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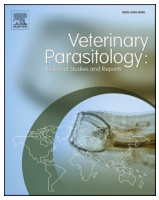

Original Article

An epidemiological survey of equine piroplasmosis in donkeys and horses in Malawi

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ABSTRACT

Equine piroplasmosis (EP) is a tick-borne disease caused by *Theileria equi*, *Theileria haneyi*, and *Babesia caballi* in equids, such as horses, donkeys, mules, and zebras. A comprehensive understanding of the epidemiology of *T. equi* and *B. caballi* is vital for EP management. The present study surveyed *T. equi* and *B. caballi* infections in donkeys and horses in Malawi. Blood samples were collected from 185 equines, including 178 donkeys in Lilongwe ($n = 136$) and Dedza ($n = 42$) districts, and seven horses in Lilongwe district. The blood samples were used to measure hematocrit values and prepare thin smears and blood spots on FTA cards. Microscopic examination of the blood smears detected *T. equi* in 91 equines (49.2 %), including 88 donkeys (49.4 %) and three horses (42.9 %), while *B. caballi* was not detected. Screening of DNA samples extracted from FTA cards with species-specific PCR assays detected *T. equi* in 156 (84.3 %) equines, including 152 (85.4 %) donkeys and four (57.1 %) horses, whereas all animals were negative for *B. caballi*. We found that the mean hematocrit value of infected donkeys (28.1 %) was significantly lower (P value = 0.0004) than that of uninfected donkeys (31.9 %). Additional analysis of *T. equi*-positive DNAs with the genotype-specific PCR assays detected all five genotypes (A, B, C, D, and E) in donkeys and four genotypes (A, B, C, and D) in horses. In summary, the present study, the first to report the *T. equi* infection in Malawi, suggests the need for EP control due to its potential clinical significance.

1. Introduction

Equine piroplasmosis (EP) is a tick-borne disease caused by two protozoan parasites, *Theileria equi* and *Babesia caballi*, in several equine species, including horses, donkeys, mules, and zebras (Onyiche et al., 2019; Qablan et al., 2013). *Theileria equi* comprises five genotypes (A–E), and a recent study characterized an isolate within genotype C as a distinct species, known as *Theileria haneyi* (Knowles et al., 2018). Consequently, the causative agents of EP include three parasite species: *T. equi*, *B. caballi*, and *T. haneyi*. The asexual reproduction of *T. equi* and *B. caballi* within the erythrocytes of infected equines, causes a progressive hemolysis, leading to anemia and other related clinical signs, such as fever, jaundice, hemoglobinuria, and in some cases, death (Friedhoff and Soulé, 1996). Consequently, EP causes significant economic losses to equine industry, due to the costs associated with treatment and tick control, loss of production, and mortality (Rothschild, 2013). Once infected, *T. equi* can persist for life in equines, while *B. caballi* infection can be cleared within a few years (Brüning, 1996). Importantly, the animals infected with *T. equi* and *B. caballi* become chronic carriers, which act as a source of infections for tick vectors, thereby facilitating

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the spread of EP (Rothschild, 2013; Tamzali, 2013; Ueti et al., 2008; Wise et al., 2014).

Both *T. equi* and *B. caballi* are primarily transmitted through competent tick vectors, with iatrogenic transmission contributing to a lesser extent (de Waal and van Heerden, 2004; Ueti et al., 2008; Wise et al., 2014). In addition, *T. equi* has been reported to undergo vertical (transplacental) transmission from mares to foals (Allsopp et al., 2007). The competent tick vectors capable of transmitting these parasites include several species of the genera *Amblyomma*, *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Scoles et al., 2011; Scoles and Ueti, 2015). In general, EP is endemic in countries where competent tick vectors are found.

Malawi is a landlocked country located in south-eastern Africa with an economy based on agriculture and livestock production, which contributes approximately 11 % to the country's national GDP (Ministry of Agriculture Malawi, 2021). The equines bred in Malawi mainly include donkeys with an estimated population of 14,000 heads, whereas the horse population is below 100 (National Statistical Office Malawi, 2007). Donkeys are mainly used to pull carts for transporting agricultural products and ploughing fields, unlike horses, which are used for tourism- and recreational-related activities. Similar to other livestock, both donkeys and horses are managed under extensive systems, which make them prone to tick infestations (de la Fuente et al., 2023). Previous studies conducted in Malawi detected ticks and several tick-borne pathogens in livestock, including cattle, sheep, and goats (Berggren, 1978; Chatanga et al., 2021, 2022). However, the prevalence of *T. equi* and *B. caballi* among equines in Malawi remains uninvestigated. Therefore, the present study aimed to determine the infection status of *T. equi* and *B. caballi* in the donkey and horse populations in Malawi.

2. Materials and methods

2.1. Study area and blood sampling

The present study surveyed donkeys and horses in two districts of Malawi, namely Lilongwe and Dedza, located in the central region. With an altitude of 690 m above sea level, the region is known for its tropical savanna climate (Kottek et al., 2006). The mean annual temperature is around 22.4 °C, with an annual precipitation ranges from 118 mm to 143 mm.

