Received: 2 November 2024 | Accepted: 20 March 2025

DOI: 10.1111/evj.14505

ORIGINAL ARTICLE

Equine Veterinary Journal WILEY

Prevalence and molecular epidemiology of the novel equine parasite *Theileria haneyi* in China

Guangpu Yang[1] | Yongyan Chen[1] | Kewei Chen[1] | Zhe Hu[1] | Jingkun Li[2] | Jingfei Wang[1] | Wei Guo[1] | Xiaojun Wang[1,3] | Cheng Du[1]

[1]State Key Laboratory for Animal Disease Control and Prevention, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China

[2]Department of Biostatistics, School of Public Health, Harbin Medical University, Harbin, China

[3]Institute of Western Agriculture, the Chinese Academy of Agricultural sciences, Changji, China

Correspondence
Xiaojun Wang and Cheng Du, State Key Laboratory for Animal Disease Control and Prevention, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China.
Email: wangxiaojun@caas.cn and ducheng@caas.cn

Funding information
National Key Research and Development Program of China, Grant/Award Number: 2022YFD1800200; Xinjiang Talent Development Fund, Grant/Award Number: ZZYD2023010; Tianchi Talent Introduction Plan, Grant/Award Number: IWA2023

Abstract

Background: Equine piroplasmosis (EP), caused by *Theileria equi* (*T. equi*) and *Babesia caballi* (*B. caballi*), is a tick-borne disease with significant economic impacts on the equine industry. *Theileria haneyi* (*T. haneyi*), a newly identified pathogen of EP, is globally distributed but has not been reported in China, where its epidemiological patterns remain undefined.

Objectives: To develop diagnostic techniques for *T. haneyi* and to assess its prevalence and risk factors in China.

Study Design: Assay development and cross sectional survey.

Methods: A nested PCR (nPCR) nucleic acid diagnostic technique targeting the chromosome 1 single-copy (chr1sco) open reading frame (ORF) was developed. This method was applied to 1318 equine nucleic acid samples. Additionally, an iELISA serological diagnostic method was established based on the EMA11 gene. This method was utilised in a cross-sectional analysis of 2627 equine samples. Logistic regression analysis was performed to identify significant risk factors.

Results: The nPCR assay showed an 11.76% positive detection rate, while iELISA indicated a 16.41% seroprevalence. Both assays were successfully applied for the epidemiological investigation of *T. haneyi*. Logistic regression analysis identified host species, age, altitude, soil type, and forest and grassland coverage as the main risk factors influencing seropositivity. Phylogenetic analysis of 18S rRNA from 29 positive samples confirmed the presence of *T. haneyi* in China.

Main Limitations: Data on clinical signs were not collected. The logistic regression model's performance metrics were not calculated.

Conclusions: This study provides the first evidence of *T. haneyi* infection in China and establishes a scientific basis for understanding its prevalence and geographical distribution.

KEYWORDS

B. caballi, epidemiological investigation, horse, piroplasmosis, *T. equi*, *T. haneyi*

Guangpu Yang, Yongyan Chen, Kewei Chen, and Zhe Hu contributed equally to this work.

1 | INTRODUCTION

Equine piroplasmosis (EP), considered an economically significant tick-borne disease in horses, is widely distributed globally, including most tropical and subtropical areas.[1–3] EP causes an illness in all equid species (horses, donkeys, mules, and zebras) characterised by fever, haemolytic anaemia, jaundice, inappetence, and hemoglobinuria.[4] Besides tick-borne transmission, direct transmission of parasites from horse to horse through blood products and transplacental transmission is possible, although uncommon.[5,6] EP is considered a notifiable disease by the World Organisation for Animal Health (WOAH) and is commonly thought to be caused by the eukaryotic hemoparasites *Theileria equi* (*T. equi*) and *Babesia caballi* (*B. caballi*).[4,7] In 2018, a third pathogen causing EP was identified in Texas, USA, known as *T. haneyi*, which appears to be expanding its global distribution. Compared with *T. equi*, *T. haneyi* causes milder clinical disease in experimentally infected horses and is capable of superinfection with *T. equi*.[8,9]

There is significant global variation in disease severity, and the parasite- and host-specific factors associated with variations in virulence are not well understood. The control of EP may be influenced by the genotypic diversity of *T. equi* and *B. caballi*.[10] Comparative genomic analysis of *T. equi* and *T. haneyi* has revealed that *T. haneyi* has undergone genomic reduction. Specifically, the genome of *T. haneyi* is ~2 Mbp smaller than that of *T. equi*.[8] The consequences of genomic reduction remain largely unknown, except for the loss of susceptibility to imidocarb dipropionate (ID).[11] The antiparasitic drug ID resolves the majority of equine infections of *T. equi*, but *T. haneyi* does not appear to be susceptible to ID, and co-infection of horses with *T. equi* and *T. haneyi* reduces the efficacy of ID against *T. equi*.[12] Despite genomic differences, *T. haneyi* also possesses the equine merozoite antigen (EMA) superfamily, which was previously thought to be unique to *T. equi*.[13] The EMA is an important antigen that induces specific neutralising antibody responses in infected animals. Interestingly, genomic analysis revealed that the *T. haneyi* genome lacks the *ema1*, 3, and 4 genes, but contains three novel *ema* family members, designated *ema11-13*.[8] Notably, EMA11 is unique to *T. haneyi* and has the potential to differentiate *T. haneyi* from *T. equi* infections.[13] Furthermore, the *T. equi* serological assays approved by the WOAH and the United States Department of Agriculture (USDA) are based on the equine immune response to EMAs. The EMA1-based *T. equi* competitive ELISA can detect a broad range of global isolates.[14,15] However, despite the antigenic cross-reactivity observed with Western blotting, the assay is unable to detect antibodies against *T. haneyi* in infected horses.[8,9] This indicates that further research and development of new diagnostic tools are needed to differentiate between *T. equi* and *T. haneyi* infections. The PCR has proven effective in detecting low parasitemia associated with *T. haneyi* infections.[8] While the 18S rRNA sequence is suitable for identifying certain species,[16] nucleic acid tests based on this sequence cannot differentiate between *T. equi* and *T. haneyi*.[8] Fortunately, a 2118 bp *chr1sco* gene of *T. haneyi* has no homology to sequences in other *Theileria* spp.[8]

Prevalence surveys in the Gambia, Nigeria, and South Africa have also reported co-infection of horses with *T. equi* and *T. haneyi* or triple infection with *T. equi*, *T. haneyi*, and *B. caballi*.[17–19] The *T. haneyi* capacity for superinfection with *T. equi* complicates the diagnosis and treatment of EP. Currently, there are no commercial kits available for the differential diagnosis between *T. equi* and *T. haneyi*.

Globally, research on the prevalence and genetic diversity of *T. haneyi* is quite limited.[18–20] A positive infection rate of 2.7% was found in 300 horse nucleic acid samples from Nigeria using nPCR.[21] A total of 176 horse serum samples were collected from the United States, Germany, Mexico, France, Ireland, Puerto Rico, the Netherlands, and other locations, of which 100 (56.8%) were determined to be positive for *T. haneyi* using an indirect enzyme-linked immunosorbent assay (iELISA).[22] However, epidemiological statistics regarding the prevalence of this new species are limited.

