently described *T. haneyi*, which represents a cryptic *Theileria* species. Recently, Bhoora *et al.*[1] observed that the OIE recognized serological assays for the detection of *Theileria* parasites in equids, which has been compromised due to genetic diversity of genes encoding antigens – employed in the serological methods.

Furthermore, *BC*48 is the most widely used antigen in ELISA for the serodiagnosis of *B. caballi*, while *EMA*-1 and *EMA*-2 are employed as serodiagnostic antigen for *T. equi*.[18,38] *EMA*-2 ELISA is much more advantageous due to its ability to detect *T. equi*-specific antibodies in horses 6–12 days earlier compared with *EMA*-1 ELISA.[39] We also speculate that low level or possibly no expression of *BC*48 and *EMA*-2 in the infected animals could be responsible for the likely reason that some results were IFAT-positive and ELISA-negative. Therefore, further studies by sequencing *BC*48 and *EMA*-2 genes from blood DNA samples that are IFAT-positive and ELISA-negative and comparing them with the sequence data from the USDA strain from which the *B. caballi* and *T. equi* ELISA were developed will provide more insights to clarify some of the observations regarding the outcome of the results using both serological techniques.

Conclusion

In conclusion, this study has provided more insight into the positive rates of Nigerian equids to antibodies of *B. caballi* and *T. equi*. We observed that the

seroprevalence of *T. equi* was higher compared to infection with *B. caballi* in both horses and donkeys. Comparatively, IFAT detected more antibodies to both piroplasmids compared to ELISA, highlighting the importance of using different diagnostic approaches to detect piroplasmids in endemic areas due to differences in diagnostic accuracy. The findings from this study will be useful in the design and implementation of a risk-based control strategy aimed at minimizing infection of equids in Nigeria to both *B. caballi* and *T. equi*. Finally, further studies analyzing the genetic diversity based on *18S* rRNA, multilocus sequence typing (MLST) schemes and whole genome sequences as well as analyzing ecological factors in relation to infections could be helpful in delineating the extent to which genetic diversity ends.

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Ethical approval and informed consent

Ethical permission to carry out this study was approved by the NWU with ethics number NWU-01242-19-A9 in line with the guidelines of the university.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

ThankGod E. Onyiche http://orcid.org/0000-0001-6738-2291
Bumduuren Tuvshintulga http://orcid.org/0000-0001-6353-0939
Lehlohonolo Mofokeng http://orcid.org/0000-0002-0274-1828
Oriel Thekisoe http://orcid.org/0000-0003-0700-4787

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