Article
Detection of *Theileria orientalis* Genotypes from Cattle in Kyrgyzstan

Sezayi Ozubek 1,*, Mehmet Can Ulucesme 1, Veli Yilgor Cirak 2 and Munir Aktas 1

1 Department of Parasitology, Faculty of Veterinary Medicine, University of Firat, Elazig 23119, Turkiye
2 Department of Parasitology, Faculty of Veterinary Medicine, Bursa Uludag University, Bursa 23119, Turkiye
* Correspondence: sozubek@firat.edu.tr

Abstract: The ikeda and chitose genotypes of *Theileria orientalis*, which for many years were thought to be benign, cause a disease that results in significant economic losses in the cattle industry. This study was carried out in order to determine the genotypes of *T. orientalis* in cattle in Kyrgyzstan, and 149 archived DNA samples known to be *T. orientalis* were analyzed by the PCR amplification of the major piroplasm surface protein (MPSP) gene region. Single-Strand Conformation Polymorphism (SSCP) analysis was performed to uncover the nucleotide changes in the archived DNA samples, and 15 samples showing different band profiles were subjected to sequence analysis. As a result of the sequence analysis, it was seen that the samples belonged to the buffeli and chitose A genotypes. In order to identify mixed genotypes, PCR was performed using primers specific for these genotypes, and buffeli (type 3), chitose (type 1) and buffeli+chitose were found to be positive in 26.2%, 2% and 71.8% of samples, respectively. As a result of this study, we showed the presence of buffeli (type 3) and chitose (type 1) genotypes of *T. orientalis* in cattle in Kyrgyzstan. Comprehensive epidemiological studies are needed to understand the clinical infections caused by the pathogenic chitose A and to determine the geographical distribution and different genotypes of *T. orientalis*.

Keywords: *Theileria orientalis*; genotype; cattle; PCR

1. Introduction

Bovine theileriosis, caused by *Theileria* species (Apicomplexa: Piroplasmida; Theileriidae), is an important tick-borne disease of cattle in tropical and subtropical regions of the world and causes serious economic losses [1,2]. It is known that *Theileria parva* and *Theileria annulata*, which are also known as transforming species, are highly pathogenic species for cattle. Other species of *Theileria* that infect cattle, *Theileria mutans*, *Theileria taurotragi*, and members of the *Theileria orientalis* complex, frequently cause benign infections [3–5]. It has been observed that *Theileria orientalis*, long thought to be benign, has different genotypes, some of which cause clinical cases and adversely affect the cattle industry [6]. According to sequence variations in the *major piroplasm surface protein* (MPSP) gene, 11 genotypes, type 1 (chitose), type 2 (ikeda), type 3 (buffeli), type 4–8 and N1-N3, have been reported in various region in the world. Of these genotypes, ikeda and chitose cause clinical cases of oriental theileriosis in cattle. The transmission of *T. orientalis* occurs through the feeding of infected ticks of the *Haemaphysalis* genus [7,8]. It has also been reported that *T. orientalis* can be spread mechanically among cattle via blood-sucking flies and arthropods [9]. Recently, it has been shown that sheep can be effective in spreading *T. orientalis*; healthy sheep can be infected with the pathogenic genotype ikeda, and ticks can become infected by feeding on sheep [10].

In a molecular survey we conducted in 2019, we reported that *T. orientalis* is the most common blood parasite (32.8%; CI 28.5–37.3) in cattle in Kyrgyzstan [11]. In this study, we aimed to determine which genotypes of *T. orientalis* are present in cattle in Kyrgyzstan.
2. Materials and Methods

Archived DNA samples used in this study and detailed information from the investigated provinces has been documented previously [11]. Briefly, this study was carried out between December 2012 and June 2013 on 454 cattle in eight provinces (Karaşar, Kayyngdy, Kızıl-Töbö, Kopuro Bazar, Moldovanovka, Sokuluk, Tamga, and Tokmok) located around the Chu valley and Issyk Kul Lake in Kyrgyzstan (Figure 1). All DNA samples were tested using the reverse line blot assay for bovine Theileria and Babesia species to determine the frequency of piroplasm distribution, and 149 DNA samples identified as containing T. orientalis [11] were used for genotype analysis.