In China, the potential host and ecological risk factors associated with the prevalence of *T. haneyi* infection in equids have not been evaluated yet. To better understand the distribution and genetic diversity of *T. haneyi* in China, our goals were to develop an iELISA for detecting *T. haneyi*-positive horses using the EMA11 protein, which is specific to *T. haneyi*. This research provides a novel diagnostic antigen to differentiate between *T. equi* and *T. haneyi* infections, thereby improving the accuracy of EP diagnosis. Additionally, equine whole blood samples were collected from various regions across China and analysed using nPCR technology. By excluding samples co-infected with *T. equi* and *T. haneyi*, the study focused on the 18S rRNA sequence of *T. haneyi* to elucidate its distribution and intraspecific genetic diversity in China. These findings are of significant importance for controlling the spread of *T. haneyi* in China.

2 | MATERIALS AND METHODS

2.1 | Study area and sample collection for seroprevalence analysis

Blood samples were randomly collected from local equids (horses and/or donkeys) in various provinces of China between May 2019 and May 2020. The samples from individual horses were collected into sterile, anticoagulant-free tubes (Figure 1). The seroprevalence analysis was carried out in four different geographic areas of China (the north, the south, the northwest, and the Tibetan plateau), which have different climates, altitudes, soil types, and forest and grassland coverage. Because there were no previous studies in China to provide data on the prevalence of *T. haneyi* across the country, the minimum sample size was pre-determined using a random sampling method according to the formula below[3] assuming an expected prevalence of 50%, 95% confidence level, and 10% desired precision.

$$n = p^{*}(1-p)^{*}(1.96/d)^{2}$$

where n is the sample size, p is the expected prevalence, and d is the desired precision. Although the calculated minimum sample size was 97 animals, the sample size was increased to 2627 to increase the power of our statistical analysis.

FIGURE 1 The geographic locations of the sampling sites in this study are distributed across various provinces in China. The map illustrates the distribution of all the sampling sites, including 22 sera sampling sites and 11 whole blood sampling sites.

To identify risk factors related to *T. haneyi*, data on the following variables were registered at blood collection: species (horse; donkey); age (<3 years; between 3 and 7 years; ≥7 years); gender (male; female); and coat colour (light; dark). Furthermore, the geographic location of the animals included in the study was established, allowing other related variables to be evaluated. These were geographic area within China (north; south; northwest; Tibetan plateau); altitude (≤100 metres above sea level [m asl]; 100–1000 m asl; 1000–2000 m asl; >2000 m asl); soil type (ferralsol, siallite, udult, primitive soil, pedocal); and forest and grassland coverage (>74%; 45%–74%; <45%). The website of the National Earth System Science Data Center, National Science & Technology Infrastructure of China (http://www.geodata.cn) was used to obtain the altitude and other geographical information about each area from which a sample was taken, as well as soil type and forest and grassland coverage.

2.2 | Expression, purification, and verification of recombinant EMA11

EMA11 protein was selected as a diagnostic antigen of *T. haneyi* in our study, as reported in previous research.[22] Briefly, the nucleotide sequence of EMA11 was codon-optimised for expression in *Escherichia coli* (Tsingke, China) and was successfully cloned into pET-32a. After the transformants were cultured in ampicillin-supplemented Luria–Bertani (LB) broth, induced with isopropyl β-D-1--thiogalactopyranoside (IPTG), and then centrifuged at 8000*g* at 4°C for 10 min, cells were lysed with brief sonication to obtain a clear lysate. After that, the EMA11 proteins were purified with affinity chromatography using a Ni-nitrilotriacetic acid (NTA) agarose column system (Genscript, China) according to the manufacturer's protocol. The total protein in the sample was quantified using Nanodrop (Implen, Germany). The recombinant EMA11 protein was analysed with SDS-PAGE and stored at −20°C for serological studies.

We next examined whether sera from *T. haneyi* infected horses recognise the recombinant protein expressed by prokaryotic cells. Briefly, 0.1 mg of recombinant purified EMA11 was loaded into each lane and separated on an ExpressPlus™ PAGE Gel (Genscript, China). The gel was then electro-transferred to a nitrocellulose membrane and blocked with 5% non-fat milk. After blocking, the membrane was incubated for 2 h at room temperature with positive and negative horse serum (1:200). After washing in PBST, the membrane was incubated for 1 h at room temperature with secondary anti-horse IgG HRP (Biodragon, China, 1:5000). The immune complexes were revealed using an enhanced chemiluminescence method (ECL; Alphabio, Israel).

2.3 | Development of iELISA based on EMA11

The checkerboard titration method was used to optimise the iELISA.[23] In brief, Stripwell microplate 96-well microtiter ELISA plates (Costar, USA) were coated overnight at 4°C with 100 μL of recombinant purified EMA11 (25, 50, 100, and 200 ng/well) in PBS. After that, excess antigen was removed, plates were washed twice with PBST, and were blocked

with 200 μL/well of skimmed milk at 37°C for 2 h. After three washes with PBST, following the blocking step, serial dilutions (1:50, 1:100, 1:200, and 1:400) of *T. haneyi*-positive or negative horse sera were added to the plates and incubated at 37°C for 1 h. After three washes with PBST, rabbit anti-horse IgG-HRP (1:15,000) (KPL, USA) was added to each well, and the plates were incubated at 37°C for 0.5 h. Plates were then washed three times by PBST, and then developed with 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB)-ELISA Substrate Solution (Biopanda, China). The enzymatic reaction was stopped via the addition of 50 μL of TMB Stop Solution (0.2 M H_2SO_4) to each well, and plates were read at 450 nm using an ELISA plate reader (Biotek, USA). To discriminate between positive and negative samples, the cut-off value was calculated as the average of negative samples plus three standard deviations.[22] Negative serum samples, confirmed by Western blot (WB) and nPCR, were used as control sera. To verify the specificity of this iELISA method, we tested sera positive for *T. haneyi*, *T. equi*, *B. caballi*, *Equine infectious anaemia virus* (EIAV), *Equine influenza virus* (EIV), *Trypanosoma evansi* (*T. evansi*), *Burkholderia mallei* (*B. mallei*), *Streptococcus equi* (*S. equi*), *Equine arteritis virus* (EAV), *Equine herpesvirus* (EHV), and *Salmonella abortus equi* (*S. abortus equi*), as well as sera negative for *T. haneyi* serum, all of which were kept in our laboratory.

2.4 | Data analyses

Statistical analysis of data was performed using the SPSS software version 20.0 (SPSS Inc.). All risk factors were individually screened by cross-tabulation for association with the outcome variables (status of *T. haneyi* seropositivity in equids) using Pearson's Chi-square tests.[3] Moreover, to adjust for possible confounders, we included all variables in a multi-factor logistic regression analysis, which was able to facilitate the identification of independent risk factors for *T. haneyi* seropositivity in equids. The logistic model was developed in a stepwise forward approach, using a likelihood ratio test at each step with 0.10 as the significance level for removal or entry. Any variable with a p value ≤0.05 was considered statistically significant and was retained in the final model. Model fit was assessed with Hosmer and Lemeshow goodness-of-fit tests.

2.5 | Study area and sample collection for the molecular prevalence analysis

From May 2019 to May 2020, a total of 1318 equine whole blood samples were collected from the jugular vein of each animal using sterile K2-EDTA vacuum tubes (Solarbio) in 11 provinces in China (Figure 1). The samples were stored at −20°C until DNA extraction.

