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Original article

Prevalence and genetic diversity of *Theileria equi* from horses in Xinjiang Uygur Autonomous region, China

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ABSTRACT

Theileria equi is a tick-borne intracellular apicomplexan protozoan parasite that causes equine theileriosis (ET). ET is an economically important disease with a worldwide distribution that significantly impacts international horse movement. Horses are an essential part of the economy in Xinjiang which is home to ~10% of all the horses in China. However, there is very limited information on the prevalence and genetic complexity of *T. equi* in this region. Blood samples from 302 horses were collected from May to September 2021 in Ili, Xinjiang, and subjected to PCR examination for the presence of *T. equi*. In addition, a Bayesian latent class model was employed to estimate the true prevalence of *T. equi,* and a phylogenetic analysis was carried out based on the 18S rRNA gene of *T. equi* isolates. Seventy-two horses (23.8%) were PCR positive. After accounting for the imperfect PCR test using a Bayesian latent class model, the estimated true prevalence differed considerably between age groups, being 10.8% (95%CrI: 5.8% - 17.9%) in ≤ 3-year-old horses and 35.7% (95%CrI: 28.1% - 44.5%) in horses that were > 3 year-old. All *T. equi* isolates had their 18S rRNA gene (430bp) sequenced and analyzed in order to identify whether there were multiple genotypes of *T. equi* in the Xinjiang horse population. All of the 18S rRNA genes clustered into one phylogenetic group, clade E, which is thus probably the dominant genotype of *T. equi* in Xinjiang, China. To summarize, we monitored the prevalence of *T. equi* in horses of Xinjiang, China, with a focus on the association between age and the occurrence of *T. equi* by Bayesian modelling, accompanied by the genotyping of *T. equi* isolates. Obtaining the information on genotypes and age structure is significant in monitoring the spread of *T. equi* and studying the factors responsible for the distribution.

1. Introduction

Theileria equi is a tick-borne intra-erythrocytic hemoprotozoan that causes equine theileriosis (ET) (Wise et al., 2013). In endemically affected regions, ET causes significant morbidity and economic loss (Rothschild and Chantal, 2013; Scoles and Ueti, 2015), occurring in acute, sub-acute, and chronic forms. The acute form is characterized by fever, hemolytic anemia, edema, and, occasionally death (Manna et al., 2018). In non-endemic countries, regulatory testing for ET is required before horses are imported, restricting the international movement of horses and further increasing the economic losses associated with ET (Bishop et al., 2020). A particular issue with the disease (for both testing and morbidity/mortality) is that horses that experienced acute infection are likely to become asymptomatic carriers for life and serve as a reservoir for tick transmission (Knowles et al., 2018; Sears et al., 2019). Therefore, highly sensitive and specific diagnostic tests are required to detect these asymptomatic reservoirs and prevent further transmission between animals.

Polymerase chain reaction (PCR)-based methods that can detect small amounts of protozoal DNA are useful in identifying asymptomatic

Abbreviation: ET, equine theileriosis; PCR, Polymerase chain reaction; CI, confidence interval; CrI, credible interval; qPCR, quantitive PCR.

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Table 1
The distributions of *T. equi* in horses in different age and sex groups, the strength of association is measured using prevalence ratio and attributable fraction among the exposed with 95% confidence interval (CI).

		T. equi detected by PCR			
		Positive	Negative	Total	Proportion
Age	> 3-year old	64	146	210	30.5%
	≤ 3-year-old	8	84	92	8.7%
Prevalence ratio: 3.5 (95%CI: 1.75, 7.01)					
Attributable fraction among the exposed: 71.47% (95%CI: 42.95%, 85.73)					
Sex	Female	68	194	262	26%
	Male	4	36	40	10%
Prevalence ratio: 2.6 (95%CI: 1, 6.72)					
Attributable fraction among the exposed: 61.47% (95%CI: 0.18%, 85.13%)					

