

Molecular phylogenetic studies on clinical bovine piroplasmosis caused by benign *Theileria* in Shaanxi Province, China

Jing Wang^{1,2,*}, Jiyu Zhang², Zhen Zhu², Xuzheng Zhou², Bing Li²

¹Department of Veterinary Pharmaceutical, Jiangsu Agri-animal Husbandry Vocational College, Taizhou 225300, China

²Lanzhou Institute of Husbandry and Veterinary Pharmaceutical Science, Chinese Academy of Agricultural Sciences, Key Laboratory for Veterinary Drug Innovation, Ministry of Agriculture, Lanzhou 730050, China

A group of benign *Theileria* species, which are often referred to as *T. orientalis*/*T. buffeli*/*T. sergenti* group, has low pathogenicity in cattle. Herein, we report on *Theileria* spp. in cattle on a farm from China. Based on phylogenetic analysis of the major piroplasm surface protein gene sequences, we detected 6 genotypes that were categorized as Types 1, 2, 3, 4, and 5 as well as an additional Type 9 genotype. The new epidemiological features of the *T. orientalis*/*T. buffeli*/*T. sergenti* parasites in China indicate a greater diversity in the genetics of these species than had been previously thought.

Keywords: *MPSP* gene, *Theileria* spp., genotype, phylogeny

A group of benign *Theileria* species transmitted by *Haemaphysalis* ticks is often referred to as *T. orientalis*/*T. buffeli*/*T. sergenti* group. The parasite's presence is characterized by anemia, jaundice, depression, abortion, mortality, and the presence of *Theileria* in blood films [4]. Benign *Theileria* group parasites are widespread among cattle in subtropical and temperate zones [6], and the parasite can eventually lead to severe economic losses in endemic areas. For simplicity, *T. orientalis* is used throughout this paper to denote this benign *Theileria* group. Herein, we report on *Theileria* spp. in cattle on a farm in the Shaanxi Province, a region in which disease outbreaks have occurred.

The exact taxonomic status of *T. orientalis* has been debated for many years. The 18S rRNA data have shown that parasites in this group can be divided into at least 8 types, A, B, B1, C, D, E, H, and *T. buffeli* (Warwick). However, the number of identified types is increasing and there is a lack of consensus on their nomenclature [12,13]. Recently, the gene encoding the major piroplasm surface protein (*MPSP*) was considered a highly useful marker in revealing the phylogeny of *Theileria* parasites [3].

The nomenclature for the benign *Theileria* group has not reached consensus to date. In 1995, Kubota *et al.* [7] divided the

group into at least four types, Ikeda (I), Chitose (C), Thai, and Buffeli (B), based on the allelic forms of the *MPSP* gene. However, Kim *et al.* [6] proposed a different classification approach based on *MPSP*, in which there were 6 genotypes, designated Types 1 to 6. Subsequently, 5 additional genotypes, including Types 7, 8, and N1 to N3, were added to the list of previously described genotypes [3,5].

Theileriosis caused by the benign *Theileria* group is widely reported from countries neighboring China. However, there are few reports on the occurrence of benign *Theileria* in China. Liu *et al.* [8] and He *et al.* [2] have reported that *Theileria* spp. are present in Hubei Province of China. In addition, surveys have revealed that at least five genotypes of *T. orientalis* (Types 2, 3, 6, 7, and 8) exist in China [3].

