Ticks and Tick-borne Diseases 10 (2019) 528–532

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

The first molecular detection and genetic diversity of *Babesia caballi* and *Theileria equi* in horses of Gansu province, China

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ARTICLE INFO

Keywords:
Babesia caballi
Theileria equi
Molecular epidemiology
Genetic diversity
China

ABSTRACT

Equine piroplasmosis, caused by *Theileria equi* and *Babesia caballi*, is an economically important tick-borne disease worldwide. In the current study, 242 blood samples were randomly collected from horses in Zhangye city of Gansu province, China. The presence and genetic diversity of piroplasms were evaluated with a nested PCR assay, gene sequencing and phylogenetic analysis. The results showed that seventy-five (31.0%) samples were positive for piroplasms. Sequences analysis showed that seventy-three (30.2%) were positive for *T. equi*, and seven (2.9%) for *B. caballi*, five of which (2.1%) were infected with *T. equi* and *B. caballi*. Phylogenetic analysis revealed two *T. equi* genotypes (C and E) and one *B. caballi* genotype (A). The molecular epidemiological and genetic diversity results provide important epidemiological data for control of equine piroplasmosis caused by *T. equi* and *B. caballi* in China.

1. Introduction

Equine piroplasmosis (EP) is an important tick-borne disease with a worldwide distribution, caused by *Theileria equi* and *Babesia caballi* (Rothschild, 2013; Wise et al., 2013; Database, 2018). Infected animals present with varying clinical manifestations, such as high fever, lethargy, anorexia, peripheral edema, petechiation of the mucous membranes, hemolysis, tachycardia, and pigmenturia, which impacts the health of horses, particularly in terms of their working capacity (Scoles and Ueti, 2015).

The taxonomy of EP agents has been controversial since their discovery, particularly *T. equi*. *Theileria equi* was initially named *Piroplasm equi* (but reclassified as *B. equi*) in South Africa in 1901 (Bowhill, 1905). Based on its extra-erythrocytic stage in equine peripheral blood mononuclear cells, *B. equi* was considered an invalid name and was renamed as *T. equi* in 1998 (Mehlhorn and Schein, 1998). Recently, according to the results of molecular phylogenetic investigations and genomic analysis, *T. equi* should be classified between the genus of *Babesia* and *Theileria* or as a new genus (Allsopp and Allsopp, 2006; Allsopp et al., 2007; Uilenberg, 2006; Kappmeyer et al., 2012). However, additional information is needed to make a suitable classification for this parasite.

The small subunit ribosomal RNA (18S rRNA) gene has been widely used for species identification, phylogenetic, and genotype studies in *T. equi* and *B. caballi* (Criado-Fornelio et al., 2003; Nagore et al., 2004; Bhoora et al., 2009; Kouam et al., 2010; Qablan et al., 2013; Seo et al., 2013; Hall et al., 2013; Veronesi et al., 2014; Braga et al., 2017; Peckle et al., 2018; Vieira et al., 2018). Initially, only two genotypes were identified in these two parasites, genotype A and B. Shortly afterward, three additional *T. equi* genotypes (C, D, E) were identified in South Africa (Bhoora et al., 2009), Sudan (Salim et al., 2010), Jordan (Qablan et al., 2012), and the USA (Knowles, 2018), and genotype B of *B. caballi* was divided into two clades (B1 and B2) (Bhoora et al., 2009). *Babesia caballi* genotype B2 was also named genotype C (Qablan et al., 2013). Currently, five *T. equi* 18S rRNA genotypes (A, B, C, D, E) and three *B. caballi* (A, B1, B2) are identified (Bhoora et al., 2009; Manna et al., 2018; Munkhjargal et al., 2013; Qablan et al., 2013).

In 1955, the first occurrence of EP caused by *B. caballi* was described in China. It has since been reported sporadically several provinces across China (Li, 1985; Xu et al., 2003; Chahan et al., 2006; Gong et al.,

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https://doi.org/10.1016/j.ttbdis.2019.01.003
Received 15 August 2018; Received in revised form 22 November 2018; Accepted 11 January 2019
Available online 16 January 2019
1877-959X/

2010; Wang et al., 2014). Recently, horse deaths resulting from EP have been reported from local veterinary stations in Zhangye city, Gansu province, northwestern China. However, there is little information available relating to the molecular epidemiology and genotypes of *T. equi* and *B. caballi* across China. Thus, in the current study, the molecular epidemiological status of these parasites was investigated in this region of China.