carriers (Alhassan et al., 2005; Schwint et al., 2008) and have been widely used in epidemiological studies of ET in endemic areas (Heim et al., 2007; Motloang et al., 2008; Ros-García et al., 2013). It is likely that at least some areas of China are endemic to ET (Zhao et al., 2020), but there is only limited data on prevalence. For example, the Xinjiang Uygur Autonomous region has ~10% of all horses in China and is considered to be an area where ET is endemic (Wang and Zou, 2020). However, as far as the authors' are aware, the only published data on prevalence is the study by Zhang, who sampled 723 horses in Ili, Xinjiang (Zhang et al., 2017). They reported that 40.8% of horses tested positive for *T. equi* using a PCR assay and suggested that older animals were more susceptible to infection with *T. equi*. However, that study did not report how animals were selected for sampling, with the difference in prevalence between counties within Ili being explained by horses being infected in one county and not the others. In addition, the calculation of prevalence assumed that the PCR test was perfect (100% specificity and sensitivity). This is not likely to have been the case, potentially resulting in bias due to misclassification (false positives or/and false negatives). Thus, in addition to collecting and testing more samples from horses in Xinjiang for *T.equi* using PCR, we also need to estimate the true prevalence of *T. equi* infection after accounting for the sensitivity and specificity of the imperfect PCR test (Rosales et al., 2013).

Additionally, we need to better understand the genetic diversity of *T. equi* in Xinjiang. This is largely unknown and addressing this knowledge gap will help develop robust diagnostic and preventive strategies (Qablan et al., 2013). The 18S rRNA gene has been widely used for phylogenetic studies of *T. equi* given its low nucleotide substitution rates and variable regions with high taxonomic information (Peckle et al., 2017). As such, *T. equi* isolates have been classified into five clades: clades A to E. Three different genotypes of *T. equi* (A, B, C) were initially reported in South Africa (Bhoora et al., 2009), with subsequent studies identifying two additional genotypes (D and E) of *T. equi* in Sudan (Salim et al., 2010) and Jordan (Qablan. et al., 2012), respectively. As far as we know, there are only limited reports of the *T. equi* genotypes present in China. Wang et al. identified that genotypes C and E were present in Gansu province (Wang et al., 2019), while Zhao et al. reported the presence of genotypes A and E in equids in Jilin province (Zhao et al., 2020). Thus, the main objectives of this study were to 1) estimate the age-specific true prevalence of *T. equi* in Xinjiang region, and 2) explore the different genotypes of *T. equi* present in that region.

2. Material and methods

2.1. Blood sample collection

Blood samples were taken from 302 horses in Ili, Xinjiang, China, between May and September 2021. Horses were sampled on a convenience basis on five breeding stations, three private farms and one racecourse. The sample size calculation was based on the sampling distribution of the estimated apparent prevalence. The previous data in this area were used for the expected prevalence set at 25% and the margin of error was set at 0.05, and at the 95% confidence level, the minimum sample size required 289 horses. The age and sex of all horses present on each of the nine establishments were recorded (239 from the breeding stations, 42 on the private farms and 21 horses from the racecourse). In all horses, blood samples were taken from the external jugular vein into EDTA-coated vacutainer tubes. Samples were kept at 4 °C until transferred to the laboratory within 24 hours of sampling.