2.6 | DNA extraction and PCR amplification

Genomic DNA was extracted from an anticoagulant whole blood sample using a blood DNA extraction kit (TIANGEN) according to the manufacturer's recommendations, and was subsequently stored at −20°C until further use. Referring to Knowles's study,[8] we made some modifications to the nPCR protocol. Primers for the nPCR derived from extracted parasite DNA were based on the 2118 bp *chr1sco* gene from the newly generated draft genome sequence for *T. haneyi*. This gene has no homology to sequences in other *Theileria* spp. The primers used in the nPCR were: external-forward 5′-CCATACAACCCACTAGAG-3′, external-reverse 5′-CTGTCATTTGGGTTTGATAG-3′, internal-forward 5′-GACAACAGAGAGGTGATT-3′, and internal-reverse 5′-CGTTGAATGTAATGGGAAC-3′. The first round of PCR reactions was set up in a final volume of 20 μL containing 2 μL of DNA extraction, 250 nM of each primer, and 10 μL of 2× PCR reaction premix (GenStar, China). The PCR amplification conditions were as follows: initial denaturation at 98°C for 2 min, 30 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s with the final extension at 72°C for 8 min. The conditions and PCR reaction used for the second round of amplifications were identical to those used for the first round of PCR, except that amplification was conducted for 35 cycles. Following the second round of amplifications, the PCR products were determined using 1.5% agarose gel electrophoresis, and visualised under a UV transilluminator after staining with ethidium bromide. Amplicons from positive PCR products were purified using a Gel Extraction Kit and cloned into pMD-18T-Simple Vector (TaKaRa, Japan). Following transformation, the precisely identified amplicons were sequenced by the TSINGKE Biotechnology Company (China). To verify the specificity of this nPCR method, we tested nucleic acids positive for *T. haneyi*, *Salmonella Dublin* (*S. Dublin*), *Salmonella typhimurium* (*S. typhimurium*), *E. coli*, EHV-4, EHV-1, *S. abortus equi*, *T. equi*, *B. caballi*, and the nucleic acid negative for *T. haneyi*, all of which were kept in our laboratory. The sensitivity of nPCR was tested under the conditions described above. The *T. haneyi* genomic DNA from the positive sample (2.04×10^2 copies/μL) was diluted with PBS with a 10-fold gradient into seven different concentrations.

2.7 | Sequencing and phylogenetic analysis

Because 18S rRNA gene sequences cannot be used to distinguish between *T. equi* and *T. haneyi*, before we investigated the evolution of the *T. haneyi* 18S rRNA, we excluded samples with mixed infections of *T. haneyi* and *T. equi*. The *ema-1* of *T. equi* has no homology to sequences in other *Theileria* spp.[24] So, samples positive for *T. haneyi* were amplified initially using *ema-1* primers: external-forward 5′-TCGACTTCCAGTTGGAGTCT-3′, external-reverse 5′-AGCTCGACCCAGTTATCACC-3′, internal-forward 5′-CTCGACCACATCACTATCGA-3′, and internal-reverse 5′-GTCCTTCTTGAGAACGAGGT-3′.[25] The thermal cycling conditions are consistent with the nPCR protocol for *T. haneyi*, yielding a 218 bp fragment. Samples with *ema-1* positive bands were infected with both *T. equi* and *T. haneyi* parasites and were excluded from this analysis. Finally, samples positive for *T. haneyi* but negative for *T. equi* were amplified using 18S rRNA

primers,[26] and cloned into a pMD-18T-Simple Vector for sequencing analysis. The 18S rRNA sequences directly pair-ended sequenced by Sanger method. Afterwards, sequences were subjected to BLAST analysis for determining the identity with the sequences deposited in the nucleotide database. All nucleotide sequences acquired in this study were initially aligned using ClustalW and subsequently subjected to manual refinement and editing utilising the Bioedit software, version 7.0.9. Twenty-nine representative nucleotide sequences of *T. haneyi* were obtained from this study, and these were analysed together with 18 sequences of *T. equi* from the GenBank database to reconstruct a phylogenetic tree of a region of the hypervariable V4 region of the 18S rRNA gene. The evolutionary history of this region was inferred with Maximum Likelihood following Model Selection (ML) analysis. A Tamura-Nei + G model was selected and 1000 bootstrapping replicates were run. Based on the 18S rRNA, *T. equi* has been identified to possess five genotypes, ranging from A to E.[27–30] Our phylogenetic tree for *T. haneyi* was constructed using the 18S rRNA gene sequences obtained in this study, combined with those of equine *Theileria* previously published in GenBank (Table S1).

3 | RESULTS

3.1 | Establishment of iELISA based on EMA11

The nucleotide sequence of *ema11* was codon-optimised for *Escherichia coli* expression, and was successfully cloned into a pET-32a vector (Figure 2A). The purified EMA1 protein with a molecular weight of about 50 kDa was obtained after its expression, and the SDS-PAGE verification is displayed in Figure 2B. A large amount of purified EMA1 protein with a molecular weight of about 50 kDa was obtained. Western blotting was used to evaluate the antigenicity of the recombinant EMA11 with sera from horses. *T. haneyi* positive sera reacted well with the purified EMA11 protein, whereas negative sera from uninfected horses did not (Figure 2C). The best discrimination between positive and negative sera was obtained using 200 ng/well of antigen with a serum dilution of 1:200, and the optimal cutoff value was 0.5 (OD450 nm). Therefore, these conditions were used for the EMA11-based iELISA. One *T. haneyi*-negative serum and several serum samples positive for different pathogens were also examined.

