Comparative Immunology, Microbiology and Infectious Diseases 65 (2019) 132–136

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

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

# Genetic diversity of *Theileria orientalis* from cattle in Turkey

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ARTICLE INFO

*Keywords:*
*Theileria orientalis*
Cattle
Genotype
PCR
SSCP

ABSTRACT

*Theileria orientalis* is usually a benign parasite but some genotypes cause infection and economic losses to the cattle industry. This study was carried out to determine *T. orientalis* genotypes in cattle. *T. orientalis* positive 63 sample were analyzed by amplifying the MPSP gene region by PCR. As a result of the SSCP analysis, samples with different band profiles were sent to the sequence analysis and genotypes were determined. *T. orientalis* genotype-specific PCR was performed to determine the mix genotypes. Type 1 (chitose), type 3 and type 1-type 3 mix were found positive 11.1%, 46%, and 17.5% respectively. In addition, phylogenetic analysis was performed to separate the chitose genotypes, and two samples were found in chitose A, one sample was found in chitose B. Although chitose A genotype is suggested to be more pathogenic than chitose B, but there is little evidence for this. As a result of this study, we showed the presence of pathogenic genotype *T. orientalis* in Turkey. Therefore, extensive epidemiological studies are required to understand the geographic distribution, different genotypes and clinical pathologies of *T. orientalis*.

## 1. Introduction

Bovine theileriosis caused by *Theileria* species is one of the most important diseases of cattle in various regions of the world and causes severe economic losses [1]. *Theileria* parasites can be divided into two groups of host cell transforming and non-transforming species. Transforming species such as *Theileria parva* and *T. annulata*, are highly pathogenic and cause death whereas non-transforming species for example *T. orientalis*, are believed to cause asymptomatic disease [2]. The taxonomic classification of *T. buffeli/orientalis/sergenti* group has been discussed together for years because it is complex with similar morphologies, serology and vector transmission. Based on various biological differences, these three parasites are classified as a species, *T. orientalis* [2,3]. Recently, *T. orientalis* has emerged as an agent that can lead to clinical outbreaks of theilerosis leading to losses in the cattle industry [4–10].

Major Piroplasm Surface Protein (MPSP) is a major immunodominant protein that is expressed in large numbers on the surface of *T. orientalis* piroplasm in infected erythrocytes. Several variants of this protein were observed among the *T. orientalis* isolates. Sequence variations in the MPSP gene have been used to determine the genetic diversity of *T. orientalis* [10,11]. To date, type 1 (chitose), type 2 (ikeda), type 3 (buffeli), type 4–8 and N1-N3 genotypes have been identified based on MPSP gene sequences [2,12]. Chitose and ikeda genotypes have caused significant morbidity, economic losses and mortality in cattle in the Asia-Pacific region. Oriental theileriosis is generally characterized by pyrexia, hemolytic anemia, productivity losses and abortion in dairy and beef cattle [4–10,12].

*T. orientalis* prevalence in different regions of the Turkey has been determined between 0.9% and 13.6% by molecular techniques [13–15]. Very little information is known about *T. orientalis* genotypes in cattle from Turkey. The aim of this study was to investigate different genotypes of *T. orientalis* from cattle in Turkey.

## 2. Material and methods

### 2.1. Study area and blood sample collection

The study was conducted using 63 *T. orientalis* positive cattle in four different provinces (Tokat, Yozgat, Elazığ, Kırklareli) in Turkey. All samples were collected between 2005 and 2012 years. Blood samples (3 mL) were collected in EDTA tubes and, based on behavior and appearance, cattle were healthy at the time of sampling.

### 2.1.1. Isolation of genomic DNA and PCR amplification

Genomic DNA was extracted from 200 μl anticoagulated blood by a

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https://doi.org/10.1016/j.cimid.2019.05.015
Received 5 February 2019; Received in revised form 11 May 2019; Accepted 13 May 2019
0147-9571/

commercial kit (Vivantis, CA, USA) following the manufacturer's instructions and stored at − 80 °C until further analysis. MPSP gene of *T. orientalis* was amplified by nested PCR from genomic DNA using the primers MPSP-F/MPSP-R [16] and MPSPAJ-F/MPSP-AJ-R1 [17] described previously. Five microliters of PCR product were visualized using UV transillumination in a 1.4% agarose gel stained with jel red and the remaining products were stored at 4 °C until used in the Single-strand conformation polymorphism (SSCP).

### *2.1.2. Single-strand conformation polymorphism (SSCP) and Phylogenetic analysis*

To show sequence variation among cattle *T. orientalis* MPSP amplicons, SSCP analysis was performed [18]. Briefly, the mixture formed with 8 μL of a positive PCR product and 12 μL of denaturing buffer (10 mM NaOH, 0.05% bromophenol blue, 95% formamide, and 0.05% xylene cyanole) was denatured at 95–100 °C for 10 min. The mixture was placed on ice and loaded into 5 μL of gel from each sample. The band profiles were visualized using silver staining. Samples have different band profile directly sequenced for MPSP genes. Chromatograms were edited with Chromas Lite v. 2.1.1 (www.technelysium.com.au). MPSP gene sequences of all *T. orientalis* genotype were used for the phylogenetic analysis. A phylogenetic tree was constructed based on a maximum likelihood phylogeny with Tamura 3- parameter model and Kimura 2-parameter model (1000 bootstrap replicates). All phylogenetic analysis were conducted using MEGA 7.0 [19–21].