Sampling was conducted between March and May 2023. Only animals that were older than six months, non-pregnant (in the case of females), and not undergoing treatment were sampled. A total of 178 donkeys were sampled in three Extension Planning Areas (EPA) in two districts: Mitundu EPA ($n = 64$) and Mkwinda EPA ($n = 72$) in Lilongwe district, and Linthipe EPA ($n = 42$) in Dedza district. Of the sampled donkeys, 70 were males, 108 were females, 58 were $<$ 3-year-old, and 120 were $\geq$ 3-year-old. A total of seven horses were sampled at Likuni EPA in Lilongwe district. Briefly, after sterilizing the venipuncture site with a methylated spirit swab, 3 mL of blood was collected from the jugular vein of each animal into a sterile EDTA vacutainer tube. Approximately 40 µL of whole blood from each animal was added on a well of Whatman FTA card (Whatman FTA Elute, GE Healthcare life sciences, Chicago, IL, USA), air-dried, and then stored at room temperature.

2.2. Measurement of hematocrit value

To measure the hematocrit value, each blood sample was loaded into a micro-hematocrit capillary tube (Fisher Scientific, Pittsburgh, PA, USA) and sealed at one end with a sealant. The capillary tube was centrifuged at 3000 rpm for 1 min using a micro-hematocrit centrifuge (Hawksley, Sussex, UK), and then read using a micro-hematocrit centrifuge reader (VIN, Davis, CA, USA) to measure the hematocrit value.

2.3. Microscopic examination of thin blood smears for B. caballi and T. equi infections

Thin blood smears were prepared from a drop of the collected blood samples, air-dried, fixed in absolute methanol for 3 min, and then stained with 10 % Giemsa solution for 12 min (Barcia, 2007; Houwen, 2002). The stained smears were air-dried at room temperature and then observed under a light microscope (CX33, Olympus, Tokyo, Japan), using 100x objective lens and oil immersion, for detecting *T. equi* and *B. caballi* within erythrocytes.

2.4. PCR screening for B. caballi and T. equi infections

DNA samples were extracted from the dried blood spots on FTA cards, following the manufacturer's instructions with minor modifications that included using two 3-mm discs and eluting DNA with 40 µL double distilled water. All DNA samples were subjected to previously described PCR assays based on *18S rRNA* and rhoptry-associated protein 1 gene (*rap-1*) sequences to detect *T. equi* and *B. caballi* infections, respectively (Ahedor et al., 2023b; Alhassan et al., 2005). The PCR assay used for detecting *T. equi* is capable of amplifying *18S rRNA* from all genotypes (A–E), as well as *T. haneyi*. In brief, a 10-µL PCR reaction mixture was prepared by adding 1 µL of 10× PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 1 µL of 2 mM each dNTP (Applied Biosystems), 0.5 µL of 10 µM each forward and reverse primer (Table 1), 0.1 µL of 5 U/µL Taq DNA polymerase (Applied Biosystems), 5.9 µL of molecular grade water, and 1 µL of DNA template. The DNA samples extracted from the *in vitro* cultures of *T. equi* and *B. caballi* (Tuvshintulga et al., 2016) were used as the positive controls, while molecular-grade water was used as a negative control. The reaction mixture was then subjected to a pre-denaturation step at 95 °C for 5 min, followed by 40 cycles that included a denaturation step at 95 °C for 30 s, an annealing step at 51 °C (*T. equi*) or 55 °C (*B. caballi*) for 30 s, and an extension step at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The PCR products were resolved on a 1.5 % agarose gel, stained with Midori Green Extra (NIPPON Genetics, Tokyo, Japan), and then visualized under UV illumination. Samples that produced bands of the expected sizes (Table 1) were classified as positive.

2.5. Genotyping of T. equi

All DNA samples that tested positive for *T. equi* infection were screened using *18S rRNA*-based PCR assays specific for the genotypes A, B, C, D, and E of *T. equi*, as described previously (Ahedor et al., 2023a). Briefly, a 10-µL reaction mixture, consisting of 0.1 µL of proof reading KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan), 5.0 µL of 2× buffer for KOD FX Neo (TOYOBO), 0.5 µL of 10 µM each forward and reverse primer, 1.0 µL of DNA template, and 2.9 µL of molecular grade water, was prepared. Following a pre-denaturation at 94 °C for 2 min, the reaction mixture underwent 40 cycles, each consisting of a denaturation step at 98 °C for 10 s, an annealing step at 70 °C, 66 °C, 66 °C, 64 °C and 72 °C, respectively for genotypes A, B, C, D and E, for 30 s, and an extension step at 68 °C for 30 s. PCR products were resolved, stained, and then visualized as described above. Samples producing bands of the expected sizes (Table 1) were considered as positive. To minimize contamination, all PCR procedures were performed using filter tips and in physically separated areas for pre- and post-PCR work. Multiple negative controls were included in each PCR run to ensure the reliability of the results.

2.6. Cloning and sequencing

The amplicons from *T. equi* genotype-specific PCR assays were cloned and sequenced, as described previously (Ahedor et al., 2023a). Briefly, two amplicons for genotypes A and B and three amplicons for genotypes C, D, and E from the specific PCR assays were gel-extracted

Table 1
List of primers used in diagnostic and genotyping PCR assays.