2. Material and methods

2.1. Sample collection and DNA extraction

From March to June in 2017and 2018, a total of 242 healthy horses ranging from 1 to 19 years in age (mean 8.4) belonging to 11 different farms in Zhangye were sampled. Blood samples were randomly collected from these horses into EDTA-coated vacutainer tubes and transferred to the laboratory in iceboxes. The protocol used in this study and all animal handling were approved by the Animal Ethics Committee of the Lanzhou Veterinary Research Institute, CAAS (Permit No. LVRIAEC-2018-001). Genomic DNA was extracted from 200 µl of each blood sample according to the manufacturer's instructions (QIAGEN DNA Blood Mini-Kit, Germany). The concentration was determined with a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA samples were stored at -20∘C until use.

2.2. Nested PCR amplification and sequencing

A nested PCR (nPCR) assay was used to detect and amplify the piroplasm 18S rRNA gene from genomic DNA of the horse blood samples. In the first-round of PCR amplification, a set of primers (Piro1-S: 5´-CTTGACGGTAGGGTATTGGC-3´, Piro3-AS: 5´-CCTTCCTTTAAGTGA TAAGGTTCAC-3´) was used to amplify 18S rRNA gene fragments (˜1, 369 bp for *B. caballi* and ˜1399 bp for *T. equi*) (Yang et al., 2014). Genomic DNA isolated from *B. motasi* was used as the positive control and distilled water as the negative control. In the second PCR reaction, 2 µl of each PCR product from the first PCR amplification was used as template. The primers (Piro-A: 5´-ATTACCCAATMCBGACACVGKG-3´ and Piro-B: 5´- TTAAATACGAATGCCCCCAAC -3´) were made with minor modifications on the basis of multiple sequence alignments of *T. equi* and *B. caballi* 18S rRNA gene sequences deposited in GenBank, and used to amplify 425 bp and 397 bp fragments of *T. equi* and *B. caballi* 18S rRNAs, respectively (Olmeda et al., 1997; Niu et al., 2017;). The PCR amplifications were conducted according to the previous report by Yang et al. (Yang et al., 2014). PCR products were analyzed on a 1.0% agarose gel containing gold view dye (SolarBio, Beijing, China) in Tris-acetate-EDTA (TAE) buffer at 120 V for 30 min and visualized under UV light. Positive PCR products were purified using a gel DNA purification kit (ZYMO, USA). The purified DNA fragments were cloned into pClone007 Simple vectors (TSINGKE Biological Technology, China) and then transformed into *Escherichia coli* JM109 competent cells. For each amplicon, three positive clones were selected by PCR amplification with the primer pair M13 F and M13R and sequenced using the BigDye Terminator Mix (TSINGKE Biological Technology, China).

2.3. PCR amplification of piroplasm 18S rRNA gene fragments

To identify the species of piroplasm infecting horses, long fragments of the 18S rRNA genes were amplified from 75 positive samples using a nested PCR assay. A set of primers, PIRO-F (5´-GAAAYTGCGAATGGC-TCATTAM-3´) and PIRO-R (5´-CACCGGATCACTCGATCGGTAGG- 3´), was used in the first-round PCR to produce 1600 bp fragments. In the second-round PCR, another pair of primers, PIRO-nest F (5´-GGATAA-CCGTGSTAATTSTAGGGC-3´) and PIRO-nest R (5´-GTGTGTACAAAGG GCAGGGACG-3´), was used to amplify approximately 1,400bp fragment. The conditions of PCR amplification, DNA fragment purification, and gene cloning of gene were as detailed above. For each amplicon, three positive clones were sequenced using the BigDye Terminator Mix (TSINGKE Biological Technology, China).

2.4. Sequence analysis

All sequences determined in the present study were subjected to blast analysis on NCBI website using the BLASTn program (Altschul et al., 1990). Cloning vector sequences were manually trimmed.

To assess the genetic diversity of *T. equi* and *B. caballi* detected in horses, a phylogenetic tree of the 18S rRNA gene V4 region was constructed using the MEGA 7.0 software. The 18S rRNA sequences obtained in this study and those of piroplasms previously registered in GenBank were aligned using ClustalW in the MegAlign software (DNAStar, USA). The consensus sequences were trimmed manually to 280 positions in the final data set and distance matrices were inferred by the Kimura 3-parameter model using Neighbor-Joining in MEGA 7.0 software (Kumar et al., 2016). Representative *T. equi* and *B. caballi* 18S rRNA sequences for distinct genotypes were registered into the GenBank database under accession numbers: MH651211–MH65122.