### *2.1.3. Theileria orientalis genotype-specific PCR assays*

*Theileria orientalis* genotype-specific PCR was performed to determine the mix genotypes. For this purpose, TSB-TSR [22], TSC-TSR [23] and TSI-TSR [24] primer pairs were used for the diagnosis of buffeli, chitos and ikeda genotypes, respectively.

## 3. Result

Nested PCR targeting the MPSP gene of *T. orientalis* generated amplification products of the expected size (approximately 344 bp) in DNA samples. SSCP analysis of individual aliquots of the 63 amplicons displayed five distinct profiles. The nucleotide sequences were registered in GenBank under the accession numbers MK415829-MK415834.

Using a number of nucleotide sequences obtained in this study, a phylogenetic tree was constructed with 42 sequences of the 11 *T. orientalis* genotype previously reported. A maximum likelihood phylogenetic analysis based on a Tamura 3-parameter model divided *T. orientalis* MPSP gene sequences into 11 genotypes as shown in Fig. 1. Result of the phylogenetic analysis to separate chitose genotypes showed that one sample, MK415832 accession number, was found in the chitose B cluster whereas two samples, MK415829 and MK415833 accession numbers, were found in the chitose A cluster (Fig. 2).

Genotype-specific PCR was performed to determine mix genotypes among samples, and 17.5% was found positive for type 1-type 3 mix. Only type 1 and type 3 were found positive were 11.1% and 46%, respectively (Table 1).

## 4. Discussion

Tick-borne diseases, especially theileriosis, are a major threat to livestock production worldwide and constitute economic losses [25]. The most important *Theileria* species leading to infection in cattle are *T. parva* and *T. annulata*, which cause East Coast fever and Tropical theileriosis respectively [2]. No information was available on the outbreaks of *T. orientalis* and related losses in cattle until the last ten years. However, in the recent years, clinical outbreaks of *T. orientalis* have been increasingly observed throughout Asia-Pacific region. Oriental theileriosis has been linked to a pathogenic genotype of the parasite such as chitos (Type 1) and ikeda (Type 2) [2,4–10,16]. In addition, the type 7 genotype has been associated with clinical outbreaks of theilerosis in India [26]. 11 genotypes were found in studies based on the MPSP gene sequences, which are abundantly expressed on the surface of the piroplasm in the infected erythrocytes and proved to be a good marker in phylogenetic and diversity studies [4–10,26,27].

Tropical theileriosis caused by *T. annulata* in cattle leads to serious problems in Turkey [28–30]. There are studies about the existence of *T. orientalis* in Turkey, but there is little information about genotypes. In this study, the existence of type 1 and type 3 genotypes in cattle has been reported. The presence of type 3 based on MPSP gene region in cattle was demonstrated by phylogenetic analysis in Turkey [13]. The presence of type 1 and type 3 was reported using genotype specific primers in Turkey. However, phylogenetic and sequence analysis was not performed in same study [14]. Type 3 (buffeli) genotype of *T. orientalis* is known as low pathogenicity, whereas type 1 (chitose) genotype leads to clinical infections and associated yield losses in cows. Type 2 (ikeda) or type 1–2 mix genotypes lead to clinical cases [31], while only type 1 genotype usually leads to subclinical infection [32]. Although some studies suggest that only type 1 is a direct cause of disease, the pathogenicity of type 1 is still less than the pathogenesis of type 2 [5,6,33]. In this study, genotypes of type 1 (11.1%), type 3 (46%) and type 1-type 3 mix (17.5%) of *T. orientalis* are shown by sequence, phylogenetic analysis and genotype specific PCR. Jenkins et al. [34], reported that chitose genotypes are divided into two groups such as chitose A and chitose B. Chitose A genotype was found to be more pathogen than chitose B, but A genotype almost always appeared with the ikeda genotype [34]. Therefore, there is little evidence that chitose A is a direct cause of clinical disease. In this study, in the phylogenetic analysis of chitose genotypes, MK415832 accession number sample in chitose B, MK415829 and MK415833 accession number samples were found to be in the chitose A genotype. In contrast to the other study, the chitose A genotype was not found to be mixed with the ikeda genotype.

Recently, live cattle have been imported to Turkey from various countries. According to the 2017 data, 17% of cattle imported into the Turkey comes from Australia [35]. Australia is one of the largest live cattle exporters in the world and they export animals mostly to Asia and the Middle East [36]. Recently, *T. orientalis* related abortions and death have seen in the pregnant dairy cattle imported from Australia [9,10]. In Australia, the pathogen genotypes of *T. orientalis* (ikeda and chitose) were observed to cause clinical cases in cattle and 624 liters of milk loss per year [8]. In order to control the spread of *T. orientalis*, tests, quarantine and prevention programs in the importing countries should be strengthened and stress factors should be eliminated [1,9,10].