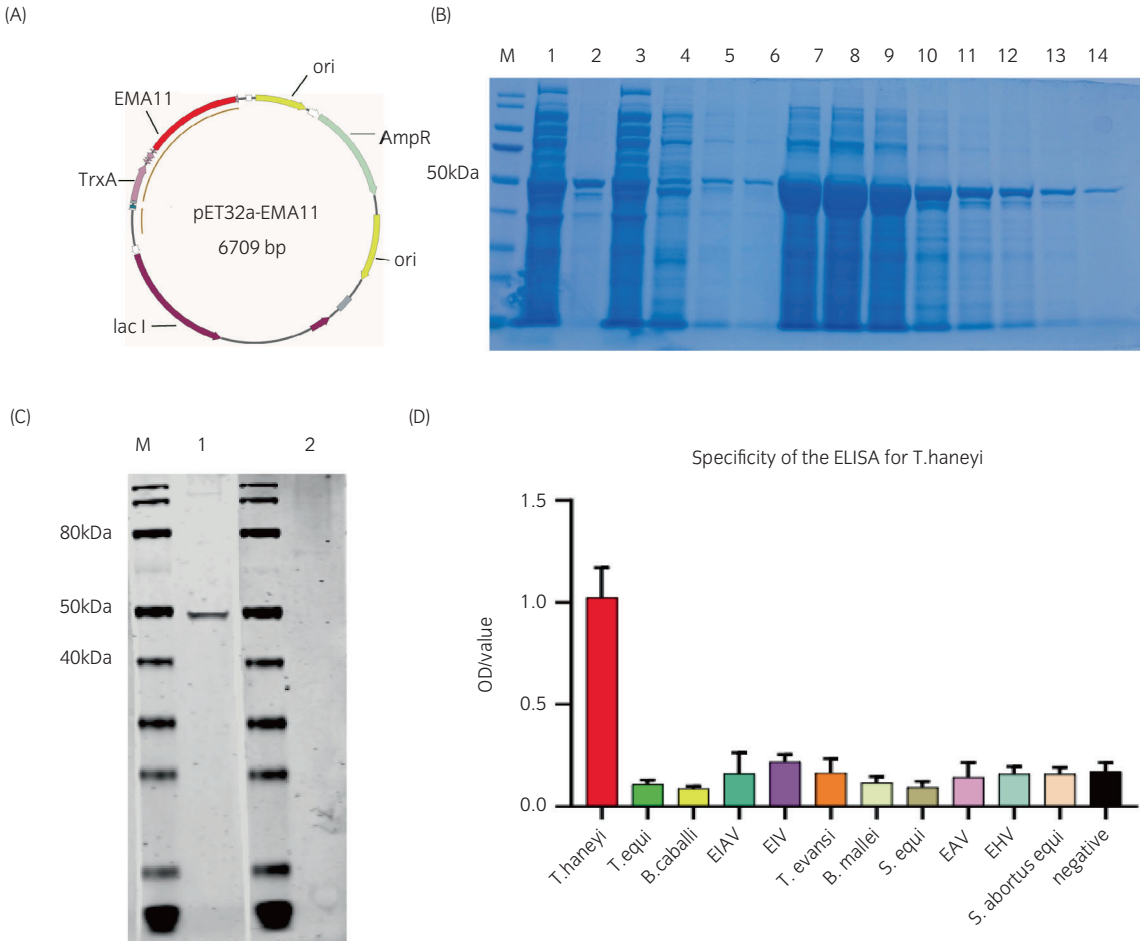

FIGURE 2 Establishment of iELISA based on EMA11. (A) Codon-optimised sequence of EMA11 was cloned into pET32a in frame with the T7 promoter and fused with the 6His tag originating the plasmid pET32a-EMA11. (B) SDS-PAGE analysis of purified recombinant EMA11 using a 12% acrylamide gel. Lanes: M, protein markers; 1, bacterial fluid; 2, supernatant; 3, precipitation after ultrasound; 4 to 6, wash; 7 to 14, elution of purified protein pET32a-EMA11. (C) Expression of recombinant EMA11 in BL21 cells detected by serum. Lanes: 1, *T. haneyi* positive serum; 2, *T. haneyi* negative serum. (D) The specificity test of iELISA for *T. haneyi* detection.

The only serum sample with an OD value above the threshold value was found to be that sample positive for *T. haneyi* (Figure 2D). These data indicated that iELISA has good specificity to *T. haneyi*.

3.2 | Seroprevalence of *T. haneyi* in equids and analysis of risk factors

Of the 2627 sampled animals, 431 (16.41%) were found to be positive for antibodies against *T. haneyi*. Among the 22 provinces sampled in this study, seven had a seroprevalence of <10%. In contrast, three provinces showed seropositivity rates exceeding 30%, with one province reaching the highest rate of 55%. Information pertaining to sample collection and seropositivity of *T. haneyi* in equids is listed in (Table 1).

Potential host and ecological risk factors for *T. haneyi* seropositivity were initially investigated using univariate analysis (Table 2). We found that neither animal coat colour ($p = 0.3$) nor gender ($p = 0.4$) had an association with *T. haneyi* seropositivity in equids. The other two host characteristics that were potential factors associated with *T. haneyi* seropositivity were species ($p = 0.003$) and age ($p < 0.001$). The rate of seropositivity in the donkey group (19.57%) was higher than that in the horse group (14.92%). The rate of seropositivity in animals <3 years old (24.38%) was higher than that in animals that were 3–7 years old (13.22%), followed by those ≥7 years old (11.14%).

Four ecological factors were associated with *T. haneyi* seropositivity: geographical area ($p = 0.02$), altitude ($p < 0.001$), soil type ($p < 0.001$) and forest and grassland coverage ($p < 0.001$). The rate of seropositivity in north China (18.89%) was higher than that in the south (15.67%) followed by the Tibetan Plateau (13.91%), and then the northwest (12.89%).

The rate of seropositivity at altitudes of 100–1000 m (19.46%) was higher than that in the <100 m group (18.02%), followed by those in the >2000 m (13.07%) and 1000–2000 m groups (12.75%).

Soil type also had an influence on the rate of seropositivity in the equine serum samples. The highest rate of seropositivity was found in the siallite group (22.70%), followed by the ferralsol group (18.53%), the pedocal group (14.73%), the udult group (13.47%) and the primitive soil group (8.78%). The rate of seropositivity in the <45% forest and grassland coverage group (23.18%) was higher than that in the 45%–74% forest and grassland cover group (13.69%), followed by the >74% group (13.22%).

In our final multivariate analysis model, the results indicated that samples from female donkeys in medium-altitude areas with low forest and grassland coverage, as well as siallite or pedocal soils, had the highest seroprevalence of *T. haneyi* (Table 3). In the species category, the odds ratio (OR) for a seropositive status in donkeys was 7.758 (95% confidence intervals [CI], 1.900–31.680, $p = 0.004$) when compared with horses. The OR for seropositive status in male animals was 0.576 (95% CI, 0.336–0.987, $p = 0.05$) when compared with females. The OR for seropositive status of animals in the 100–1000 m altitude group was 8.309 (95% CI, 3.875–17.815, $p < 0.001$) when compared with the <100 m group. In the soil type category, the OR for a seropositive status in the siallite soil group was 29.041 (95% CI, 12.033–70.088, $p < 0.001$) when compared with the udult soil group, and the OR for seropositive status of animals in the pedocal soil group was 10.087 (95% CI, 1.024–99.370, $p = 0.05$) when compared with the udult soil group. The OR for seropositive status in animals from areas with 45%–74% forest and grassland coverage was 0.277 (95% CI, 0.070–0.734, $p = 0.01$) when compared with the group from areas with <45% forest or grassland coverage.

TABLE 1 Seroprevalence of *T. haneyi* in 22 provinces and regions of China.

Provinces or regions	Number of samples	Positive of samples	Prevalence (%)	95% CI
Anhui (N)	180	8	4.44	1.42–7.46
Beijing (N)	192	14	7.29	3.61–10.97
Gansu (N)	203	23	11.33	6.97–15.69
Guangdong (S)	264	40	15.15	10.83–19.48
Guangxi (S)	32	3	9.38	0.72–19.47
Guizhou (S)	142	17	11.97	6.63–17.31
Heilongjiang (N)	32	3	9.38	0.72–19.47
Henan (N)	111	60	54.05	44.79–63.33
Hubei (S)	25	5	20	4.32–35.68
Jilin (N)	60	0	0	0
Jiangsu (S)	68	8	11.76	4.11–19.42
Liaoning (N)	100	19	19	11.31–26.69
Inner Mongolia (NW)	332	48	14.46	10.67–18.24
Ningxia (NW)	48	1	2.08	1.96–6.12
Qinghai (TP)	72	5	6.94	1.07–12.82
Shandong (N)	128	37	28.91	21.05–36.76
Shanxi (N)	51	14	27.45	15.20–39.70
Sichuan (S)	88	28	31.82	22.09–41.55
Tianjin (N)	60	33	55	42.41–67.59
Tibet (TP)	104	18	17.31	10.04–24.58
Xinjiang (TP)	169	25	14.79	9.44–20.15
Zhejiang (S)	166	22	13.25	8.09–18.41
Total	2627	431	16.41	14.99–17.82

Note: The sampling sites in this province are categorised by geographic region: N (North), S (South), NW (Northwest), and TP (Tibetan Plateau).