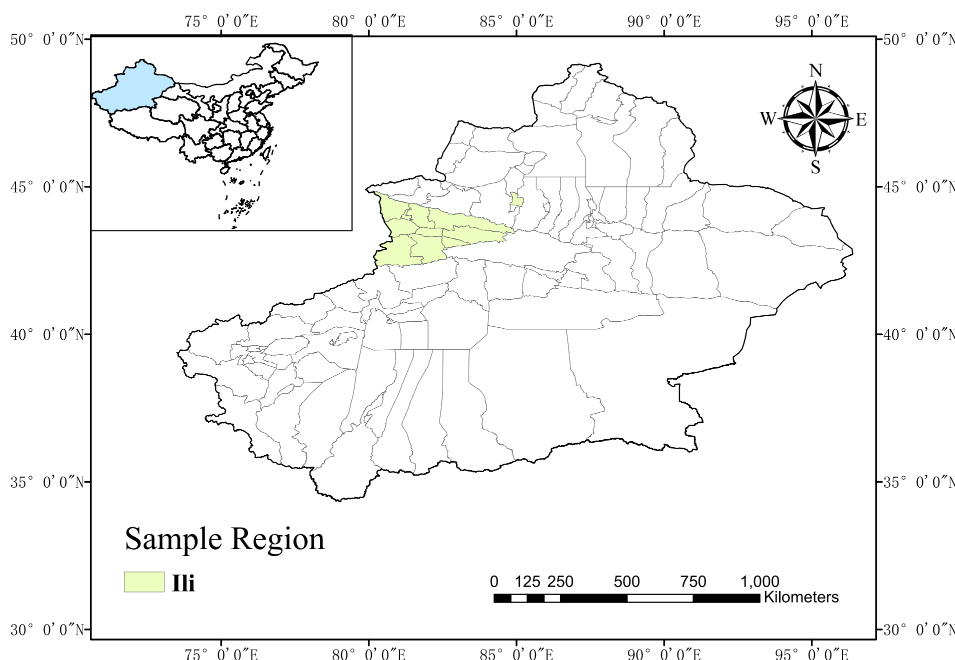

Fig. 1. Map of the study area.
Scale bar=100 km

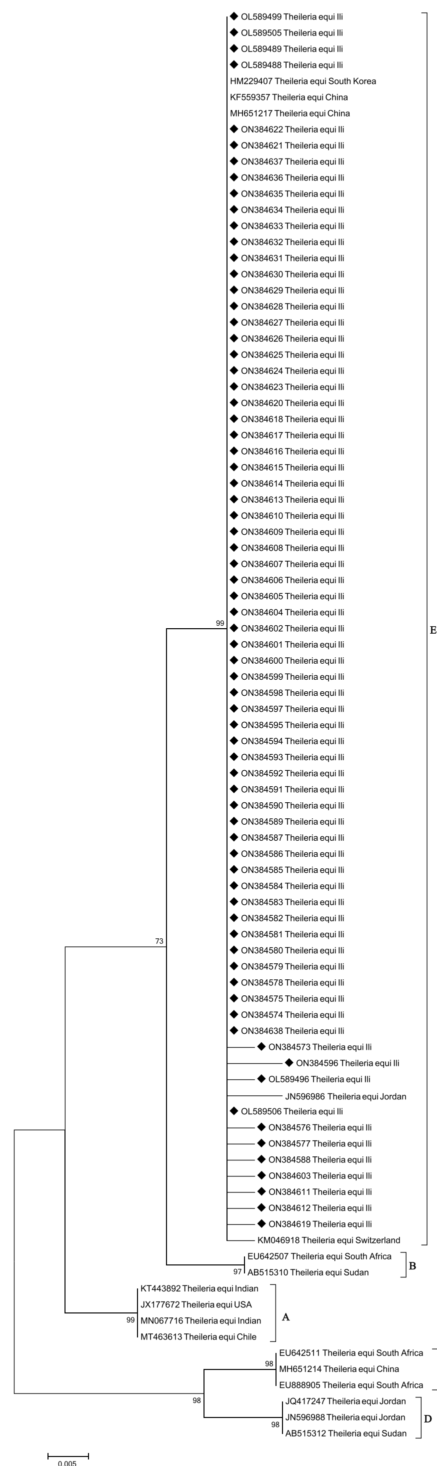

Fig. 2. Molecular phylogenetic analysis of *T. equi* isolates from horses based on the 18S rRNA gene sequence comparison.
Seventy-two sequences (430bp) are identified in this study and indicated with black rhombuses, and the rest of the sequences are obtained from the GenBank database. The phylogenetic analysis was performed using the Kimura model. The five groups (A, B, C, D, and E) represent the different *T. equi* genotypes. All the isolates in Ili, Xinjiang belong to genotype E.