3. Results

3.1. Nested PCR detection of equine piroplasms

The results of the nPCR assay showed that the piroplasms prevalence was 31.0% (75/242) in the horses. The sequencing results showed that approximately 397 bp and/or 425 bp fragments were obtained from 75 samples. Furthermore, based on sequence analysis, it was determined that the prevalence of *T. equi* and *B. caballi* infections were 30.2% (73/242) and 2.9% (7/242), respectively. Five horses (2.1%) were co-infected with *B. caballi* and *T. equi*; whereas two (0.8%) and 68 (28.1%) horses were singly infected with *B. caballi* and *T. equi*, respectively (Table 1). No other piroplasms were determined in these blood samples.

3.2. Sequences analysis of the T. equi and B. caballi 18S rRNA genes

Theileria equi and *B. caballi* 18S rRNA genes (˜1, 400 bp) were successfully amplified from positive blood samples to further confirm the nPCR results. Sequence analyses showed that the sequences belonged to *B. caballi* and *T. equi* and were the same as those of short sequences (Figs. 1 and 2).

The results of the phylogenetic analysis of 18S rRNA gene sequences

Table 1
Detection results of *T. equi* and *B. caballi* in horse blood samples.

Pathogen species detected	Positive No (%)	No of positive samples for *T. equi* and *B. caballi* genotypes
One pathogen		
T. equi	68 (28.1)	3 (*T. equi* genotype C and E), 60 (*T. equi* genotype E), 5 (*T. equi* genotype C),
B. caballi	2 (0.8)	2 (*B. caballi* genotype A)
Two pathogens		
T. equi + *B. caballi*	5 (2.1)	5 (*B. caballi* genotype A), 1 (*T. equi* genotype C), 4 (*T. equi* genotype E)
Total	75 (31.0)	

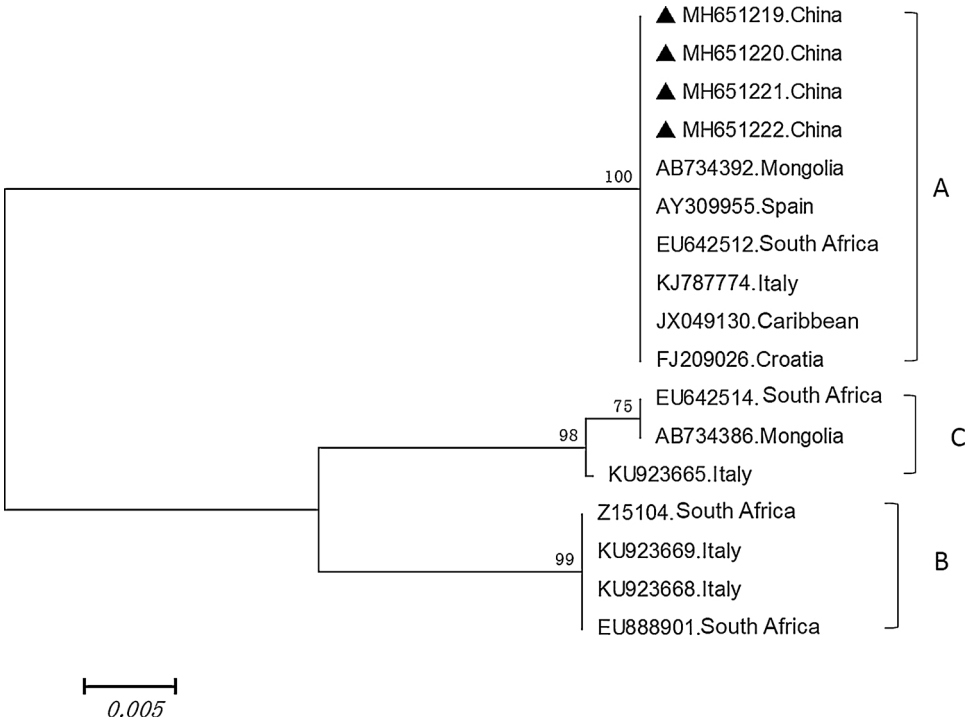

Fig. 1. Phylogenetic tree of *B. caballi* inferred from partial sequences of the 18S rRNA gene. Numbers above the branches demonstrate bootstrap support from 1000 replications. The newly generated sequences in present study were indicated with bold triangles. A, B, and C represent genotypes.

classified the isolated *T. equi* as genotypes C and E (Table 1). *Theileria equi* 18S rRNA gene from China shared 99.2% to 99.7% sequence similarity to each other (MH651211-MH651218). Sequences of *T. equi* genotype C (MH651214-MH651216, MH651218) showed 99.4–99.9% sequence identify to those from South Africa (EU642511). Sequences of *T. equi* genotype E (MH651211-MH651213, MH651217) shared 99.9% nucleotide sequence similarity with that of *T. equi* isolated from horses in Spain (AY534882).