Purpose	Target gene	Primer sequence (5′ → 3′)	Product size (bp)	Reference
Screening	*T. equi 18S rRNA*	F: TCGAAGACGATCAGATACCGTCG R: TGCCTTAAACTTCCTTGCGAT	435	Alhassan et al., 2005
Screening	*B. caballi rap-1*	F: CCAACCGCTGACCCTTC R: CTTCAGCTTCATGTACCACTTCTT	544	Ahedor et al., 2023b
Genotyping	*T. equi* genotype A *18S rRNA*	F: CGTTGCGGCTTGGTTGGGTTTCGATTA R: GCAAAGTCCCTCTAAGAAGCGGA	692	Ahedor et al., 2023a
Genotyping	*T. equi* genotype B *18S rRNA*	F: GTGGTCCTTCGCTATGTCGAGTGGTCC R: CGCAAAGTCCCTCTAAGAAGCGATGGT	718	Ahedor et al., 2023a
Genotyping	*T. equi* genotype C *18S rRNA*	F: TGTATCGTTATCTTCTGCTTGACAGTTTGG R: GTCCCTCTAAGAAGCAGTGTAGAACATAAC	714	Ahedor et al., 2023a
Genotyping	*T. equi* genotype D *18S rRNA*	F: TGTATCGTTTTCCTCTGCTTGACAGTTGGA R: GTCCCTCTAAGAAGCAGTGTAGAACTAG	719	Ahedor et al., 2023a
Genotyping	*T. equi* genotype E *18S rRNA*	F: ATCGTGGTTCTTCGCTATGTCGAGTGATCT R: CGCAAAGTCCCTCTAAGAAGCGATAAC	722	Ahedor et al., 2023a

F, forward primer; R, reverse primer.

using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), cloned into a PCR 2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA, USA), and then sequenced using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Tokyo, Japan). The resulting sequences were analyzed with basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch) to determine the identity scores shared with the homologous sequences registered in the GenBank. The *T. equi 18S rRNA* sequences generated in the present study were registered with the DNA Data Bank of Japan (DDBJ) under the accession numbers LC853091 - LC853103.

2.7. Phylogenetic analysis of T. equi 18S rRNA sequences

The Malawian *18S rRNA* sequences determined in the present study, along with the *T. equi 18S rRNA* sequences representing each of the five genotypes (A–E) from the GenBank database, were aligned using a MAFFT online version (https://mafft.cbrc.jp/alignment/server/index.html) (Katoh et al., 2019). The MEGA X software (Kumar et al., 2018) was then used to analyze the resulting 657 bp alignment to predict the best-fitting substitution model. Finally, a maximum likelihood phylogeny was constructed based on general time reversible substitution model, incorporating the invariant plus gamma-distributed sites (GTR + I + G), along with 1000 bootstrap replicates for assessing the reliability (Felsenstein, 1981; Gatto et al., 2006).

2.8. Statistical analyses

The positive rates of *T. equi* infection in donkeys were assessed with an 'N-1' chi-squared test (https://www.medcalc.org/calc/comparison_of_proportions.php), and *P* values were calculated to determine whether the differences in positive rates were statistically significant among sexes, age groups, and sampling districts (Richardson, 2011; Campbell, 2007). Microsoft Excel was used to determine standard deviations (SD) and *P* value (Student's *t*-test) for the mean hematocrit values of infected and non-infected donkeys. A *P* value less than 0.05 indicated statistical significance for the observed differences.

3. Results

3.1. Microscopy and PCR results and hematocrit alterations

In the present study, 185 equines, including 178 donkeys and 7 horses, were screened for the infections with *T. equi* and *B. caballi*, using microscopy and PCR assays. Microscopic examination of Giemsa-stained thin blood smears detected *T. equi* in 88 donkeys (88/178, 49.4 %) and three horses (3/7, 42.9 %) (Table 2). Of 136 and 42 donkeys sampled in Lilongwe and Dedza districts, 69 (50.7 %) and 19 (45.2 %), respectively, were *T. equi*-positive. Different morphological forms of *T. equi*, including

Table 2
Microscopic and PCR detection of *Theileria equi* from donkeys and horses surveyed in Malawi.

Host[a]	District	No. sample	No. microscopy-positive (%)	No. PCR-positive (%)
Donkey	Lilongwe	136	69 (50.7)	117 (86.0)
	Dedza	42	19 (45.2)	35 (83.3)
	Sub-total	178	88 (49.4)	152 (85.4)
Horse	Lilongwe	7	3 (42.9)	4 (57.1)
Total		185	91 (49.2)	156 (84.3)

[a] All donkeys and horses surveyed in the present study were negative for *Babesia caballi* in both microscopy and PCR assay.

Maltese cross, ring, and irregular forms, were observed (Fig. 1). However, all surveyed equines were negative for *B. caballi* in microcopy. All surveyed animals were also negative for *B. caballi* infection in PCR assay. By contrast, 152 donkeys (85.4 %), including 117 (86.0 %) from Lilongwe and 35 (83.3 %) from Dedza, were tested positive for *T. equi* infection by PCR (Table 2). Of the surveyed horses, 4 (57.1 %) tested positive. All microscopy-positive equines were also PCR-positive.