2.2. DNA extraction and PCR detection

Two hundred μL of EDTA blood was taken from each sample, and total genomic DNA was extracted using the Genomic DNA kit (TransGen, Beijing, China) according to the manufacturer's instructions. Extracted DNA was then stored at -20 °C until PCR analysis. The presence of *T. equi* 18S rRNA gene fragments was detected using the specific primers Beq F and Beq R (Kumar et al., 2020). The target PCR amplification product was approximately 430 bp. The PCR mixture consisted of 12.5 μL of 2 × Taq PCR MasterMix II (TIANGEN, Beijing, China), 10 pmol of each primer, 2 μL of template DNA, and DNase/RNase-free water. This was added to the 25 μL PCR reaction system. The cycle conditions were as follows: an initial denaturation for 10 min at 96°C, followed by 36 cycles of 1 min at 96°C, 1 min at 60.5°C and 1 min at 72°C with a final extension of 10 min at 72°C. All positive PCR products were extracted and purified using the TIAgel Midi Purification Kit (TIANGEN, Beijing, China). The products were cloned into the pMD^{TM}19-T vector cloning kit (Takara, Shiga, Japan). The extraction of plasmids from the transformed bacteria was performed using the E.Z.N.A. Plasmid DNA Mini Kit (OMEGA Bio-Tek, Norcross, Georgia, USA). The reconstructed plasmids were sequenced using Sanger (Sangon Biotech, Shanghai, China).

2.3. Phylogenetic analysis

The sequencing results obtained in this study were subjected to blast analysis on the NCBI website using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic tree of the 18S rRNA gene was constructed by trimming and aligning the sequences with the partial deletion option using Clustal W in MEGA 7.0. The 18S rRNA phylogenetic tree of *T. equi* contains 89 nucleotide sequences, of which 72 sequences were obtained in this study. The rest were retrieved from the GenBank database. The evolutionary tree was constructed using the Kimura model with two parameters for both transitions and transversions. Model selection between different candidate models was not performed (Abadi et al., 2019). The parameters were estimated using the maximum likelihood estimation, after which the bootstrap support values were calculated using 1000 replicates (Harrison and Langdale, 2006). Sequences were grouped according to their branches (A-E).

2.4. Statistical analysis

2.4.1. Exploratory data analysis

A one-way table was used to study the frequency of each level within a categorical variable (i.e. sex and age). Levels with a limited number of observations were grouped with adjacent levels. For example, a small number of horses ≥ 10-year-old was observed; thus, the variable 'age' was re-categorised to 0, 1, 2, …, ≥ 10. The crude non-linear relationship between age and the occurrence of *T. equi* was visualised using a univariable generalised additive model (a binomial family with a logit link). Based on this model, 'age' was then dichotomised as young (≤ 3-year-old) and adult (> 3-year-old). The associations between dichotomised 'age', 'sex' and the occurrence of *T. equi* were tested using either a chi-square test or a Fisher's exact test. The crude strength of association between any predictor and the presence/absence of *T. equi* was measured using prevalence ratio and attributable fraction among the exposed with 95% confidence interval (CI). A contingency table was constructed to study the association between sex and age measured using the risk ratio with its 95%CI. Both age and sex were then included in a multivariable logistic regression model with presence/absence of *T. equi* as the outcome. A backwards selection algorithm was applied to retain variables with p-value < 0.05. This exploratory data analysis was performed using R (version 4.1.2).

2.4.2. Bayesian modelling

The finalised model identified whether age and/or sex affected the apparent prevalence of *T. equi*, but this did not account for the potential misclassification bias introduced by an imperfect PCR test (Koop et al., 2013). Therefore, a Bayesian latent class model that corrects the misclassification bias was constructed. The observed test result for the ith animal was modelled as a realisation of a Bernoulli random variable, where p_i – the probability that the animal was test positive – was a