Sequences from *B. caballi* from China were identified as genotype A and were identical to each other, also sharing 99.7% sequence similarity with *B. caballi* isolated from Spain (AY309955) and South Africa (EU642512).

4. Discussion

Compared with *B. caballi, T. equi* is the predominant parasite in some EP-endemic areas, as confirmed by several studies using molecular approaches. The prevalences of *T. equi* and *B. caballi* range from 5.4% to 59.7% and from 0% to 19.3%, respectively (Heim et al., 2007; Motloang et al., 2008; Gallusova et al., 2014; Mahmoud et al., 2016; Sumbria et al., 2016). A high prevalence of *B. caballi* has also been described in several countries, including Italy (Laus et al., 2013) and Mongolia (Munkhjargal et al., 2013; Mans et al., 2015). In 2017, Zhang et al. (2017) reported an average positivity of *T. equi* of 40.8% in 723 horses from Xinjiang province, northwest China. In the current study, *T. equi* and *B. caballi* were detected in equine horse blood samples with a positive rate of 30.2% and 2.9%, respectively. The discrepancies in the prevalence of these two parasites across these studies could be explained by the sampling time, sampling sites, climate conditions, the distribution of tick vectors in the sampling regions, and the tools used in the studies. In addition, *B. caballi* infections are efficiently eliminated by the host immune system, in contrast to the life-long persistence of *T. equi*, making the former harder to detect (Bruning, 1996).

To identify the species of piroplasms infecting horses, a pair of primer (Piro1-S: 5´-CTTGACGGTAGGGTATTGGC-3´, Piro3-AS: 5´-CCT TCCTTTAAGTGATAAGGTTCAC-3´) was used to amplify a long fragment of 18S rRNA genes from positive samples. However, only 12 samples were successfully amplified. The low concentration of *Babesia* spp. and *Theileria* spp. DNA in the field samples might be explain this result. Therefore, we designed two other primer pairs, which were effective in amplification of the 18S rRNA gene from *T. equi*, *B. caballi*, *Babesia bovis*, *Babesia bigemina*, *Babesia ovate*, *Babesua motasi*, *Theileria annulata*, *Theileria orientalis*, *Theileria ovis* and had no cross reaction with *Anaplasma ovis* or *Anaplasma phagocytophilum*.

Phylogenetic analysis revealed that there were two *T. equi* 18S rRNA genotypes and one *B. caballi* genotype in Gansu province. Our results indicated that these genotypes of *T. equi* and *B. caballi* might be associated with outbreaks of EP in this region. Previous studies reported that *T. equi* genotype E was closely related to clinically fatal cases of EP in Greece and Spain (Kouam et al., 2010; Nagore et al., 2004). Although the prevalence of *T. equi* genotype E was relatively high in this region, more information is needed to elucidate the association of this genotype with the clinical outcomes of EP. The clinical significance of the different genotypes with regard to *Theileria orientalis* and *Babesia canis rossi* had ever been investigated (Matjila et al., 2009; Kamau et al., 2011; Eamens et al., 2013). However, the pathogenic mechanism between18S rRNA gene genetic variation and clinical status remain unknown.

To our knowledge, this is the first molecular epidemiological and genetic diversity study of *T. equi* and *B. caballi* in horses in China, and has identified two genotypes of *T. equi* and one of *B. caballi*. The prevalence of the *T. equi* genotype E was relatively high in this region of China, which could be responsible for the outbreak of EP in this area. To develop effective measures to prevent this disease, more *T. equi* and *B. caballi* molecular epidemiology data are required to help determine the clinical significance between EP and the *T. equi* and *B. caballi* genotypes.

Conflict of interests

The authors declare no conflict of interests.