This study investigated the genetic diversity of the benign *Theileria* parasites in Huanglong county, which is located in the northern part of Shaanxi Province, China, by analyzing blood samples gathered from cattle at a farm where there was an outbreak of clinical piroplasmosis following a severe tick infestation (species unknown) in August 2013. Blood samples were collected from 10 cattle showing appropriate clinical signs: marked pallor in the oral and genital mucosa, anorexia, and fever (rectal temperature with high readings $\leq 42^{\circ}\text{C}$). The

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*Corresponding author: Tel: +86-13951140263; Fax: +86-523-86663808; E-mail: kenhtsjj@163.com

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diagnoses were made based on clinical signs and a light microscopic examination of Giemsa-stained blood smears. To confirm the species of the piroplasm, we conducted polymerase chain reaction (PCR) with primer set 989/990 [11] from the 18S rRNA gene sequences of *Theileria* species. Subsequently, 5 randomly selected PCR-positive samples were cloned and sequenced. In order to further identify the types of *Theileria* present, the *MPSP* genes were examined according to a previously described method [10] using *MPSP*-specific primers from the *T. orientalis* group. All PCR products were cloned and sequenced for confirmation by performing sequencing analysis.

One type of polymorphic *Theileria*-like parasite was identified in the erythrocytes in the anticoagulant-treated blood samples by performing light microscopy-based examinations. Dot-shaped, pyriform or irregular-shaped parasites were seen, and there were generally 2 to 4 parasites per erythrocyte (Fig. 1). The rate of parasitemia reached 13.7%.

The obtained 18S rRNA gene sequences were compared with other *Theileria* spp. available in GenBank by using the BLASTN algorithm. The results indicated that the pathogens from the sampled cattle belonged to the benign *T. orientalis* group. There were at least three types of *Theileria* detected: Type A (Chitose), Type B (Ikeda), and Type C (Buffeli) (Table 1). The 18S rRNA gene sequences were submitted to GenBank under accession Nos. KJ020544 to KJ020548.

Based on our PCR analyses, 9 of the 10 blood samples showed amplification with both the 989/990 and the *MPSP*-F/R primer pairs. The *MPSP* genes of all PCR-positive samples were cloned and sequenced. Representative sequences were registered in the GenBank under the assigned accession Nos.

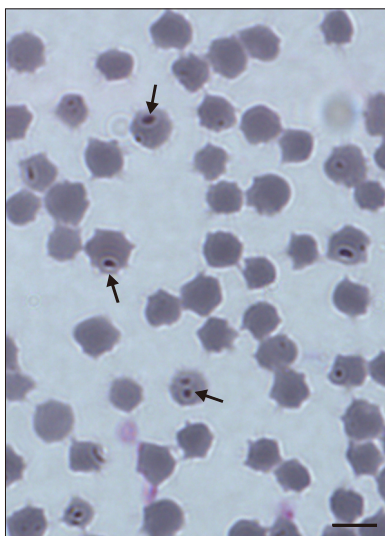


Fig. 1. Giemsa-stained blood smear from an infected calf. The arrows point out different polymorphisms of *Theileria orientalis*. Scale bar = 10 μ m.

KJ020544 to KJ020562. When the BLAST analysis was conducted, sample numbers 7 to 9 (KJ020550), 1 to 10 (KJ020551), and 8 to 13 (KJ020557) showed a high identity with the registered database sequence for *Theileria* Type 5 accession No. AB560825 [5]. Sample numbers 3 to 5 (KJ020552) and 8 to 11 (KJ020556) clustered with the classical benign Type 3 EU584237 isolated in Hubei Province, China; and this type is commonly found on Cheju Island, Korea and on Okinawa Island, Japan. Samples 10 to 22 (KJ020558) clustered with the unclassified *T. buffeli* accession No. AF236095 from Brisbane, Australia [1,3].

Moreover, by using the MEGA5 program [14], a neighbor-joining phylogenetic tree was constructed from 32 bovine *Theileria MPSP* gene sequences, including 14 sequences cloned in this study and 18 sequences from the GenBank database. From the phylogenetic tree, we detected 6 *MPSP* genotypes that were categorized as *MPSP* Types 1, 2, 3, 4, and 5 as well as an additional Type 9. The phylogenetic tree indicated that all sequences belonged to 6 of the 9 previously designated types (Fig. 2). *T. orientalis* Types 6, 7, and 8, which have been reported in China, were not detected in this study [3].