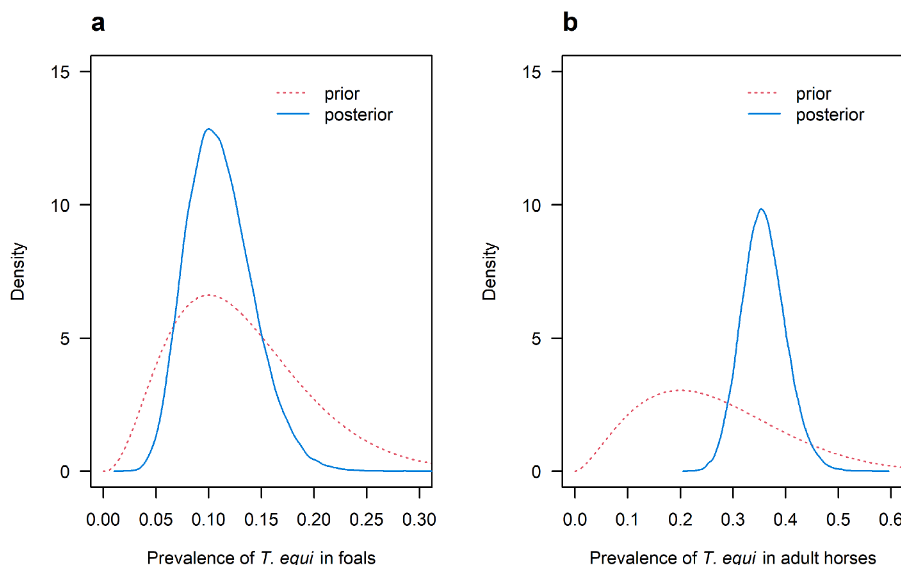

Fig. 3. Contrast priors to posteriors for prevalence of *T. equi* in young stock and adult horses obtained using a Bayesian latent class model in Ili, Xinjiang, China.

Table 2
Effect of sensitivity analysis scenarios on estimated age-specific prevalences of *T. equi.* The results are presented as posterior median (95% credible interval).

Prevalence (%)	Sensitivity analysis scenarios			
	Model[a]	Scenario 1[b]	Scenario 2[c]	Scenario 3[d]
≤ 3-year-old	10.8 (5.8, 17.9)	10.5 (5.6, 17.7)	10.9 (5.6, 18.6)	10.9 (5.8, 18.1)
> 3-year old	35.7 (28.1, 44.5)	34.4 (27.1, 43.9)	35.7 (28.3, 44.7)	36.3 (28.8, 45.3)

[a] priors of the main model
[b] a more diffuse but optimistic prior for sensitivity of the PCR test
[c] a more diffuse but increased estimate of prior for the prevalence in foals
[d] a more diffuse but increased estimate of prior for the prevalence in adult horses

function of the probability that the animal was infected with *T. equi* (π_i), the PCR sensitivity (η) and the PCR specificity (θ) based on the law of total probability (eq 1). Thus, p_i and π_i are analogous to the apparent and true prevalence terms at the population level. In the last step, the relationship between π_i and predictors x_i were described using logistic regression.

For this Bayesian analysis, we needed to incorporate previous understanding of PCR sensitivity and specificity into the data analysis (i.e. creating prior distributions). For this study, the specificity of the PCR test was not considered to be stochastic, but fixed at 1. This was because all PCR positives were also confirmed by sequencing, indicating that no false positives were found in the sample. Therefore, prior distributions were required only for PCR sensitivity and the vector of the regression coefficients ($\boldsymbol{\beta}$). Previous studies have reported that qPCR tests have a sensitivity for detecting *T. equi* of > 0.95 (Bhoora et al., 2010; Lobanov et al., 2018). As qPCR is generally more sensitive than the PCR used in this study, we chose a conservative mode for sensitivity of 0.85, and we were 95% certain that actual sensitivity was > 0.75 (0.05-quantile $=$ 0.75); this corresponds to a beta (46.35, 9) distribution. Informative priors for the "$\boldsymbol{\beta}$" were difficult to specify; therefore, they were induced indirectly by specifying prior distributions for the prevalence of *T. equi* in both age groups (Yang et al., 2019). Informative but diffuse priors were used. The best-estimated prevalence for young stock ($\widetilde{p}_0$) was 10%, and we were 95% certain it was smaller than 25% (0.95-quantile $=$ 0.25), corresponding to beta (3.44, 22.99); for adult horses, our best estimate ($\widetilde{p}_1$) was 20% and we were 95% certain it was less than 50%, corresponding to beta (2.64, 7.55). A complete model structure was presented as the following:

$$y_i \sim \text{Bern}(p_i),$$

$$p_i = \pi_i\eta + (1-\pi_i)(1-\theta) \quad (1)$$

$$\text{logit}(\pi_i) = \beta_0 + \beta_1 x_i \quad (2)$$

$$\beta_0 = \text{logit}(\widetilde{p}_0) \quad (3)$$

$$\beta_1 = \text{logit}(\widetilde{p}_1) - \beta_0 \quad (4)$$

$$\eta \sim \text{beta}(46.35,\ 9),$$

$$\theta = 1,$$

$$\widetilde{p}_0 \sim beta(3.44,\ 22.99),$$

$$\widetilde{p}_1 \sim beta(2.64,\ 7.55).$$

A sensitivity analysis was undertaken to identify whether our priors were driving our outcomes or whether it was the data. For this analysis, we used moderately different (but still biologically plausible) priors that were more diffuse (Johnson et al., 2019). We ran three sensitivity analysis scenarios. Firstly, while holding the other priors unchanged, a more diffuse prior beta (22.99, 3.44) was used for the sensitivity. This prior suggests that the most likely value for the sensitivity is 0.9 while keeping the 0.05-quantile at 0.75. Secondly, a more diffuse prior beta (2.56, 15.03) was specified for the prevalence in young stock (mode $=$ 10%, 0.95-quantile $=$ 30%) while keeping the other priors the same as they were in the main analysis. Finally, the prior prevalence in adult horses was changed to beta (3.29, 6.33), suggesting that our best estimate increased to 30% while we allowed the upper bound (0.95-quantile) to go as high as 60%.

The model was constructed using JAGS. After discarding 5000 iterations as burn-in, three MCMC chains with different sets of initial stochastic parameter values were run 20000 iterations (Additional file 1: Figure S1). Model convergence was ensured by examining trace plots as well as Gelman and Rubin's convergence diagnostic (Gelman and Rubin, 1992).

3. Results

3.1. Preliminary data analysis

Of the 302 horses sampled, 210 (69.5%) were adults (> 3 years) and 92 (30.5%) young stock (≤ 3 years), and there were 40 males (13.2%) and 262 females (86.8%). Seventy-two horses (23.8%) were identified as PCR positive (Additional file 2: Figure S2). The data showed a strong association between age and sex, the probability of being older (> 3 years) in female horses was 15.88 (95%CI: 4.11, 61.38), higher than that probability in male horses. Both age and sex were significantly associated with PCR results in the univariable analysis with a significance level set at 0.05 (Table 1). However, after accounting for age, the *p*-value for the association between the presence/absence of *T. equi* and sex was 0.954 (odds ratio: 0.96, 95%CI: 0.26 – 3.94). As removal of sex as a predictor variable did not markedly influence the regression coefficient for age (Additional file 3: Table S1), the final model included age as the only predictor variable.

3.2. Sequence analysis

Fig. 1.

Seventy-two sequences of the *T. equi* 18S rRNA gene (430bp) obtained in this study were deposited in GenBank (Additional file 4: Table S2). The *T. equi* 18S rRNA gene sequences fell into one genotype group designed as clade E (Fig. 2). Genotype group E contains 77 sequences, including the 72 sequences identified in this study, and the other four existing sequences in Genbank that are Jordan (1) - JN596986, Switzerland (1) - KM046918, China (2) - KF559357 and MH651217, and Korea (1) - HM229407. These sequences represent all five known clades.

3.3. Bayesian analysis

Based on the Bayesian modelling, after accounting for the sensitivity of the PCR test, the prevalence of *T. equi* infection in youngstock (≤ 3 years) and adult horses (> 3 years) was estimated at 10.8% (95% Credible Interval [CrI]: 5.8% - 17.9%) and 35.7% (95%CrI: 28.1% - 44.5%), respectively. The prevalence ratio (adult:youngstock) was estimated at 3.31 (95%CrI: 1.92 - 6.36). The posterior distributions of the age-specific prevalences were different from their prior distributions indicating the data provided sufficient information to update our prior knowledge (Fig. 3). The sensitivity analysis suggested that little variation in the posterior distributions was found when the priors were changed (Table 2).

4. Discussion

In this study, 302 samples were examined to determine the prevalence of *T. equi* in horses in Xinjiang using the specific PCR assay. Of those 302 samples, the proportion which was PCR positive was 23.8%. This result is similar to previous surveys (unpublished data) of *T. equi* prevalence conducted in Ili, which found the apparent prevalence of *T. equi* to be 24% (2013), 31.7% (2015), 14.8% (2017), and 13.9% (2018). However, direct comparison with such data is difficult, as our study showed apparent prevalence is strongly affected by the age structure of the sampled population and age was not recorded in those previous surveys. Except for using PCR assay, an ELISA test had been used in an early study which found 28/70 horses infected with *T. equi* in Ili, Xinjiang (Xuan et al., 2002).

