Research Square

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Detection and isolation of pathogenic Theileria orientalis Ikeda genotype from confined dairy cattle, in Hebei, China

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Abstract

Background

Theileria orientalis is a group of benign pathogenic of cattle parasites with a cosmopolitan distribution, and has been classified into 11 genotypes through MPSP gene phylogenetic analysis. In China, *T. orientalis* is the most prevalent *Theileria* species, with several genotypes, but few fatal cases have been reported. In June 2020, dairy cattle in Zhangjiakou, Hebei Province, showed clinical symptoms of piroplasmosis, causing many animals to die. In the present study, we confirmed the infection of *T. orientalis* was responsible for the disease and isolated the *T. orientalis* Ikeda genotype from the field blood samples.

Methods

Fifty-one blood samples were collected from ill and recovered animal. Meanwhile, 12 samples were collected from animals without any clinical symptoms. Blood smears were prepared from blood samples and detected with microscope. Genomic DNA was extracted and tested by PCR method. The MPSP gene was sequenced from the *Theileria* positive samples detected by both methods, to identify the *T. orientalis* genotypes. Parasites were isolated by inoculate the mixed positive blood samples into the experimental cattle and identified by MPSP sequence analysis.

Results

Blood smears and PCR detection results confirmed *T. orientalis* infection with a 66.7% positive rate of collected blood samples. The MPSP sequences analysis revealed parasite genotypes 1 (Chitose) and 2 (Ikeda). In animal experiments, only *T. orientalis* genotype 2 (Ikeda) was obtained, indicating that the Ikeda type was predominant and responsible for the disease.

Conclusions

The present study firstly identified pathogenic *T. orientalis* Ikeda in dairy cattle in China, and isolated the parasite from experimental animal. Although many *T. orientalis* genotypes are present in China, the possibility of *T. orientalis* genotype 1 and 2 infections in confined dairy cattle should considered to avoid additional economic losses.

Background

Theileria orientalis is a complex group of benign *Theileria* species. Historically, these species were referred to as *T. sergenti* in Japan, *T. buffeli in* Australia and *T. orientalis* in Europe, depending on their geographical origin [1]. However, the classification of this *Theileria* group remains in dispute. Based on

serological and morphological comparisons, Uilenberg et al. suggested that *T. orientalis* and *T. sergenti* from cattle and *T. buffeli* from buffalo should be considered a single species and proposed *T. orientalis* as the name for these parasites [2, 3]. With the development of molecular research methods, an increasing number of genes, such as 18S RNA, internal transcribed spacers (ITS) and major piroplasm surface protein (MPSP) gene sequences, have been used for the phylogenetic analysis of *Theileria* species [4–10]. Among these genes, MPSP gene, which exists in all *Theileria* species and is expressed at the intraerythrocytic stage, is the most common molecular marker used for the phylogenetic analysis of *Theileria* parasites [1, 5, 11–13], which has resulted in the *T. orientalis* group into 11 genotypes (types 1–8 and types N1-3) [14]. In contrast to *T. annulata* and *T. parva*, which show high pathogenicity, *T. orientalis* is traditionally recognized as a benign pathogen of bovines. However, an increasing number of oriental theileriosis outbreak cases have been reported in both beef and dairy cattle, indicating that *T. orientalis* infection also affects the development of the cattle industry. Through genotype analysis, type 1 (Chitose) and type 2 (Ikeda) have been found to show pathogenicity, these genotypes occur mainly in Australia, New Zealand, and India and have recently been reported in the USA [15–21]. Genotype N2 has also been reported cause death in Asian water buffaloes, but only one such case has been recorded in India [22].

In China, *T. orientalis* is the most prevalent *Theileria* species and is distributed in at least 20 provinces [23–26]. Based on MPSP analysis, 10 genotypes, including types 1–5, 7, 9 and N1-3, have been confirmed to infect beef or dairy cattle and yaks [24, 27–29]. However, all the genotypes of *T. orientalis* found in China have been identified from field-collected blood samples or ticks, and no report has addressed the pathogenicity of the identified *T. orientalis* genotypes.