A significant difference was observed in mean hematocrit values between the *T. equi*-infected and non-infected donkeys. The mean hematocrit value of infected donkeys (28.05 %, SD ± 4.2) was significantly lower (*P* value = 0.0004) than that of non-infected donkeys (31.9 %, SD ± 2.4). Furthermore, mean hematocrit values were comparable (*P* = 0.937) between microscopy-positive (27.8 %) and PCR-positive but microscopy-negative (28.1 %) donkeys.

3.2. Risk factor analyses

To identify the potential risk factors associated with *T. equi* infection in donkeys, the positive rates as determined by PCR assay were analyzed, based on the sexes (male and female), age groups (<3-year-old and ≥ 3-year-old), and sampled districts (Lilongwe and Dedza). However, our analyses revealed no statistically significant correlation (*P* > 0.05) between the *T. equi*-positive rates and the analyzed risk factors

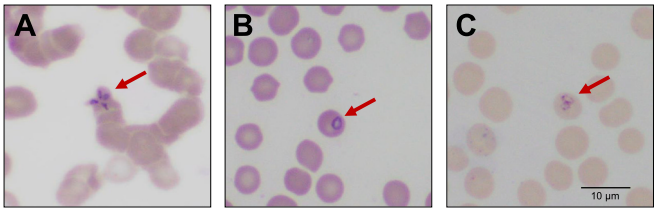

Fig. 1. Micrographs of *Theileria equi* detected in donkeys in Malawi. A. Maltese-cross form; B. ring form; and C. irregular pyriform.

Table 3
Positive rates of *T. equi* infection in donkeys, based on sexes, age groups, and sampling locations.

Factors	No. donkeys	No. positive (%)	*P* value[a]
Sex			0.6
Male	70	61 (86.1)	
Female	108	91 (83.2)	
Age			0.5
<3-year-old	58	48 (82.8)	
≥3-year-old	120	104 (85.0)	
Location			0.6
Lilongwe	136	117 (86.0)	
Dedza	42	35 (83.3)	

[a] The *P* values indicated that the *T. equi*-positive rates in donkeys were comparable between sexes (males and females), age groups (<3-year-old and ≥ 3-year-old), and location (Lilongwe and Dedza districts).

(Table 3).

3.3. Genotyping of T. equi

Of 156 *T. equi*-positive DNA samples, genotype-specific PCR assays detected at least one genotype in 152 samples (Table 4). The PCR results further revealed that the donkeys were infected with all five genotypes (A-E), whereas the horses were infected with four genotypes (A, B, C, and D). The genotype D was the most common, detected in 138 animals (138/152, 90.8 %), followed by the genotypes A, C, B, and E, which were detected in 80 (52.6 %), 48 (31.6 %), 32 (21.1 %), and 3 (2.0 %), respectively. In terms of isolate counts, among the 301 *T. equi* isolates identified from 152 equines, 45.8 % (138/301) were genotype D, 26.6 % (80/301) were genotype A, 15.9 % (48/301) were genotype C, 10.6 % (32/301) were genotype B, and 1.0 % (3/301) were genotype E. Among the 152 positive animals, 56 (36.8 %) had infections with single genotypes, with the genotype D being the most common (Table 4). The remaining 96 animals were co-infected, with 51 (33.6 %), 38 (25.0 %), six (3.9 %), and one (0.7 %) were infected with two, three, four, and five genotypes, respectively. The most common combination of genotypes infected with two, three, and four genotypes were, A + D, A + C + D, and A + B + C + D, respectively (Table 4).

To verify the results from genotype-specific PCR assays, two amplicons from the genotypes A and B and three amplicons from the genotypes C, D, and E were sequenced. The resultant *18S rRNA* sequences were then compared to those registered in the GenBank database. We found that the Malawian sequences representing the genotype A (accession numbers LC853091 and LC853092), B (LC853093 and LC853094), C (LC853095–LC853097), D (LC853098–LC853100), and E (LC853101–LC853103) shared high identity scores with previously reported sequences of the respective genotypes: 100 % with KY952226, 99.86 % with EU642507, 99.86 % with EU888905, 99.85 % with LC670600, and 99.72–100 % with KF559357. Additionally, the Malawian sequences for each genotype clustered within the corresponding clades in the phylogenetic tree (Fig. 2).

Table 4
Single and co-infections of *Theileria equi* genotypes in donkeys and horses surveyed in Malawi.

Type of infection	Genotype(s)	No. positive (%[a])
Single infection	A	7 (4.6)
	B	2 (1.3)
	C	2 (1.3)
	D	45 (29.6)
	Total	56 (36.8)
Double infections	A + B	1 (0.7)
	A + C	2 (1.3)
	A + D	31 (20.4)
	B + D	8 (5.3)
	C + D	9 (5.9)
	Total	51 (33.6)
Triple infections	A + B + D	9 (5.9)
	A + C + D	22 (14.5)
	A + D + E	1 (0.7)
	B + C + D	6 (3.9)
	Total	38 (25.0)
Quadruple infections	A + B + C + D	5 (3.3)
	A + C + D + E	1 (0.7)
	Total	6 (3.9)
Quintuple infection	A + B + C + D + E	1 (0.7)
	Total	1 (0.7)
Total		152

[a] Expressed as a percentage of the number of animals (152) tested positive for at least one genotype.