In this study, we detected an additional sequence (KJ020558), which has been classified as Type 9, that was not clustered with the *T. orientalis* sequences of Types 1 to 8 or N1 to N3 that have been reported in Vietnam [2] (data not shown). Therefore, based on phylogenetic analysis, we describe an additional genotype (Type 9) that is similar to a previously reported *MPSP* gene that was sequenced in Brisbane, Australia [3]. Based on the phylogenetic tree analysis, many of the sequences from this

Table 1. Comparison of gene types based on 18S and major piroplasm surface protein (*MPSP*) genes

Animal No.	18S gene type/ GenBank accession No.	<i>MPSP</i> gene type/ GenBank accession No.
1	-	Type 5/KJ020551
3	-	Type 3 (Buffeli)/KJ020552
5	Type B (Ikeda)/ KJ020548	Type 2 (Ikeda)/KJ020553, KJ020549
6	Type C (Buffeli)/ KJ020546	Type 3 (Buffeli)/KJ020555, Type 1 (Chitose)/KJ020554
7	-	Type 5/KJ020550
8	Type A (Chitose)/ KJ020547	Type 3 (Buffeli)/KJ020556, Type 5/KJ020557
9	Type C (Buffeli)/ KJ020545	Type 5/KJ020560
10	Type B (Ikeda)/ KJ020544	Type 3 (Buffeli)/KJ020559, Type 9/KJ020558
4,045	-	Type 2 (Ikeda)/KJ020561, Type 4/KJ020562

-, not detected.