On a dairy cattle farm in Zhangjiakou, Hebei Province, that maintains approximately 4,000 cows, the clinical symptoms of anemia appeared in June 2020, and 50–60 animal deaths per year have been recorded. After eliminating all other possible diseases, the animals were suspected of being infected with piroplasms. Blood samples were collected from ill or treated animals and from randomly selected health animals. Blood smears showed different pleomorphic characteristics that conformed to those of *Theileria* species. PCR detection and MPSP gene analysis confirmed mixed infections with *T. orientalis* genotypes 1 and 2 in animals. Although different genotypes of *T. orientalis*, including types 1 and 2, are present in China, clinical cases (especially the fatal cases) have not been reported. The present study showed that infection with genotypes 1 and 2 of *T. orientalis* in dairy cattle can lead to animals death and results in an important impact on animal health and economic losses.

Materials And Methods

Blood samples

In June 2020, the manager of a dairy cattle farm in Zhangjiakou, Hebei Province, on which approximately 4,000 cattle are reared, contacted our laboratory and described a disease characterized by anemia, weakness, reduced production and abortions. Some severe cases of the disease led to death of the

animals. Approximately 50-60 cattle per year died from this disease. The disease could occur throughout the year, especially after animals had given birth. All other diseases caused by bacteria or viruses with the above clinical symptoms were excluded, and piroplasmosis was suspected. The ill animals were treated with anti-parasite medicines, but the disease could not be completely eliminated. A total of 63 blood samples were collected from cattle with clinical symptoms or animals that had recovered from the disease and 12 samples were collected from healthy animals. Blood was collected from the caudal artery of each animal into the 9 ml Vacuette tube containing K_3EDTA as an anticoagulant (Greiner Bio-One GmbH, Austria). Some of the blood was used to produce blood smears and stained with Giemsa to investigate the morphology of the pathogens in red blood cells.

DNA extraction and PCR detection

DNA was extracted from 300 μL blood samples by using the QIAamp DNA Mini Kit (QIAGEN) according to the provided instructions. The extracted DNA was eluted into 100 μL of RNase-free water, and the concentration was determined with a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

T. annulata is the only *Theileria* species that causes animals death in China. A multiplex PCR method was performed to amplify the cytochrome b gene and internal transcribed spacers of *T. annulata* and *T. orientalis*, respectively [30]. 2 μL of extracted DNA in a 25 μL reaction volume was used to amplify the target sequence, which is 393 bp for cytochrome b and 818 bp for ITS. The PCR products were electrophoresed on a 1.0% agarose gel containing 10 μL of Goldview (SolarBio, China) in Tris-acetate-EDTA (TAE) buffer and visualized under UV light.

Amplification and sequencing of the MPSP gene

The samples, that were positive for *T. orientalis*, were subjected to the amplification of the MPSP gene. The primers used for the MPSP gene were 5'-CACGCTATGTTGTCCAAGAG-3' (Ts-U) and 5'-TGTGAGACTCAATGCGCCTA-3' (Ts-R), as previously reported [31]. PCR was performed in a 25 μL total volume containing 12.5 μL of Premix Taq DNA Polymerase (TaKaRa, Dalian, China), 1 μM of each primer and 2 μL of genomic DNA. The thermal cycling program was as follows: 95°C for 3 min; 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 40 s and extension at 68°C for 1 min; and a final extension at 68°C for 5 min. The positive PCR products were cloned into pGEM-T Easy vectors (Promega Corporation, Madison, USA), which were then transformed into *Escherichia coli* JM109 (TaKaRa, China). At least five positive clones were sequenced to obtain consensus sequences.

Sequence analysis

The obtained MPSP sequences were aligned using the MegAlign component of the DNAStar program (DNAStar Version 4.0, Madison, USA). Following alignment with related *T.* spp MPSP sequences in GenBank, parts of the vector sequences were manually removed. The resulting sequences were then submitted to the GenBank database.