4. Discussion

The present study found that the donkeys and horses in Malawi were infected with *T. equi*, whereas *B. caballi* was not detectable. Microscopy and PCR results revealed that a significant proportion of the surveyed equines were infected with *T. equi*. *Theileria equi* does not undergo transovarial transmission in ticks, and equines are the only known reservoir host. Therefore, to maintain transmission in endemic areas, *T. equi* infection persists in the equine host, often for life. This may explain the high positive rate observed in this study (Rothschild, 2013; Tamzali, 2013; Wise et al., 2014). In contrast, *B. caballi* is transovarially transmitted in ticks and can persist across tick generations, reducing the reliance on equine reservoirs. Consequently, *B. caballi* infections can be

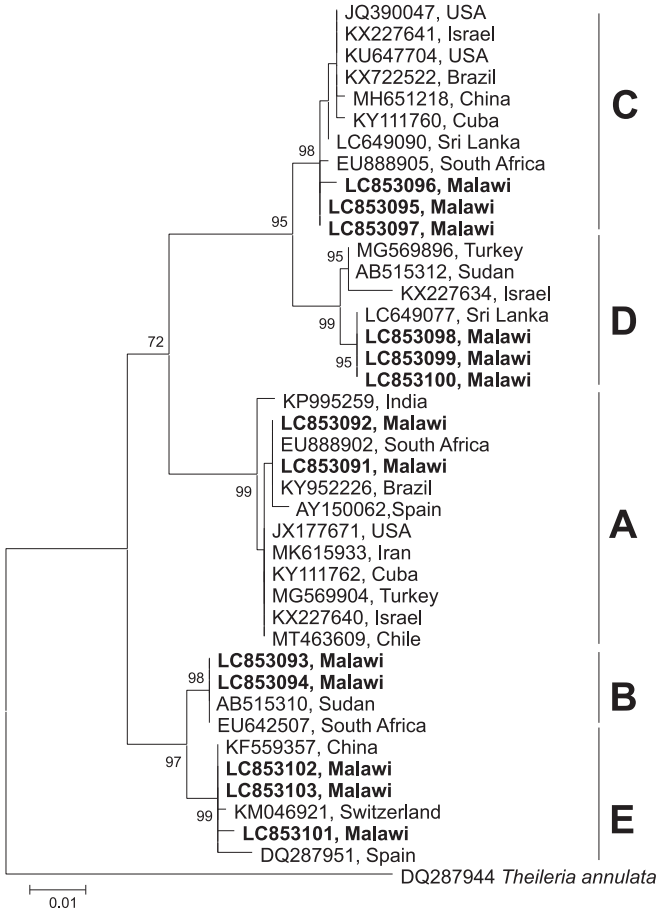

Fig. 2. Phylogenetic analysis of *T. equi 18S rRNA* sequences. The *T. equi 18S rRNA* sequences generated from the amplicons of each genotype-specific PCR assays and those retrieved from GenBank were used to construct a maximum likelihood phylogeny. The Malawian sequences (highlighted in bold) occurred in clades representing all five genotypes (A, B, C, D, and E).

naturally cleared over time, leading to lower detection rates in cross-sectional surveys (Ahedor et al., 2023b). In addition, potential polymorphisms in the primer binding regions (Bhoora et al., 2010; Rapoport et al., 2014) or the relatively low sensitivity of the single-step PCR assay used in this study may also explain the lack of *B. caballi* detection. Therefore, the negative results obtained for *B. caballi* in the present study, which employed a small sample size, do not definitively prove its absence in Malawi.

Our findings revealed no statistically significant variations in the positive rates of *T. equi* infection among the donkeys, based on sexes, age groups, and sampling sites. The comparable positive rates across demographics may be due to potentially similar levels of exposure to tick vectors, because all donkeys are managed under extensive systems (Rapoport et al., 2014). However, the small sample size might render these findings inconclusive, highlighting the need for large-scale surveys to better understand the risk factors associated with *T. equi* infection in Malawi.

The mean hematocrit value was lower in *T. equi*-infected donkeys than that in non-infected donkeys, suggesting an association between *T. equi* infection and anemia (Onyiche et al., 2022; Ueti et al., 2012). However, no correlation was observed between hematocrit levels and the microscopic detection of parasites. Hemolysis of erythrocytes, both infected and uninfected, in the infected animals may explain this observation (Wise et al., 2014). Nevertheless, our results highlight the importance of implementing effective control strategies to minimize the *T. equi* infection in equines in Malawi.

Of the 156 *T. equi*-positive samples analyzed using genotype-specific PCR assays, 152 yielded positive results for at least one genotype, while the remaining four tested negative. One possible explanation is that these negative samples could have involved co-infections with multiple genotypes, each present at low concentrations. In such cases, the combined DNA templates may have been sufficient to produce a positive result in the *T. equi*-specific screening PCR, while the low abundance of individual genotypes may have led to negative results in the genotype-specific assays. Alternatively, these results may reflect the relatively lower sensitivity of the genotype-specific PCR assays compared to the initial screening assay. Despite this, all five known genotypes (A–E) of *T. equi* were detected in the surveyed equines. In contrast, previous studies in Africa reported only genotypes A–D (Bhoora et al., 2020), with genotype E rarely documented, including a single report from Egypt (Amer et al., 2024). This discrepancy may be due to the use of pan-genotype PCR assays targeting the *18S rRNA* gene in earlier studies, where sequencing predominantly reflected the major genotype in mixed infections, thereby masking the presence of rare genotypes.