3.3 | Molecular prevalence of *T. haneyi* in equids and phylogenetic analysis

The primer design regions for the nPCR amplification of the chr1sco gene of *T. haneyi* are depicted in Figure 3A. The specificity results from the nPCR demonstrated no cross-reactivity; no PCR product was amplified from the other eight pathogen samples, confirming the assay's specificity (Figure 3B). The detection limit of the nPCR was 2.04×10^1 copies/μL (Figure 3C). The results of the nPCR assay

TABLE 2 Results of the univariate analysis of risk factors with *T. haneyi* seroprevalence.

Variables	Category	No. of samples	No. (+prevalence%)	Chi-square value	*p*
Species				8.9813	0.003
	Horse	1789	267 (14.92)		
	Donkey	838	164 (19.57)		
Age (years)				31.2830	<0.001
	<3	402	98 (24.38)		
	3–7	537	71 (13.22)		
	≥7	404	45 (11.14)		
Gender				0.8051	0.4
	Female	1021	164 (16.06)		
	Male	423	60 (14.18)		
Coat colour				0.8798	0.3
	Light	466	71 (15.24)		
	Dark	817	141 (17.26)		
Geographic area				10.3153	0.02
	North	1117	211 (18.89)		
	South	785	123 (15.67)		
	Northwest	380	49 (12.89)		
	Tibetan Plateau	345	48 (13.91)		
Altitude (m)				17.6633	<0.001
	<100	627	113 (18.02)		
	100–1000	930	181 (19.46)		
	1000–2000	894	114 (12.75)		
	>2000	176	23 (13.07)		
Soil type				37.7149	<0.001
	Ferralsol	286	53 (18.53)		
	Siallite	727	165 (22.70)		
	Udult	995	134 (13.47)		
	Primitive soil	205	18 (8.78)		
	Pedocal	414	61 (14.73)		
Forest and grassland coverage				28.4099	<0.001
	<45%	604	140 (23.18)		
	45%–74%	796	109 (13.69)		
	>74%	552	73 (13.22)		

Note: The *p* value ≤0.05 was considered significant.

TABLE 3 Results of multivariate logistic analysis of risk factors with *T. haneyi* seroprevalence.

Variables	Category	*p*	OR	95% CI
Species	Horse-vs.-Donkey	0.004	7.758	1.900–31.680
Gender	Female-vs.-Male	0.05	0.576	0.336–0.987
Altitude (m)	<100-vs.-100–1000	<0.001	8.309	3.875–17.815
Soil type	Udult-vs.-Siallite	<0.001	29.041	12.033–70.088
	Udult-vs.-pedocal	0.05	10.087	1.024–99.370
Forest and grassland coverage	<45%-vs.-45%–74%	0.01	0.227	0.070–0.734

Note: The *T. haneyi* model chi-square (χ^2)111.8824 with 11 degrees of freedom. *T. haneyi* model log likelihood 1081.662. *p* value ≤0.05 was considered significant.

showed that the molecular prevalence of *T. haneyi* was 11.76% (155/1318) in the equids sampled in this study. Information regarding the individual samples and positivity values of *T. haneyi* in equids is given in Table 4. Tianjin had the highest molecular prevalence (37.29%). However, no equine samples infected with *T. haneyi* were found in Heilongjiang Province. A total of 155 *T. haneyi chr1sco* gene sequences were successfully obtained from the identified positive samples using nPCR, and a total of thirty 18S rRNA gene sequences were successfully obtained from mono-infected *T. haneyi* positive samples using the nPCR.

The *T. equi* 18S rRNA gene samples analysed in this study fell into five major groups, representing the five genotypes A, B, C, D, and E. The *T. equi* 18S rRNA gene sequences obtained from this study suggested that the Chinese *T. haneyi* samples fell into E (Figure 4). Phylogenetic analysis revealed that the 18S rRNA sequences of *T. haneyi* from China (accessions ON428994–ON429022) formed a subclade

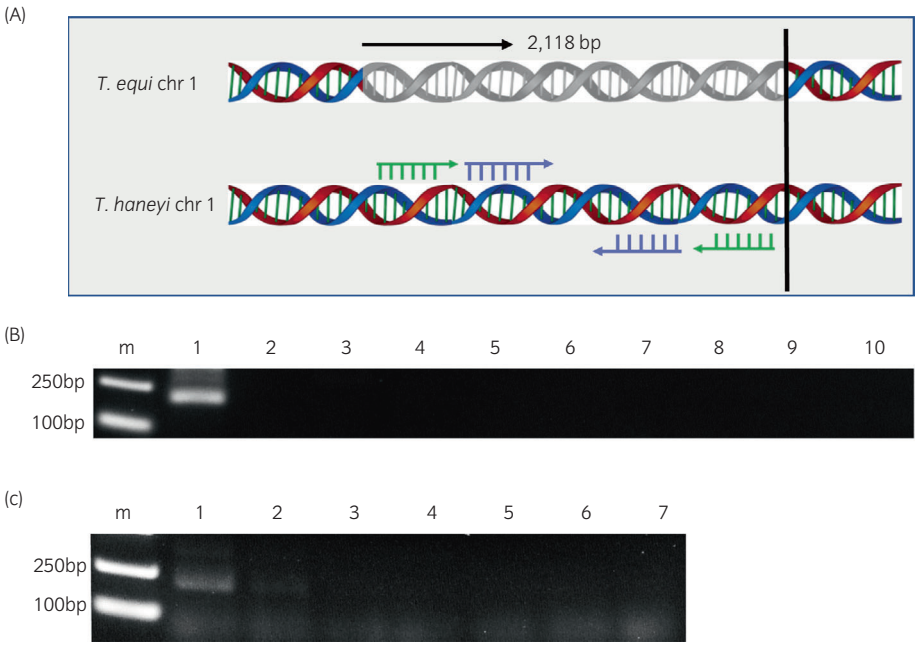

FIGURE 3 Development of nPCR method based on chr1sco gene. (A) Schematic diagram of the nPCR technology for the amplification of the chr1sco gene of *T. haneyi*. The double helix structure of *T. equi* and *T. haneyi* gene was coloured with blue and red. The missing chr1sco gene sequence of *T. equi* is coloured grey. The external primers used in this study were labelled with green arrows and the internal primers used in this study were labelled with purple arrow. (B) The specificity test of nPCR for *T. haneyi* detection. Lanes: M, marker; 1, *T. haneyi*; 2, *S. Dublin*; 3, *S. typhimurium*; 4, *E. coli*; 5, EHV-4; 6, EHV-1; 7, *S. abortus equi*; 8, *T. equi*; 9, *B. caballi*; 10, negative control. (C) The sensitive test of nPCR for *T. haneyi* detection. Template was subjected to 10-fold serial dilution. Lanes: M, marker; 1, 2.04×10^{2} copies/μL, 2, 2.04×10^{1} copies/μL, 3, 2.04×10^{0} copies/μL, 4, 2.04×10^{-1} copies/μL, 5, 2.04×10^{-2} copies/μL, 6, 2.04×10^{-3} copies/μL, 7, 2.04×10^{-4} copies/μL.

TABLE 4 Molecular prevalence of *T. haneyi* in 11 provinces and regions of China.