A phylogenetic tree was generated based on the obtained MPSP sequences and the 11 reported genotypes of the *Theileria* MPSP sequences obtained from GenBank by using the neighbor-joining algorithm in MEGA 7.0 software[32,33]. The evolutionary distances were computed using the Kimura 2-parameter method [34].

Isolation of the parasite

To isolate the pathogens causing disease in dairy cattle, 1 mL of each blood sample positive for *T. orientalis* was combined, and the mixed sample was stored in liquid nitrogen. A calf at approximately 6-12 months of age that was free of piroplasms was splenectomized. Ten days before the pathogen experiment, both microscopy and PCR methods were applied to test for piroplasm infection again. The calf that was negative for piroplasms was inoculated with the stored mixed blood sample via the jugular vein. Blood smears were obtained every day after infection to investigate the presence of *Theileria* in red blood cells.

Results

Blood Smears

According to the blood smear examination, 36 of the 51 (70.1%) blood samples collected from animals with anemia symptoms or animals treated with anti-parasite medicines were positive for piroplasms. Half of the healthy cows (6/12) exhibited a similar morphology of the parasites in their red blood cells. The parasite has showed typical *Theileria* morphological characteristics, including oval, comma, pear seed-shaped and cruciform forms (Figure 1).

PCR detection

The PCR detection results for all samples from the dairy cows were negative for *T. annulata*. Nineteen blood samples from animals with clinical symptoms or animals that had recovered from the disease were positive for *T. orientalis*. All 19 positive samples were also positive according to microscopic examination. However, all 12 blood samples from healthy cows were negative for *T. orientalis*. The results indicated that *T. orientalis* was responsible for the observed clinical signs, further analysis was conducted to determine the genotypes of the isolated organisms.

Amplification of the MPSP gene and sequence analysis

A total of 19 blood samples that were positive for *T. orientalis* according to both the blood smear analysis and PCR detection results were selected for MPSP gene amplification. Fourteen of the 19 samples (Animal Nos. 8010, 8080, 8009, 8253, 8086, 8056, 7877, 6267, 5267, 7028, 8061, 8183, 8311, and 7853) were positive according to MPSP gene amplification, and their PCR products were sequenced. A total of 17 sequences were obtained by sequencing. All the sequences contained 852 bp coding sequences, and 10 of the 17 sequences were identical. The other 7 sequences were 99.9% identical and showed only one nucleotide mutation at site 847.

Based on MPSP gene phylogenetic analysis, 10 samples were clustered with the sequences of genotype 2 (GenBank accession no.: MW752894), which is also referred to as the Ikeda type. Seven samples were grouped into the cluster of genotype 1 (Chitose) (GenBank accession no.: MW752892). These results confirmed that 3 animals exhibited mixed infection with *T. orientalis* types 1 and 2 (Figure 2).

Isolation of *T. orientalis*

After the inoculation of the blood samples into the experimental animal, parasites were detected in its red blood cells on day 12. The shape of the parasites was similar to that in the initial samples. When the infection rate reached 10%, blood was collected for parasite purification and DNA extraction. The MPSP gene was amplified and sequenced. The phylogenetic analysis results showed that the obtained *T. orientalis* Hebei isolates belonged to the type 2 (Ikeda) group. The results indicated that *T. orientalis* type 2 (Ikeda) was the predominant species and was responsible for the disease.

Discussion

T. orientalis is the most prevalent parasite in China and is distributed in most areas of the country. Traditionally, *T. orientalis* was thought to show low pathogenicity in animals, but dairy cows and exotic and hybrid cattle imported from other places are susceptive to this parasite. Since the MPSP gene was first used to genotype the *T. orientalis* group, 11 genotypes of *T. orientalis* have been identified. Among these 11 genotypes of *T. orientalis*, several types including type 1 (Chitose), 2 (Ikeda) and N2 have been found to cause outbreaks of theileriosis [20,22,35]. From recently published reports, Ikeda/type 2 *T. orientalis* is the most common type responsible for outbreaks of the disease in Australia, New Zealand, and the United States [20,36,37]. In China, many types, including type 1 (Chitose) and 2 (Ikeda), have been confirmed [24,27,29] by analyzing blood samples of grazing cattle or ticks. However, few clinical cases related to *T. orientalis* have been reported.