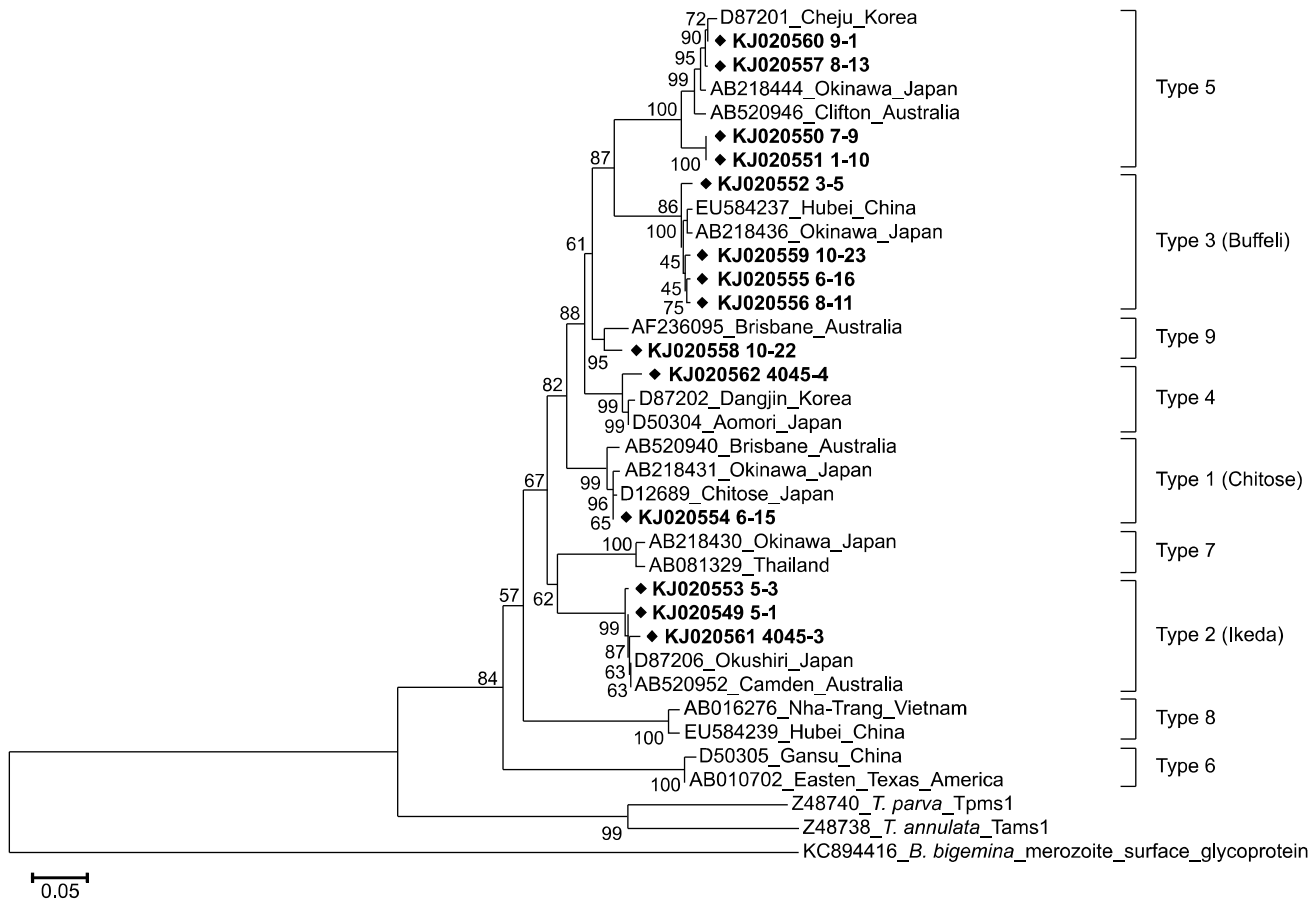


Fig. 2. Phylogenetic relationships among *Theileria* isolated in Shaanxi Province, China, based on major piroplasm surface protein (MPSP) gene sequences. The bold font indicates sequences obtained from this study. Bootstrap values are shown as percentages at each node based on 1,000 replicates. Branch lengths correlate to the number of substitutions inferred according to the scale shown.

study are very close to the Australian sequences. Piroplasmosis is not included in the List A or List B of Contagious and Parasitic Diseases for the Animals Imported from Other Countries into the People's Republic of China. By coincidence, the herd sampled in this study was imported from Australia a few months prior to this study. We speculate that the cattle were infected while in Australia. On that basis, it is essential to implement effective quarantine and control measures for vector-borne pathogens in animal populations. Note, in this study, all possible causes of regenerative anemia, such as babesiosis, anaplasmosis, and trypanosomosis were eliminated (data not shown).

Although *T. orientalis* shows a lower pathogenicity in cattle than *Theileria parva* and *Theileria annulata*, occasional disease outbreaks characterized by fever, anemia, anorexia, and reduced growth rate have been reported [9,12,13]. *T. orientalis*, of which Ikeda (Type 2) is a representative, appears to be closely associated with clinical cases reported in Japan and other Asian countries [4,12]. Ikeda (Type 2) and Chitose (Type 1) were the predominant types (accounting for 46.4% of the

cases) detected in New South Wales, Australia, the epicenter of a theileriosis outbreak [4]. Although Type 2 and Type 1 were detected in this study, they were not the predominant genotypes in our limited dataset. It is common for animals to be infected with mixed genotypes, and that feature presents challenges to the detection and control of theileriosis. After obtaining the diagnostic results, the animals received diminazene aceturate (3 mg/kg of body weight, intramuscularly) every 12 h for 7 days [15]. The therapy resulted in complete recovery of all animals within approximately 10 days; however, additional clinical cases of theileriosis were sporadically reported the following month.

The epidemiology of theileriosis in China has not been described. Basic information about the *Theileria* spp., such as their life cycles, vectors, modes of transmission, virulences, and host compatibilities require further study. Regardless, the present study demonstrates that infection by *T. orientalis* is a potentially serious problem in Shaanxi Province, China.

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Conflict of Interest

The authors declare no conflicts of interest.

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