Previous studies indicated that the genotypic diversity of *T. equi* could have implications for disease control strategies (Knowles et al., 2018; Manna et al., 2018; Sears et al., 2020). These include a stronger association of genotype A with clinical EP, as compared to other genotypes (Manna et al., 2018; Tirosh-Levy et al., 2020), the inability of cELISA to detect antibodies against *T. haneyi*-a distinct species within genotype C (Knowles et al., 2018), and the inefficacy of imidocarb dipropionate in completely clearing *T. haneyi* from the infected animals (Sears et al., 2020). The detection of genotype A in Malawi underscores the clinical significance of EP in this country (Tirosh-Levy et al., 2020; Manna et al., 2018). Although genotype C was also detected in this study, it remains unclear whether it includes *T. haneyi*; if so, this may pose additional diagnostic and therapeutic challenges in disease management (Sears et al., 2020).

The donkey population in Malawi has quadrupled in the last decade, especially in the central region, where they are used for draught power, as cattle numbers decline (Ministry of Agriculture Malawi, 2021). Maintaining a healthy donkey population is, therefore, critical for sustainable agriculture in the country. Our findings indicate that *T. equi* infection, which may result in anemia, could negatively affect donkey performance. Additionally, the detection of *T. equi* in horses, despite the small sample size, has implications for the equine industry, especially as the sector seeks to import more horses to promote tourism. Therefore, effective disease management strategies, which include surveillance, diagnosis, treatment, and identification and control of tick vectors, are essential for a sustainable equine industry in Malawi.

In conclusion, the present study revealed a substantial population of equines surveyed in Malawi are infected *T. equi*. The low hematocrit values in the infected animals highlight the importance of managing the *T. equi* infection for protecting equids' health and agriculture in Malawi.

CRediT authorship contribution statement

Elisha Chatanga: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Believe Ahedor:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Berdikulov Atabek:** Writing – review & editing, Methodology, Investigation. **Henson Kainga:** Writing – review & editing, Investigation. **Thoko Kapalamula:** Writing – review & editing, Investigation. **Tinotenda Razemba:** Writing – review & editing, Investigation. **Ryo Nakao:** Writing – review & editing, Resources, Formal analysis. **Nariaki Nonaka:** Writing – review & editing, Resources, Formal analysis. **Thillaiampalam Sivakumar:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Naoaki Yokoyama:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Ethics approval

All animal sampling procedures and use of the samples collected in this study were approved by the Animal Health and Research Ethics Committee of the Department of Animal Health and Livestock Development (DAHLD), the Ministry of Agriculture (MoA) in Malawi (approval number: DAHLD/AHC/01/2023/07). The procedures were also approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (approval number: 24–2).

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Declaration of competing interest

All authors declare no competing interests related to the present study.

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References

Ahedor, B., Otgonsuren, D., Zhyldyz, A., Guswanto, A., Ngigi, N.M.M., Valinotti, M.F.R., Kothalawala, H., Kalaichelvan, N., Silva, S.S.P., Kothalawala, H., Acosta, T.J., Sivakumar, T., Yokoyama, N., 2023a. Development and evaluation of specific polymerase chain reaction assays for detecting *Theileria equi* genotypes. Parasit. Vectors 16, 435. https://doi.org/10.1186/s13071-023-06045-z.

Ahedor, B., Sivakumar, T., Valinotti, M.F.R., Otgonsuren, D., Yokoyama, N., Acosta, T.J., 2023b. PCR detection of *Theileria equi* and *Babesia caballi* in apparently healthy horses in Paraguay. Vet. Parasitol. Reg. Stud. Rep. 39, 100835. https://doi.org/10.1016/j.vprsr.2023.100835.

Alhassan, A., Pumidonming, W., Okamura, M., Hirata, H., Battsetseg, B., Fujisaki, K., Yokoyama, N., Igarashi, I., 2005. Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. Vet. Parasitol. 129, 43–49. https://doi.org/10.1016/j.vetpar.2004.12.018.

Allsopp, M.T., Lewis, B.D., Penzhorn, B.L., 2007. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. Vet. Parasitol. 148, 130–136. https://doi.org/10.1016/j.vetpar.2007.05.017.

Amer, M.M., Galon, E.M., Soliman, A.M., Do, T., Zafar, I., Ma, Y., Li, H., Ji, S., Mohanta, U.K., Xuan, X., 2024. Molecular detection of tick-borne piroplasmids in camel blood samples collected from Cairo and Giza governorates, Egypt. Acta Trop. 256, 107252. https://doi.org/10.1016/j.actatropica.2024.107252.

Barcia, J.J., 2007. The Giemsa stain: its history and applications. Int. J. Surg. Pathol. 15, 292–296. https://doi.org/10.1177/1066896907302239.