In the present study, *T. orientalis* genotypes 1 (Chitose) and 2 (Ikeda) were identified from clinically ill or recovered dairy cattle. Some animals were still positive for both types of *T. orientalis* 4 months after treatment, suggesting that the animals remained in a chronic state and presented a potential risk of subsequent outbreak. The results of parasite isolate experiment, only genotype 2 (Ikeda) of *T. orientalis* was isolated, indicating that *T. orientalis* genotype 2 (Ikeda) is the predominant genotype responsible for the disease.

The confirmation of the clinical disease related to *T. orientalis* in dairy cattle farms is of particular concern and raises many questions. Because the analysis of the samples collected from the farm indicated that the disease could occur year round, it is not related to the lifecycle of the ticks. We checked the body surface of the cattle and the living areas, and no ticks were found. The dairy cattle were enclosed for breeding and had no possibility of contact with the outside environment. We also checked for the presence of ticks via the cloth flag method in grassy and bush areas outside of the dairy cattle farm but did not find any ticks. Because the disease has existed for many years, there must be other methods of *T. orientalis* transmission. In addition to tick transmission, flies and lice or vaccine needles

could possibly transmit *T. orientalis* [38]. *T. orientalis* positivity has been found the blood meals of mosquitoes fed on cattle, indicating that mosquitoes might be able to transmit *T. orientalis* [39]. A previous study confirmed that calves could not be infected with *T. orientlais* via vertical transmission [40]. Future works are needed to determine the presence of ticks in the local area and to clarify the transmission of *T. orientalis* in the enclosed feeding environment.

Once the disease occurred, the ill cattle were treated with some antiparasitic medicines. However, according to our detection results, some cattle were still positive for *T. orientalis*, indicating that the medicines could not eliminate the parasite completely. Buparvaquone has been successfully used for the treatment of *T. orientalis*, but its residues remain in animals for more than 100 days [38]]. In dairy cattle in particular, some medicines are forbidden to control the quality of milk. Because of the immune depression of cows after calving, oriental theileriosis is established easily after birth, often leading to death. According to our oral discussion with the farm manager, 50-60 cows per year have died because of oriental theileriosis in last three years, causing serious economic loss. To completely eliminate *T. orientalis* in confined feeding dairy cattle, it is important to identify effective medicines and clarify the lifecycle of this parasite in future studies.

Conclusion

In conclusion, we have confirmed that the mixed infection of *T. orientalis* genotype 1 (Chitose) and 2 (Ikeda) was responsible for theileriosis outbreak in confined dairy cattle in Hebei, China. Only the *T. oreintalis* Ikeda was isolated from animal experiment, indicating that the Ikeda type was the predominant parasite. The finding indicated that the oriental theileriosis not only happened in grazing cattle but also in confined dairy cattle. However, ticks were not found on the body surface of animals and environment. Future works to clarify the transmission way of *T. orientalis* in confined feeding situation will help to explain why the disease exist for many years.

Declarations

Ethical approval and consent to participate

The present animal experiment was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, CAAS (No. LVRIAEC2019-010). The procedure for acquiring the field samples was approved according to the Animal Ethics Procedures and Guidelines of China.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JL and ZL did the sample collection and the molecular genetic studies. Al and JW did the parasite isolation and purification. JL wrote the draft of the manuscript. GG, JL and HY corrected the manuscript. All authors read and approved the manuscript.