Provinces or regions	Number of samples	Positive samples	Prevalence (%)	95% CI
Anhui (N)	9	1	11.11	9.42–31.64
Guizhou (S)	72	2	2.78	1.02–6.57
Heilongjiang (N)	27	0	0.00	0
Hubei (S)	25	6	24.00	7.26–40.71
Jilin (N)	32	4	12.50	1.41–23.96
Jiangsu (S)	56	6	10.71	2.61–18.82
Liaoning (N)	18	1	5.56	5.03–16.14
Inner Mongolia (NW)	533	102	19.14	15.79–22.48
Tianjin (N)	59	22	37.29	24.95–49.63
Xinjiang (TP)	81	2	2.47	0.9–5.85
Zhejiang (S)	406	9	2.22	0.8–3.65
Total	1318	155	11.76	10.02–13.49

Note: The sampling sites in this province are categorised by geographic region: N (North), S (South), NW (Northwest), and TP (Tibetan Plateau).

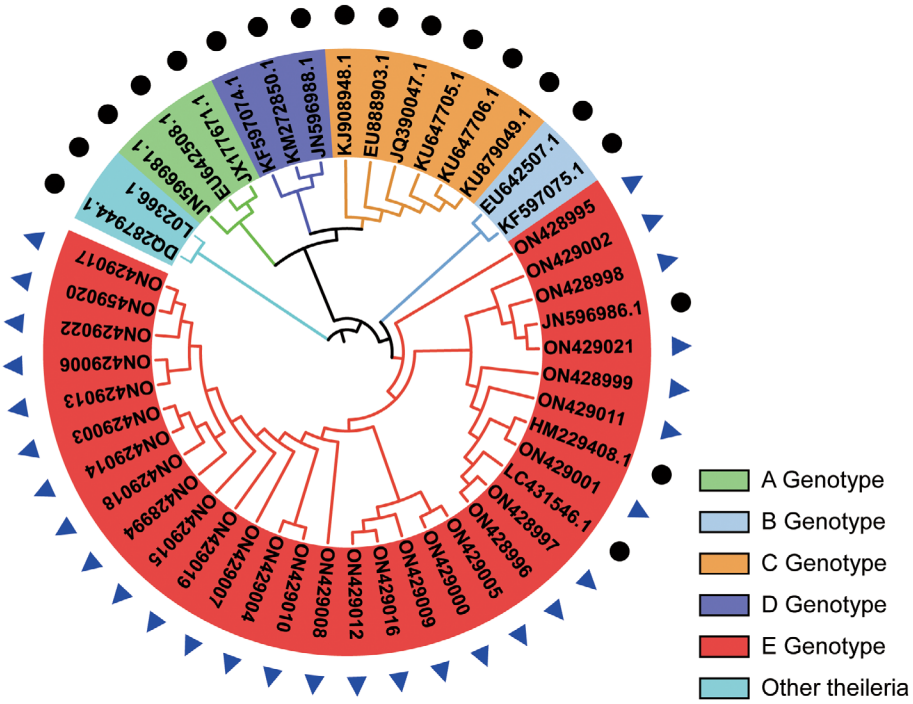

FIGURE 4 Phylogenetic analysis of the 18S rRNA sequences from *T. haneyi* isolates in this study (represented by solid triangles) with reported isolates (represented by solid circles). Different genotypes are represented with different colours. The phylogenetic tree was constructed using the Maximum Likelihood method and calculated with the Kimura 2-parameter in MEGA X software.

distinct from the Eagle Pass C-type sequences (KU647705.1, KU647706.1), despite clustering within the same major clade. The pairwise nucleotide identity between these sequences was 94.5%–95.5%.

4 | DISCUSSION

EP is an important disease that causes significant health problems and economic losses. It also negatively impacts horse trade and equestrian sports events. In China, EP has been studied in horses, but research has primarily focused on *T. equi* and *B. caballi*.[31] Neither the nucleic acid detection method based on the *T. equi* 18S rRNA gene nor the diagnostic method based on host antibodies against the *T. equi* EMA1 antigen is effective in the detection of *T. haneyi* infection.

This study conducted the first nationwide seroprevalence survey of *T. haneyi* in equids across 22 provinces and regions in China, revealing a seroprevalence rate of 16.41% (431/2627). In our univariate analyses, we found that host species and age are risk factors for EP. Global EP infection data from 1990 to 2019 showed that donkeys had a higher incidence rate than horses,[32] which is consistent with our findings. However, the seroprevalence of *T. haneyi* decreases with increasing age in horses, which is similar to previous results for *T. equi* infections.[33] Due to the small age range and the non-conformity with conventional age groups, this finding requires further research for confirmation. Generally, the prevalence of EP has been found to increase with increasing age. Infection with *B. caballi* tends to decrease with age, whereas a higher prevalence of *T. equi* has been observed compared with *B. caballi* as age increases. This increase in *T. equi* prevalence may not be linked to age-related immunity development, which could otherwise lead to parasite elimination, as seen in the case of *B. caballi*.[4] However, some studies have shown that the prevalence of EP does not significantly differ between age groups ($p = 0.233$).[29,34,35] Our findings highlight the complexity of age's impact on EP.[36] Epidemiological data from different studies around the world show conflicting results regarding the impact of sex on the seroprevalence of equine piroplasmosis.[37,38] In our study, the female equids showed higher seroprevalence of *T. haneyi* (16.06%) than did males (14.18%), which may be attributed to differing levels of sex hormones.[39]

Different reports have suggested that lighter coloured horses are less susceptible to pathologies caused or transmitted by insects.[40–42] However, in this study, we found no association between *T. haneyi* ($p = 0.3$) seropositivity in equids and host animal coat colour. Further studies are needed to understand the origins of this difference.

There is limited understanding of the influence of ecological factors on the prevalence of EP. The risk of contracting tick-borne diseases is directly linked to the timing of the questing activity and the distribution of the tick vectors. Our univariate analyses considering ecological risk factors included the factors geographical area, altitude, soil type, and forest and grassland coverage. Our previous study indicated that the highest prevalence of *T. equi* and *B. caballi* in China was found in the northwest of the country, and the lowest on the Tibetan Plateau of the country.[31] However, in this study, our data suggested that the north of China has the highest *T. haneyi* (18.89%) seroprevalence, and the northwest has the lowest (12.89%). The different seroprevalence at different altitudes may be due to the ecological distribution of ticks.[3] High altitudes of between 1500 and 3200 m are known to have an influence on the species diversity of ticks.[3] In this study, samples collected at <100 m and 100–1000 m had higher

T. haneyi seroprevalence than those collected at 1000–2000 m and >2000 m. This might be related not only to a decrease in tick distribution but also to a different tick species' spectrum. Similarly, tick distribution is also closely related to soil type.[43] We found that the samples from siallite soils had the highest seroprevalences of *T. haneyi* (22.70%). This may be due to siallite soils developing under temperate and subtropical forest or grassland vegetation, which have a strong bioaccumulation effect and support a higher density of tick vectors.[44] We therefore speculate that the ecological factors required for the presence of *T. haneyi* and *T. equi* are different.