To investigate the genetic diversity of T. orientalis, the Major Piroplasm Surface Protein (MPSP) gene was amplified by nested PCR. Briefly, the primers MPSP-F/MPSP-R [12] were used for the initial amplification of the MPSP gene in T. orientalis. Nested amplification was performed using the primers MPSPAJ-F/MPSP-AJ-R1 [13]. Ten microliters of the PCR products were used on 1.6% agarose gel for visualization and the remaining products were stored at 4 °C until use in SSCP. Single-Strand Conformation Polymorphism was performed to see possible sequence changes in the MPSP gene, and samples which had different band profiles were sent for sequence analysis. After sequence analysis, phylogenetic analysis was performed using the MEGAX program [14] and genotypes were determined accordingly. Genotype-specific PCR was then performed to determine whether infections of mixed genotype were present, using primers TSB-TSR (buffeli) [15], TSC-TSR (chitose) [16] and TSI-TSR (ikeda) [17] (Table 1). Theileria orientalis genomic DNA, previously detected by PCR and DNA sequencing (GenBank accession number MK415835), were used as positive controls in the PCR.

Figure 1. Map of Kyrgyzstan and sampling regions.

**PCR Amplification, Single-Strand Conformation Polymorphism (SSCP) and Phylogenetic Analysis**

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Table 1. The primers used in the study.

| Primer Name | Primer Sequence 5′-3′ | PCR Condition | References |
|-------------|-----------------------|---------------|------------|
| MPSP-F      | CTTTGCCCTAGGATACTTCC | 95 °C, 3 min; 95 °C, 30 s; 58 °C, 30 s; and 72 °C, 30 s (35 cycles); final extension of 72 °C, 5 min. | [12] |
| MPSP-R      | ACGGCAAGTGTTGAGAATT  |               |            |
| MPSP-AJ-F   | TTCAACTTIIIACGTCGCCAACA | 95 °C, 3 min; 95 °C, 30 s; 60 °C, 30 s; and 72 °C, 30 s (35 cycles); final extension of 72 °C, 5 min. | [13] |
| MPSP-AJ-R1  | ACGTAAAACCTTTGACTCGCTTG  |               |            |
| TSB         | CACCTTCTTACGTCTCTGCAACT | 95 °C, 3 min; 95 °C, 30 s; 55 °C, 30 s; and 72 °C, 30 s (35 cycles); final extension of 72 °C, 5 min. | [15] |
| TSC         | CACCTGCTCTGCAACCGCAGAG |               |            |
| TSR         | CACCTGCTCTGGAACCGCAGAG |               |            |
| TSI         | CACCATCGTCTGCTACCGCCGC |               |            |
| TSR         | CACCTGCTCTGCAACCGCAGAG |               |            |

3. Results

All 149 *T. orientalis* samples were amplified by nested PCR for the *MPSP* gene. The amplicons obtained from these samples were used in SSCP analysis and 15 samples with different band profiles were sent for sequence analysis (Supplementary Figure S1). The nucleotide sequences have been registered in GenBank under the accession numbers ON934520-ON934534. A maximum likelihood phylogenetic analysis based on the Tamura 3-parameter model [18] was created using the sequences obtained as a result of the sequence analysis and the sequences of 11 *T. orientalis* genotypes obtained from GenBank (Figure 2). As a result of phylogenetic analysis, it was seen that the samples belonged to buffeli and chitose genotypes. Furthermore, phylogenetic analysis was performed using the Kimura 2-parameter model [19] to determine chitose genotypes and it was seen that all samples belonged to the chitose A genotype (Figure 3). As a result of genotype-specific PCR performed to determine mixed genotypes, buffeli, chitose and buffeli+chitose were found to be 26.2%, 2% and 71.8%, respectively (Table 2).

Table 2. In this study, *T. orientalis* genotypes determined in different provinces of Kyrgyzstan.