Berggren, A., 1978. Cattle ticks in Malawi. Vet. Parasitol. 4, 289–297. https://doi.org/10.1016/0304-4017(78)90055-9.

Bhoora, R., Quan, M., Zweygarth, E., Guthrie, A.J., Prinsloo, S.A., Collins, N.E., 2010. Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. Vet. Parasitol. 169, 279–288.

Bhoora, R.V., Collins, N.E., Schnittger, L., Troskie, C., Marumo, R., Labuschagne, K., Smith, R.M., Dalton, D.L., Mbizeni, S., 2020. Molecular genotyping and epidemiology of equine piroplasmids in South Africa. Ticks Tick Borne Dis. 11, 101358. https://doi.org/10.1016/j.ttbdis.2019.101358.

Brüning, A., 1996. Equine piroplasmosis: an update on diagnosis, treatment, and prevention. Br. Vet. J. 152, 139–151. https://doi.org/10.1016/S0007-1935(96)80070-4.

Campbell, I., 2007. Chi-squared and Fisher-Irwin tests of two-by-two tables with small sample recommendations. Stat. Med. 26, 3661–3675. https://doi.org/10.1002/sim.2832.

Chatanga, E., Kainga, H., Maganga, E., Hayashida, K., Katakura, K., Sugimoto, C., Nonaka, N., Nakao, R., 2021. Molecular identification and genetic characterization of tick-borne pathogens in sheep and goats at two farms in the central and southern regions of Malawi. Ticks Tick Borne Dis. 12, 101629. https://doi.org/10.1016/j.ttbdis.2020.101629.

Chatanga, E., Maganga, E., Mohamed, W.M.A., Ogata, S., Pandey, G.S., Abdelbaset, A.E., Hayashida, K., Sugimoto, C., Katakura, K., Nonaka, N., Nakao, R., 2022. High infection rate of tick-borne protozoan and rickettsial pathogens of cattle in Malawi and the development of a multiplex PCR for *Babesia* and *Theileria* species identification. Acta Trop. 231, 106413. https://doi.org/10.1016/j.actatropica.2022.106413.

de la Fuente, J., Estrada-Peña, A., Rafael, M., Almazán, C., Bermúdez, S., Abdelbaset, A.E., Kasaija, P.D., Kabi, F., Akande, F.A., Ajagbe, D.O., Bamgbose, T., Ghosh, S., Palavesam, A., Hamid, P.H., Oskam, C.L., Egan, S.L., Duarte-Barbosa, A., Hekimoğlu, O., Szabó, M.P.J., Labruna, M.B., Dahal, A., 2023. Perception of ticks and tick-borne diseases worldwide. Pathogens 12, 1258. https://doi.org/10.3390/pathogens12101258.

de Waal, D.T., van Heerden, J., 2004. Equine piroplasmosis. In: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock. Oxford University Press, Cape Town, South Africa, pp. 425–433.

Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17, 368–376. https://doi.org/10.1007/BF01734359.

Friedhoff, K.T., Soulé, C., 1996. An account on equine babesiosis. Rev. Sci. Tech. 15, 1191–1201. https://doi.org/10.20506/rst.15.3.972.

Gatto, L., Catanzaro, D., Milinkovitch, M.C., 2006. Assessing the applicability of the GTR nucleotide substitution model through simulations. Evol. Bioinformatics Online 2, 145–155.

Houwen, B., 2002. Blood film preparation and staining procedures. Clin. Lab. Med. 22, 1–14. https://doi.org/10.1016/s0272-2712(03)00064-7.

Katoh, K., Rozewicki, J., Yamada, K.D., 2019. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. 20, 1160–1166. https://doi.org/10.1093/bib/bbx108.

Knowles, D.P., Kappmeyer, L.S., Haney, D., Herndon, D.R., Fry, L.M., Munro, J.B., Sears, K., Ueti, M.W., Wise, L.N., Silva, M., Schneider, D.A., Grause, J., White, S.N., Tretina, K., Bishop, R.P., Odongo, D.O., Pelzel-McCluskey, A.M., Scoles, G.A., Mealey, R.H., Silva, J.C., 2018. Discovery of a novel species, *Theileria haneyi* n. sp., infective to equids, highlights exceptional genomic diversity within the genus *Theileria*: implications for apicomplexan parasite surveillance. Int. J. Parasitol. 48, 679–690. https://doi.org/10.1016/j.ijpara.2018.03.010.

Kottek, M., Grieser, J., Beck, C., Rudolf, B., Rubel, F., 2006. World map of the Köppen-Geiger climate classification updated. Meteorol. Z. 15, 259–263. https://doi.org/10.1127/0941-2948/2006/0130.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.

Manna, G., Cersini, A., Nardini, R., Bartolomé Del Pino, L.E., Antognetti, V., Zini, M., Conti, R., Lorenzetti, R., Veneziano, V., Autorino, G.L., Scicluna, M.T., 2018. Genetic diversity of *Theileria equi* and *Babesia caballi* infecting horses of central-southern Italy and preliminary results of its correlation with clinical and serological status. Ticks Tick Borne Dis. 9, 1212–1220. https://doi.org/10.1016/j.ttbdis.2018.05.005.