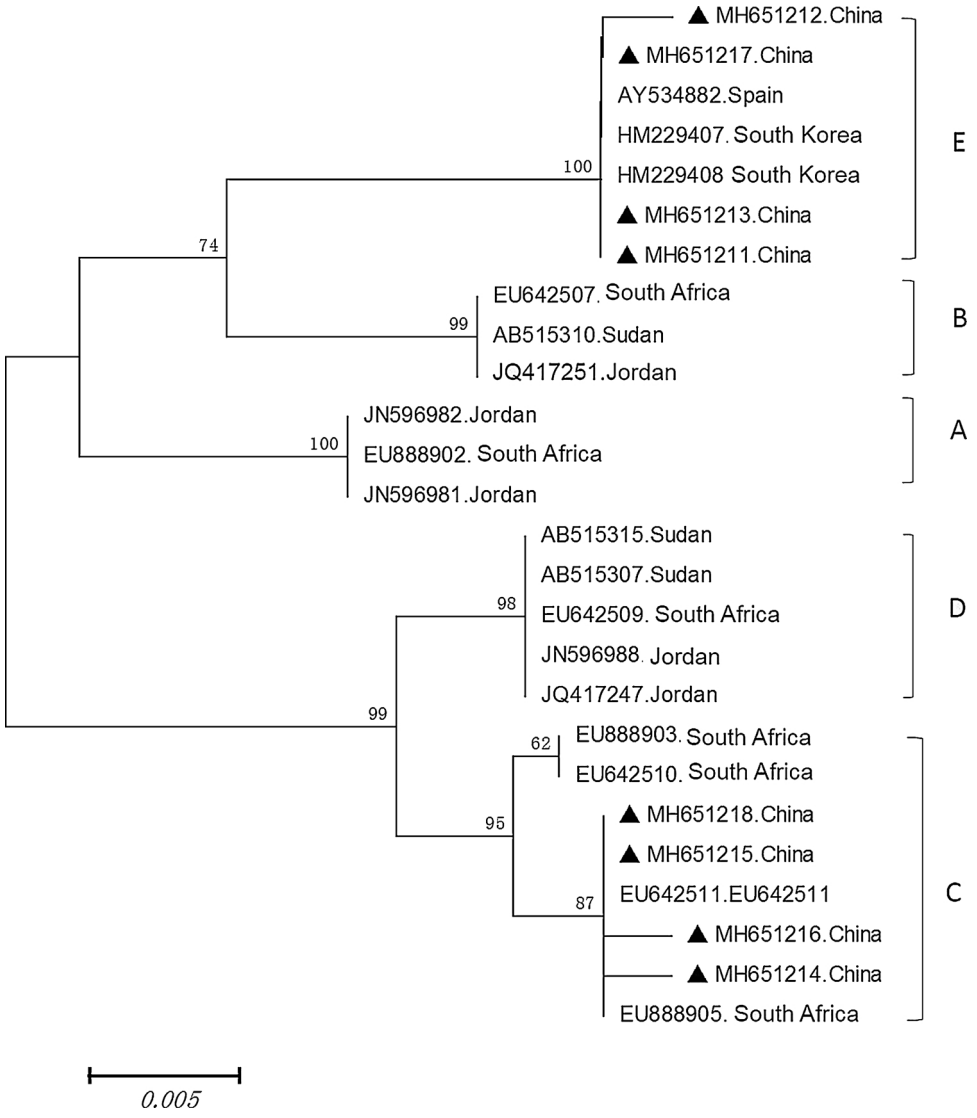

Fig. 2. Phylogenetic tree of *T. equi* inferred from partial sequences of the 18S rRNA gene. Numbers above the branches demonstrate bootstrap support from 1000 replications. The newly generated sequences in present study were indicated with bold triangles. A, B, C, D, and E represent genotypes.

Funding

This study was financially supported by National Key R&D Program of China (2017YFD0501200); The National Key Research and Development Program of China (2018YFD0502305); ASTIP (CAAS-ASTIP-2016-LVRI); 973 Program (2015CB150300); Jiangsu Co-innovation Center program for Prevention and Control of Important Animal Infectious Disease and Zoonose.

Authors' contributions

Jinming Wang wrote the draft of the manuscript. Guiquan Guan corrected the manuscript. Xiaoxing Wang, Zhi Li, Jianlin Xu, Xuan Li, and Quanjia Xiang carried out the nested PCR assays and purified the amplicons. Jifei Yang, Junlong Liu, Zhijie Liu, Youquan Li, Jianxun Luo, and Hong Yin supervised all parts of the study. All authors have read and approved the final version of this manuscript.

Ethical statement

The sample's collection was approved by the Animal Ethics Committee of the Lanzhou Veterinary Research Institute, CAAS (Permit No. LVRIAEC-2018-001). All the procedures were conducted according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Acknowledgement

Not applicable.

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