| Province       | No. of Positive Samples | Type 1 (Chitose) | Type 2 (Ikeda) | Type 3 (Buffeli) | Type 1 + Type 3 |
|----------------|-------------------------|------------------|----------------|------------------|-----------------|
| Tokmok         | 13                      | 1                | -              | 2                | 10              |
| Sokuluk        | 22                      | 1                | -              | 7                | 14              |
| Karashar       | 3                       | -                | -              | -                | 3               |
| Kyzyl-Tobö     | 3                       | -                | -              | 1                | 2               |
| Kopuro Bazar   | 19                      | -                | -              | 5                | 14              |
| Tamga          | 2                       | -                | -              | -                | 2               |
| Kayyngdy       | 27                      | -                | -              | 8                | 19              |
| Mal'dovanovka  | 60                      | 1                | -              | 16               | 43              |
| Total          | 149                     | 3 (2%)           | -              | 39 (26.2%)       | 107 (71.8%)     |
Figure 2. Phylogenetic analysis by maximum likelihood using *T. orientalis* MPSP gene sequences. The tree constructed using the Tamura 3-parameter model shows the phylogenetic relationship of the *T. orientalis* genotypes identified in this study (in bold) with other genotypes obtained from GenBank. The analysis includes 64 nucleotide sequences, and the percentage of the replica tree (1000 copies) in which related taxa clustered together in the bootstrap test are shown next to the branches.
Livestock, which is an important part of the economy of Kyrgyzstan, is at the forefront of the livelihoods for the majority of the population [20,21]. Tick-borne diseases (TBDs) have a significant economic impact on livestock worldwide. Epidemiological studies of tick-borne infections are crucial to identify tick–host–pathogen interactions; characterize pathogen transmission, occurrence, and pathogenesis; and identify new checkpoints for the control of both vectors and pathogens [22,23]. However, there are very few studies on TBDs on cattle in Kyrgyzstan. Aktas et al. [11] have reported Babesia major, T. annulata and T. orientalis in cattle in Kyrgyzstan using molecular methods (PCR-RLB). Altay et al. [24] have revealed the presence of Anaplasma capra, Anaplasma centrale, and Anaplasma phagocytophilum like-1 from cattle in Kyrgyzstan. Anaplasma capra and A. phagocytophilum are also known to cause serious infections in humans. Although there is limited information on TBD infections in farm animals in Kyrgyzstan, more comprehensive studies have been observed in countries that border Kyrgyzstan, especially China. In China, T. orientalis is the most common Theileria species, and all genotypes of T. orientalis have been identified from field-collected blood samples or ticks [25]. Anaplasma marginale and Babesia bigemina have been reported serologically in cattle in Tajikistan [26]. Live vaccines against infections caused by T. annulata and B. bigemina have been developed and applied in Uzbekistan [27,28]. There is no information about T. orientalis genotypes in Kazakhstan, Tajisiktan, and Uzbekistan located on the border of Kyrgyzstan.

Theileria orientalis, which consists of multiple genotypes and is recommended to be classified as a single species complex, can cause significant infections in cattle. Ikeda and chitose, known as virulent genotypes that cause pathogenic infections, have been reported in several countries, including Australia [29], New Zealand [30] and, more recently, the USA [31]. The molecular prevalence of T. orientalis in cattle in Kyrgyzstan was reported as 32.8% [11]. In this study, these samples have been shown to belong to buffeli and chitose genotypes. The buffeli genotype is largely considered a benign, non-pathogenic component...
of the *T. orientalis* complex [2,32]. Previous infection with the buffeli genotype has also been reported to be protective against the pathogenic ikeda genotype of *T. orientalis* [33]. The chitose genotype that causes clinical infections of *T. orientalis* is divided into two groups, chitose A and chitose B. In addition, Chitose A genotype has been found to be more pathogenic than chitose B, although there is little information [34]. In this study, all the samples defined as the chitose genotype were determined to belong to the chitose A genotype according to the phylogenetic analysis. Only two genotypes of *T. orientalis* were identified in this study; however, further investigation is warranted, including the sampling of cattle in the southeastern region, especially close to the border of China, in order to determine whether different genotypes are present in these regions.

The economic loss due to the pathogenic genotype of the *T. orientalis* amounts to millions of dollars annually. Integrated Parasite Management (IPM) is applied to reduce these economic losses [35]. In addition, occult carriers, such as sheep, have been found to be important in the spread of *T. orientalis* as a source of infection for naïve ticks [10]. It has also been reported that the buffeli genotype may be protective against pathogenic genotypes. As reported in this study, although the buffeli genotype is common in Kyrgyzstan, it should be kept in mind that there may be clinical cases originating from imported cases and the disease may spread more rapidly with the presence of the *Haemaphysalis longicornis* ticks [36]. Although a few tick species belonging to the genus *Haemaphysalis* (*Haemaphysalis punctata, Haemaphysalis erinacei*) have been reported in Kyrgyzstan [37], there are no data on *H. longicornis*, the main vector of *T. orientalis*.

5. Conclusions

In conclusion, two genotypes of *T. orientalis* in cattle have been reported in this study. Of these, the chitose A genotype is pathogenic and economically important for the cattle industry. Although the existence of ticks that can spread TBDs agents in Kyrgyzstan has been reported [37], there are no data on clinical infections caused by TBDs. In the future, detailed information about the geographical distribution, vector compatibility, different genotypes, and the clinical pathologies of *T. orientalis* can be obtained with epidemiological studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11101185/s1, Figure S1: Representative single-stranded conformation polymorphism gel showing 15 different profiles (P1–P7).

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