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Figures

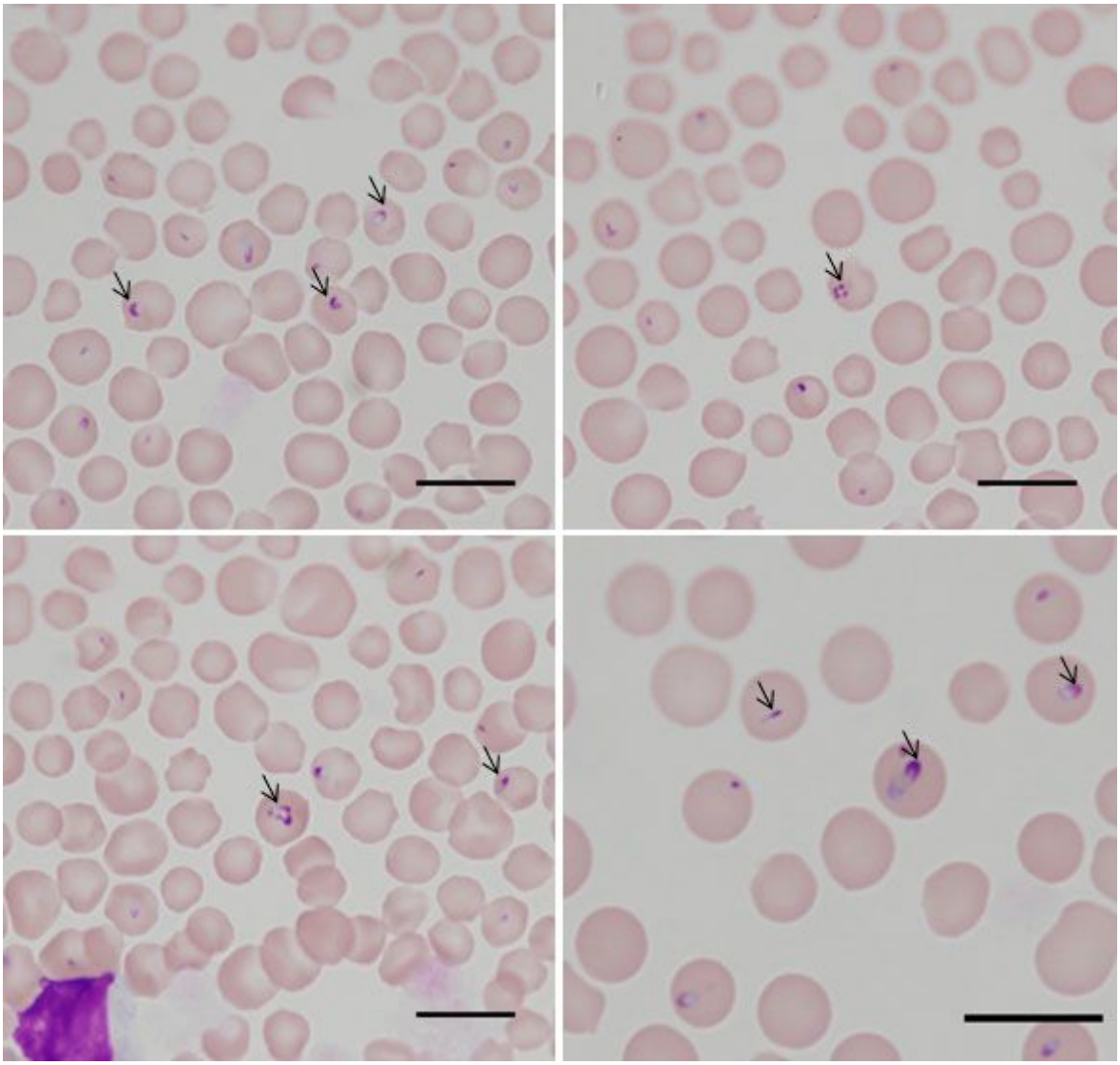

Figure 1

Blood smear of the ill dairy cattle from a farm in Hebei province, China. The intracellular piroplasms within erythrocytes are polymorphism (as indicated with arrow). Scale bar indicates 10 μm.