The nPCR method established in this study was used to test nucleic acid samples from equids in 11 provinces across China, revealing a *T. haneyi* infection rate of 11.76% (155/1318). *T. haneyi* was not discovered until recently,[8] and additional genotypes of *T. haneyi* are still being discovered due to both improvements in detection techniques and ongoing parasite evolution. Based on the 18S rRNA, *T. equi* has been identified to possess five genotypes, ranging from A to E.[27–30] *T. haneyi* has been reported as belonging to genotype C. However, the results of this study showed that the 18S RNA of *T. haneyi* from China belonged to genotype E. The genomic similarity between *T. haneyi* and *T. equi* provides a reference for studying *T. haneyi*. Previous studies have also shown that the majority of the Chinese *T. equi* strains also belong to genotype E.[31] It is likely that the *Theileria* parasite is very variable, and the strains isolated and identified to date are likely to represent only a small fraction of the existing diversity. *T. haneyi* evolved under pressure from the natural environment, the host and other factors, and we speculate that the genotype of *T. haneyi* in China has tended to evolve to genotype E under this pressure, increasing parasite adaptation and survival in China.

Serological testing (e.g., iELISA) helps identify mid-to-late-stage or past infections of *T. haneyi* in host populations. Nucleic acid testing (e.g., nPCR) is essential for early diagnosis and provides genetic insights into *T. haneyi*'s genotype distribution and evolution.[3] Combining both methods allows for a comprehensive evaluation of infection status.

Limitations of this study should be considered. Due to the complexity of the sampling environment and the farmers' inability to accurately describe clinical signs, clinical data could not be collected. Although the whole blood samples were collected in the same province, the specific locations varied, making the samples independent of each other. For these reasons, we did not compare the positive results of iELISA and PCR directly. In this study, the lack of a standardised detection method for *T. haneyi* prevented the calculation of logistic regression model performance metrics. To address this, we developed the nPCR method, which offers a practical and reproducible detection alternative. While nPCR shows clinical utility, further validation is needed for its adoption as a standard. This work emphasises the need for standardised protocols and positions nPCR as a valuable transitional tool for advancing *T. haneyi* research.

5 | CONCLUSION

The study aimed to assess the seroprevalence of *T. haneyi* in equids in China and explore the host and ecological risk factors associated with the disease. The results revealed that the epidemiological patterns of *T. haneyi* infection in China are distinct and the risk factors involved are not yet fully understood. Through a cross-sectional study, we found that the seroprevalence of *T. haneyi* was higher in the following groups: donkey, animals <3 years old, altitudes of 100–1000 m, siallite soil, and <45% forest and grassland coverage. Additionally, we uncovered the genetic characteristics of *T. haneyi* prevalent in China. These findings are significant for the development and optimisation of prevention and control strategies for equine piroplasmosis in China.

FUNDING INFORMATION

This study was supported by grants from the National Key Research and Development Program of China (2022YFD1800200), Xinjiang Talent Development Fund (ZZYD2023010) and Tianchi Talent Introduction Plan (IWA2023).

ACKNOWLEDGEMENTS

We thank the National Key Research and Development Program of China (2022YFD1800200), Xinjiang Talent Development Fund (ZZYD2023010) and Tianchi Talent Introduction Plan (IWA2023).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Guangpu Yang: Investigation; writing – original draft; validation; visualization; data curation. **Yongyan Chen:** Investigation; writing – original draft; validation; writing – review and editing; formal analysis; data curation. **Kewei Chen:** Investigation; validation; formal analysis; visualization. **Zhe Hu:** Project administration; formal analysis; resources. **Jingkun Li:** Software; formal analysis; data curation. **Jingfei Wang:** Visualization; formal analysis; resources. **Wei Guo:** Visualization; project administration; supervision. **Xiaojun Wang:** Supervision; resources; project administration; writing – review and editing; funding acquisition. **Cheng Du:** Writing – original draft; writing – review and editing; project administration; data curation; supervision; resources.

DATA INTEGRITY STATEMENT

Guangpu Yang and Dr. Du had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

ETHICAL ANIMAL RESEARCH

The animal sampling procedures were approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS).

INFORMED CONSENT

Owners gave their consent for sampling of their animals and the use of the samples in this research study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.28407389.v1.

ORCID

Xiaojun Wang https://orcid.org/0000-0003-4521-4099
Cheng Du https://orcid.org/0000-0002-1686-6189