Ministry of Agriculture Malawi, 2021. National Livestock Development Policy 2021–2026. https://faolex.fao.org/docs/pdf/mlw214429.pdf (accessed 22 January 2025).

National Statistical Office Malawi, 2007. National Census of Agricultural and Livestock 2006/07. https://catalog.ihsn.org/catalog/4578/related-materials (accessed 22 January 2025).

Onyiche, T.E., Suganuma, K., Igarashi, I., Yokoyama, N., Xuan, X., Thekisoe, O.A., 2019. A review on equine piroplasmosis: epidemiology, vector ecology, risk factors, host immunity, diagnosis and control. Int. J. Environ. Res. Public Health 16, 1736. https://doi.org/10.3390/ijerph16101736.

Onyiche, T.E., Igwenagu, E., Malgwi, S.A., Omeh, I.J., Biu, A.A., Thekisoe, O., 2022. Hematology and biochemical values in equines naturally infected with *Theileria equi* in Nigeria. Tropl. Anim. Health Prod. 54, 103. https://doi.org/10.1007/s11250-022-03095-w.

Qablan, M.A., Oborník, M., Petrželková, K.J., Sloboda, M., Shudiefat, M.F., Hořín, P., Lukeš, J., Modrý, D., 2013. Infections by *Babesia caballi* and *Theileria equi* in Jordanian equids: epidemiology and genetic diversity. Parasitology 140, 1096–1103. https://doi.org/10.1017/S0031182013000486.

Rapoport, A., Aharonson-Raz, K., Berlin, D., Tal, S., Gottlieb, Y., Klement, E., Steinman, A., 2014. Molecular characterization of the *Babesia caballi* rap-1 gene and epidemiological survey in horses in Israel. Infect. Genet. Evol. 23, 115–120. https://doi.org/10.1016/j.meegid.2014.01.033.

Richardson, J.T., 2011. The analysis of 2 × 2 contingency tables—yet again. Stat. Med. 30, 890. https://doi.org/10.1002/sim.4116.

Rothschild, C.M., 2013. Equine piroplasmosis. J. Equine Vet. Sci. 33, 497–508. https://doi.org/10.1016/j.jevs.2013.03.189.

Scoles, G.A., Ueti, M.W., 2015. Vector ecology of equine piroplasmosis. Annu. Rev. Entomol. 60, 561–580. https://doi.org/10.1146/annurev-ento-010814-021110.

Scoles, G.A., Hutcheson, H.J., Schlater, J.L., Hennager, S.G., Pelzel, A.M., Knowles, D.P., 2011. Equine piroplasmosis associated with *Amblyomma cajennense* ticks, Texas, USA. Emerg. Infect. Dis. 17, 1903–1905. https://doi.org/10.3201/eid1710.101182.

Sears, K., Knowles, D., Dinkel, K., Mshelia, P.W., Onzere, C., Silva, M., Fry, L., 2020. Imidocarb dipropionate lacks efficacy against *Theileria haneyi* and fails to consistently clear *Theileria equi* in horses co-infected with *T. haneyi*. Pathogens 9, 1035. https://doi.org/10.3390/pathogens9121035.

Tamzali, Y., 2013. Equine piroplasmosis: an updated review. Equine Vet. Educ. 25, 590–598. https://doi.org/10.1111/eve.12070.

Tirosh-Levy, S., Steinman, A., Levy, H., Katz, Y., Shtilman, M., Gottlieb, Y., 2020. Parasite load and genotype are associated with clinical outcome of piroplasm-infected equines in Israel. Parasit. Vectors 13, 267. https://doi.org/10.1186/s13071-020-04133-y.

Tuvshintulga, B., AbouLaila, M., Davaasuren, B., Ishiyama, A., Sivakumar, T., Yokoyama, N., Iwatsuki, M., Otoguro, K., Ōmura, S., Igarashi, I., 2016. Clofazimine inhibits the growth of *Babesia* and *Theileria* parasites in vitro and in vivo. Antimicrob. Agents Chemother. 60, 2739–2746. https://doi.org/10.1128/AAC.01614-15.

Ueti, M.W., Palmer, G.H., Scoles, G.A., Kappmeyer, L.S., Knowles, D.P., 2008. Persistently infected horses are reservoirs for intrastadial tick-borne transmission of the apicomplexan parasite *Babesia equi*. Infect. Immun. 76, 3525–3529. https://doi.org/10.1128/IAI.00251-08.

Ueti, M.W., Mealey, R.H., Kappmeyer, L.S., White, S.N., Kumpula-McWhirter, N., Pelzel, A.M., Grause, J.F., Bunn, T.O., Schwartz, A., Traub-Dargatz, J.L., et al., 2012. Reemergence of the apicomplexan *Theileria equi* in the United States: elimination of persistent infection and transmission risk. PloS One 7, 44713. https://doi.org/10.1371/journal.pone.0044713.

Wise, L.N., Pelzel-McCluskey, A.M., Mealey, R.H., Knowles, D.P., 2014. Equine piroplasmosis. Vet. Clin. North Am. Equine Pract. 30, 677–693. https://doi.org/10.1016/j.cveq.2014.08.008.