After accounting for the possible false negatives, the estimated true prevalences from the Bayesian modelling were 35.7% (95%CrI: 28.1% - 44.5%) in adult horses (> 3 years) and 10.8% (95%CrI: 5.8% - 17.9%) in young stock (≤ 3 years), respectively. Thus simply recording apparent prevalence is likely to markedly underestimate true prevalence, particularly in adult horses. The Bayesian analysis confirmed that age was a key risk factor, with adult horses being more likely to be infected with *T. equi* than young stock. Horses older than three years were 3.31 (95% CrI: 1.92 - 6.36) times more likely to be infected with *T. equi* (estimated true prevalence 30.5%) than those of age ≤ 3 (10.8%). This finding is consistent with other studies (Kouam et al., 2010; Rüegg et al., 2007), since the carrier state is life-long, the cumulative cases are therefore expected to be higher in older animals. In addition, our finding is probably explained by the transmission dynamics of the protozoa, with older horses having a greater chance of being bitten by hard ticks (the vector and host of *T. equi*) (Machado. et al., 2016).

In this study, we did not find an association between sex and the prevalence of *T. equi* after accounting for age, though a crude association was found in the univariable analysis. The crude effect of sex was mediated by the study population being biased towards older females. Although we did not find a clear effect of sex on *T. equi*, our data are compatible with both a considerable decrease and a significant increase in odds in female horses. This lack of clarity is also present in the literature. Some studies found that female horses were at higher risk of having *T. equi* infection while others showed that seroprevalence was not different between females and males (Onyiche et al., 2019). Further research is required on the effect of sex and the risk of *T. equi* infection. We recommend that such research should include the building of a more robust causal model to guide the data analysis.

Our study found 72 distinct 18S rRNA sequences for *T. equi*, all of which belonged to one group: genotype E. This genotype is mainly found in Asia (Munkhjargal et al., 2013; Tirosh-Levy et al., 2020) and Europe (Camino et al., 2020; Kizilarslan et al., 2015; Liu et al., 2016), but seems to be spreading within those continents, with recent testing identifying the genotype in the Philippines (Dirks et al., 2021; Galon et al., 2021). Previous genotyping studies of *T. equi* isolates derived from horses in China (i.e. Wang et al. 2019 in Gansu province and Zhao et al. 2020 in Jilin province) found that genotype E was the predominant genotype (consistent with all of our isolates being genotype E). However, in contrast to our study, they also identified that genotypes A and C were circulating in Gansu and genotype A was present in Jilin. It is thus likely that both genotypes A and C are present in Xinjiang, but our restricted sampling area in Ili, with data being only collected from nine establishments, may have limited our chances of finding other genotypes. Further studies should consider sampling sites across the province in order to better identify the genotypes of *T. equi* present in Xinjiang. Although most genotype studies of *T.equi* focus on the 18S rRNA (Tirosh-Levy et al., 2020), phylogenetic studies about β-tubulin, mitochondrial genes and equine merozoite antigen (EMA) genes should be investigated in the future.

The geographical distribution of *T. equi* has been expanding in recent years. Genotyping is likely to be of significant value in monitoring the spread of *T. equi* and the factors responsible for that spread. This paper, which to our knowledge, is one of the first reports of the genetic diversity of *T. equi* in horses of Xinjiang, China, is a start towards getting that information.

Ethics approval and consent to participate

The experimentation protocols were reviewed by the Institutional Animal Care and Use Committee of Xinjiang Agricultural University (protocol numbers: GB/T35892-2018). All animal studies were conducted following the Guidelines of the Chinese Animal Welfare Council.

Consent for publication

Not applicable.

Author statement

YZ, RL, ML, DAY, and BC contributed to the conception and design of the study. QS, CL, WH, HZ, SL, and QG performed the experiments and

carried out data curation. DAY performed the statistical analysis. YZ wrote the first draft of the manuscript, and RL, ML, and DAY edited the text. All authors contributed to manuscript revision, read, and approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2023.102193.

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