REFERENCES

1. Wise LN, Pelzel-McCluskey AM, Mealey RH, Knowles DP. Equine piroplasmosis. Vet Clin North Am Equine Pract. 2014;30(3):677–93.
2. Guidi E, Pradier S, Lebert I, Leblond A. Piroplasmosis in an endemic area: analysis of the risk factors and their implications in the control of Theileriosis and Babesiosis in horses. Parasitol Res. 2015;114(1):71–83.
3. Onyiche TE, Suganuma K, Igarashi I, Yokoyama N, Xuan X, Thekisoe O. A review on equine Piroplasmosis: epidemiology, vector ecology, risk factors, host immunity, diagnosis and control. Int J Environ Res Public Health. 2019;16(10):1736.
4. Wise LN, Kappmeyer LS, Mealey RH, Knowles DP. Review of equine piroplasmosis. J Vet Intern Med. 2013;27(6):1334–46.
5. Allsopp MT, Lewis BD, Penzhorn BL. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. Vet Parasitol. 2007;148(2):130–6.
6. Tirosh-Levy S, Gottlieb Y, Fry LM, Knowles DP, Steinman A. Twenty years of equine piroplasmosis research: global distribution, molecular diagnosis, and phylogeny. Pathogens. 2020;9(11):926.
7. Zobba R, Ardu M, Niccolini S, Chessa B, Manna L, Cocco R, et al. Clinical and laboratory findings in equine piroplasmosis. J Equine Vet Sci. 2008;28(5):301–8.
8. Knowles DP, Kappmeyer LS, Haney D, Herndon DR, Fry LM, Munro JB, et al. 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. 2018;48(9–10):679–90.
9. Sears KP, Kappmeyer LS, Wise LN, Silva M, Ueti MW, White S, et al. Infection dynamics of *Theileria equi* and *Theileria haneyi*, a newly discovered apicomplexan of the horse. Vet Parasitol. 2019;271:68–75.
10. Otgonsuren D, Amgalanbaatar T, Narantsatsral S, Enkhtaivan B, Munkhgerel D, Zoljargal M, et al. Epidemiology and genetic diversity of *Theileria equi* and *Babesia caballi* in Mongolian horses. Infect Genet Evol. 2024;119:105571.
11. Sears KP, Knowles DP, Fry LM. Clinical progression of *Theileria haneyi* in splenectomized horses reveals decreased virulence compared to *Theileria equi*. Pathogens. 2022;11(2):254.
12. Sears K, Knowles D, Dinkel K, Mshelia PW, Onzere C, Silva M, et al. Imidocarb dipropionate lacks efficacy against *Theileria haneyi* and fails to consistently clear *Theileria equi* in horses co-infected with *T. haneyi*. Pathogens. 2020;9(12):1035.
13. Wise LN, Kappmeyer LS, Knowles DP, White SN. Evolution and diversity of the EMA families of the divergent equid parasites, *Theileria equi* and *T. haneyi*. Infect Genet Evol. 2019;68:153–60.
14. Knowles DP, Perryman LE, Goff WL, Miller CD, Harrington RD, Gorham JR. A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. Infect Immun. 1991;59(7):2412–7.
15. Ueti MW, Palmer GH, Kappmeyer LS, Scoles GA, Knowles DP. Expression of equi merozoite antigen 2 during development of *Babesia equi* in the midgut and salivary gland of the vector tick *Boophilus microplus*. J Clin Microbiol. 2003;41(12):5803–9.
16. Katzer F, McKellar S, Kirvar E, Shiels B. Phylogenetic analysis of *Theileria* and *Babesia equi* in relation to the establishment of parasite populations within novel host species and the development of diagnostic tests. Mol Biochem Parasitol. 1998;95(1):33–44.
17. Coultous RM, McDonald M, Raftery AG, Shiels BR, Sutton DGM, Weir W. Analysis of *Theileria equi* diversity in the Gambia using a novel genotyping method. Transbound Emerg Dis. 2020;67(3):1213–21.
18. Bhoora RV, Collins NE, Schnittger L, Troskie C, Marumo R, Labuschagne K, et al. Molecular genotyping and epidemiology of equine piroplasmids in South Africa. Ticks Tick Borne Dis. 2020;11(2):101358.
19. Mshelia PW, Kappmeyer L, Johnson WC, Kudi CA, Oluyinka OO, Balogun EO, et al. Molecular detection of *Theileria* species and *Babesia caballi* from horses in Nigeria. Parasitol Res. 2020;119(9):2955–63.
20. Coultous RM, Phipps P, Dalley C, Lewis J, Hammond TA, Shiels BR, et al. Equine piroplasmosis status in the UK: an assessment of laboratory diagnostic submissions and techniques. Vet Rec. 2019;184(3):95.
21. Idoko IS, Edeh RE, Adamu AM, Machunga-Mambula S, Okubanjo OO, Balogun EO, et al. Molecular and serological detection of piroplasms in horses from Nigeria. Pathogens. 2021;10(5):508.
22. Bastos RG, Sears KP, Dinkel KD, Kappmeyer L, Ueti MW, Knowles DP, et al. Development of an indirect ELISA to detect equine antibodies to *Theileria haneyi*. Pathogens. 2021;10(3):270.
23. Major M, Law M. Detection of antibodies to HCV E1E2 by lectin-capture ELISA. Methods Mol Biol. 2019;1911:421–32.
24. Wise LN, Kappmeyer LS, Silva MG, White SN, Grause JF, Knowles DP. Verification of post-chemotherapeutic clearance of *Theileria equi* through concordance of nested PCR and immunoblot. Ticks Tick Borne Dis. 2018;9(2):135–40.
25. Battsetseg B, Lucero S, Xuan X, Claveria FG, Inoue N, Alhassan A, et al. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. Vet Parasitol. 2002;107(4):351–7.
26. Chen K, Hu Z, Yang G, Guo W, Qi T, Liu D, et al. Development of a duplex real-time PCR assay for simultaneous detection and differentiation of *Theileria equi* and *Babesia caballi*. Transbound Emerg Dis. 2022;69(5):e1338–49.
27. Bhoora R, Franssen L, Oosthuizen MC, Guthrie AJ, Zweygarth E, Penzhorn BL, et al. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Vet Parasitol. 2009;159(2):112–20.
28. Nagore D, Garcia-Sanmartin J, Garcia-Perez AL, Juste RA, Hurtado A. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Vet Parasitol. 2004;123(1–2):41–54.
29. Qablan MA, Obornik M, Petrzelkova KJ, Sloboda M, Shudiefat MF, Horin P, et al. Infections by *Babesia caballi* and *Theileria equi* in Jordanian equids: epidemiology and genetic diversity. Parasitology. 2013;140(9):1096–103.
30. Salim B, Bakheit MA, Kamau J, Nakamura I, Sugimoto C. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene within *Theileria equi* from horses in Sudan. Parasitol Res. 2010;106(2):493–8.
31. Chen K, Hu Z, Li J, Wang J, Liu D, Qi T, et al. Prevalence and molecular epidemiology of equine piroplasmosis in China: a neglected tick-borne disease. Sci China Life Sci. 2022;65(2):445–7.
32. Onyiche TE, Taioe MO, Molefe NI, Biu AA, Luka J, Omeh IJ, et al. Equine piroplasmosis: an insight into global exposure of equids from 1990 to 2019 by systematic review and meta-analysis. Parasitology. 2020;147(13):1411–24.
33. Ferreira EP, Vidotto O, Almeida JC, Ribeiro LP, Borges MV, Pequeno WH, et al. Serological and molecular detection of *Theileria equi* in sport horses of northeastern Brazil. Comp Immunol Microbiol Infect Dis. 2016;47:72–6.
34. Bartolome Del Pino LE, Nardini R, Veneziano V, Iacoponi F, Cersini A, Autorino GL, et al. *Babesia caballi* and *Theileria equi* infections in

horses in central-southern Italy: Sero-molecular survey and associated risk factors. Ticks Tick Borne Dis. 2016;7(3):462–9.
35. Kouam MK, Kantzoura V, Gajadhar AA, Theis JH, Papadopoulos E, Theodoropoulos G. Seroprevalence of equine piroplasms and host-related factors associated with infection in Greece. Vet Parasitol. 2010;169(3–4):273–8.
36. Elhaig MM, Selim A, Mahmoud M. Lumpy skin disease in cattle: frequency of occurrence in a dairy farm and a preliminary assessment of its possible impact on Egyptian buffaloes. Onderstepoort J Vet Res. 2017;84(1):e1–6.
37. Moretti A, Mangili V, Salvatori R, Maresca C, Scoccia E, Torina A, et al. Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: a preliminary study. Vet J. 2010;184(3):346–50.
38. Grandi G, Molinari G, Tittarelli M, Sassera D, Kramer LH. Prevalence of *Theileria equi* and *Babesia caballi* infection in horses from northern Italy. Vector Borne Zoonotic Dis. 2011;11(7):955–6.
39. Roberts CW, Walker W, Alexander J. Sex-associated hormones and immunity to protozoan parasites. Clin Microbiol Rev. 2001;14(3):476–88.
40. Epp T, Waldner C, West K, Townsend H. Factors associated with West Nile virus disease fatalities in horses. Can Vet J. 2007;48(11):1137–45.
41. Horvath G, Blaho M, Kriska G, Hegedus R, Gerics B, Farkas R, et al. An unexpected advantage of whiteness in horses: the most horsefly-proof horse has a depolarizing white coat. Proc Biol Sci. 2010;277(1688):1643–50.
42. Aharonson-Raz K, Rapoport A, Hawari IM, Lensky IM, Berlin D, Zivotofsky D, et al. Novel description of force of infection and risk factors associated with *Theileria equi* in horses in Israel and in the Palestinian Authority. Ticks Tick Borne Dis. 2014;5(4):366–72.
43. Schwarz A, Maier WA, Kistemann T, Kampen H. Analysis of the distribution of the tick *Ixodes ricinus* L. (Acari: Ixodidae) in a nature reserve of western Germany using geographic information systems. Int J Hyg Environ Health. 2009;212(1):87–96.
44. Vanwambeke SO, Sumilo D, Bormane A, Lambin EF, Randolph SE. Landscape predictors of tick-borne encephalitis in Latvia: land cover, land use, and land ownership. Vector Borne Zoonotic Dis. 2010;10(5):497–506.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Yang G, Chen Y, Chen K, Hu Z, Li J, Wang J, et al. Prevalence and molecular epidemiology of the novel equine parasite *Theileria haneyi* in China. Equine Vet J. 2025. https://doi.org/10.1111/evj.14505