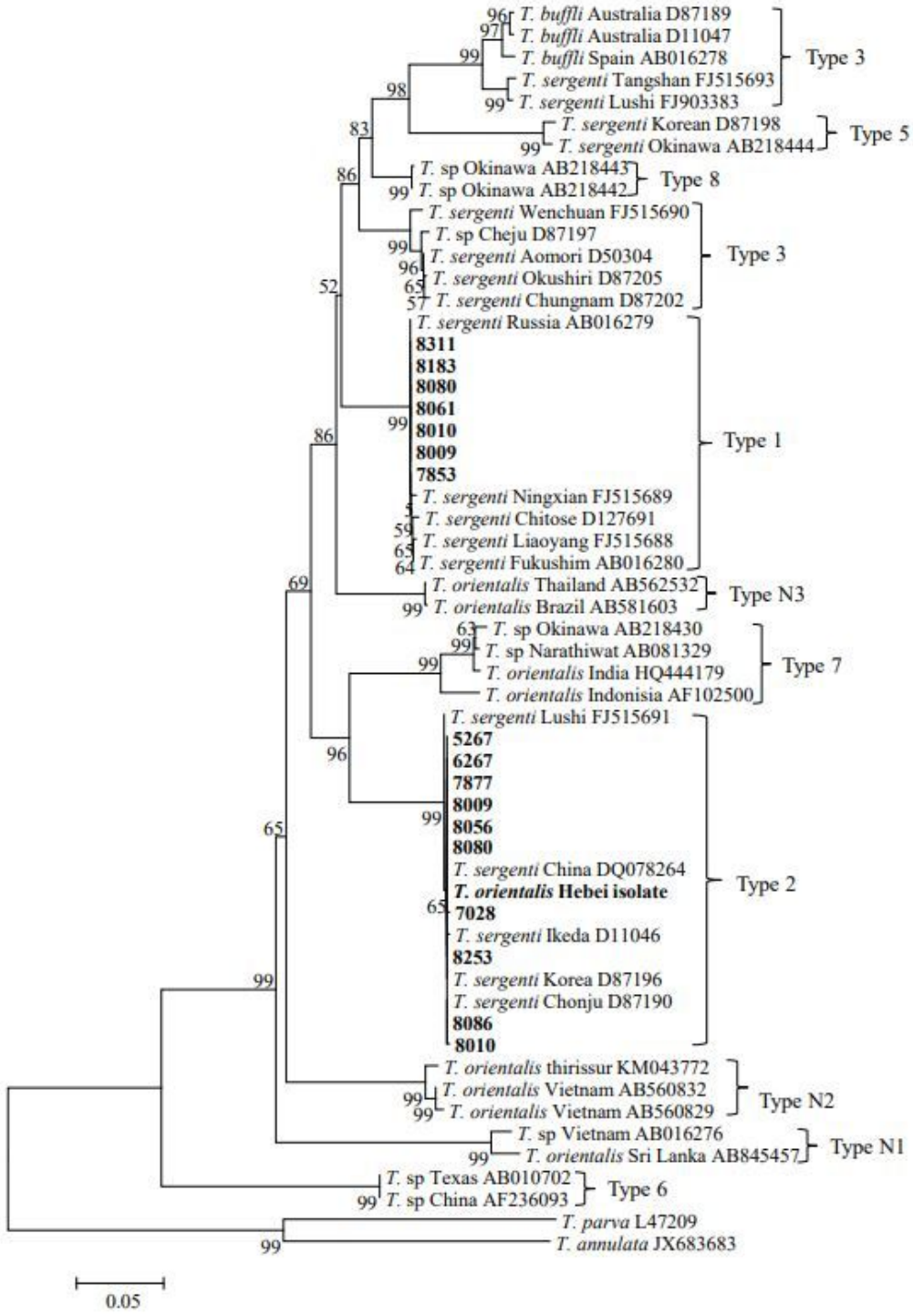

Figure 2

Phylogenetic relationship of T. orientalis genotypes (bold) identified in dairy cattle in Hebei, China, with the published genotypes for T. orientalis. Phylogenetic tree was constructed based on analysis of major piroplasm surface protein sequences by using Neighbor Joining methods from MEGA 7.0.

Supplementary Files

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