The main vector of *T. orientalis* is *Haemaphysalis longicornis* in Australia [37]. *Hae. parva*, *Hae. sulcata* and *Hae. punctata* have been reported [38], however there is no any information regarding the vector compatency of *T. orientalis* in Turkey.

As a result, it is possible that *T. orientalis* has the potential to be an ongoing problem for the livestock industry in Turkey. According to the results of this study, we showed the presence of pathogenic genotype *T. orientalis* in Turkey. Therefore, extensive epidemiological studies are required to understand the geographic distribution, vector compatency, different genotypes and clinical pathologies of *T. orientalis* in imported animals.

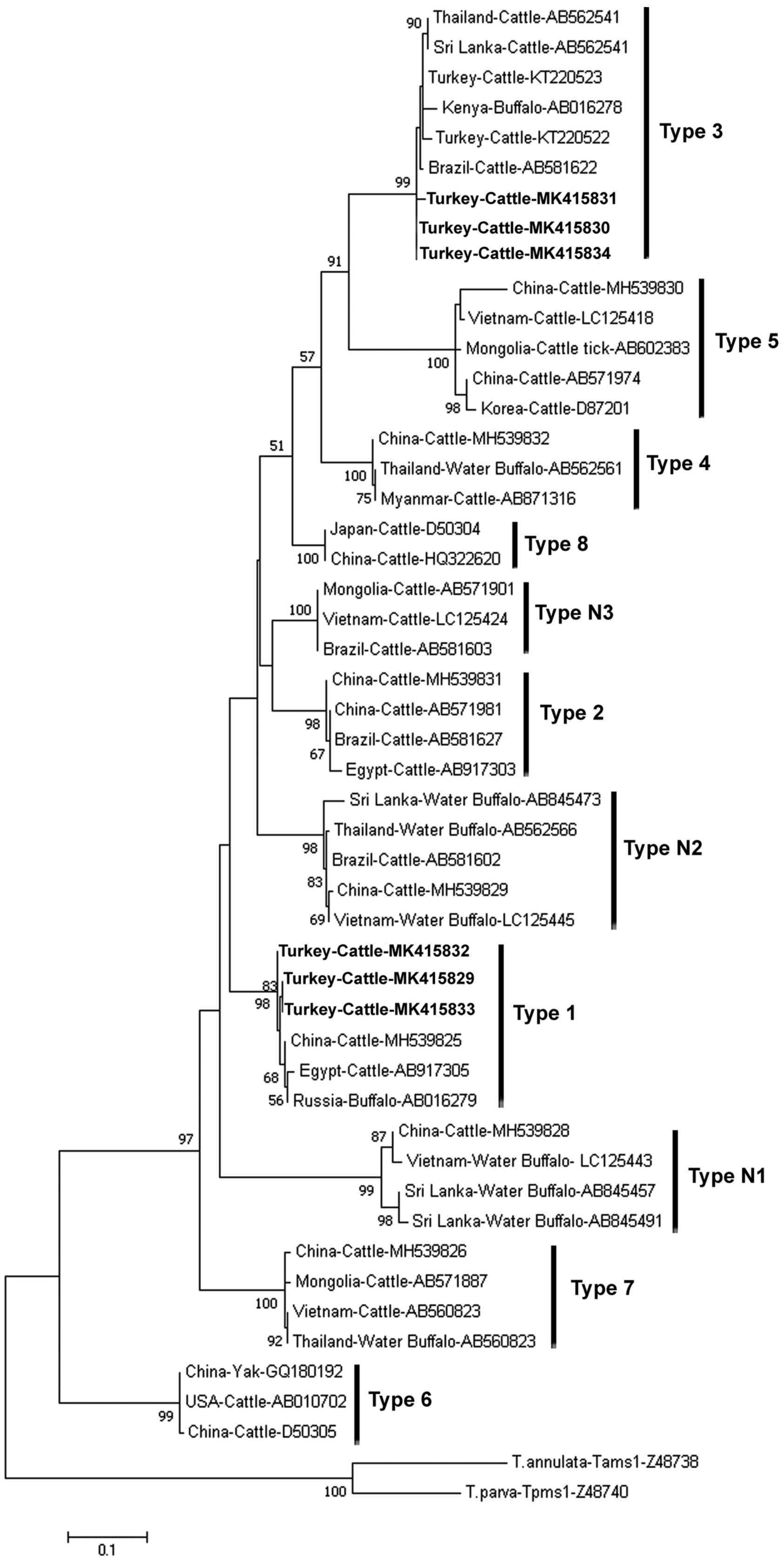

**Fig. 1.** Phylogenetic analysis by maximum likelihood using *T. orientalis* MPSP gene sequences. The evolutionary history was inferred using the Tamura 3-parameter model. The tree is shows the phylogenetic relationship of *T. orientalis* genotypes determined in this study (bold letters) with other genotypes. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 50 nucleotide sequences. All positions containing gaps and missing data were eliminated. The Tams1 gene sequence of *T. annulata* (Z48470.1) and *T. parva* (Z48738.1) were used as outgroup.

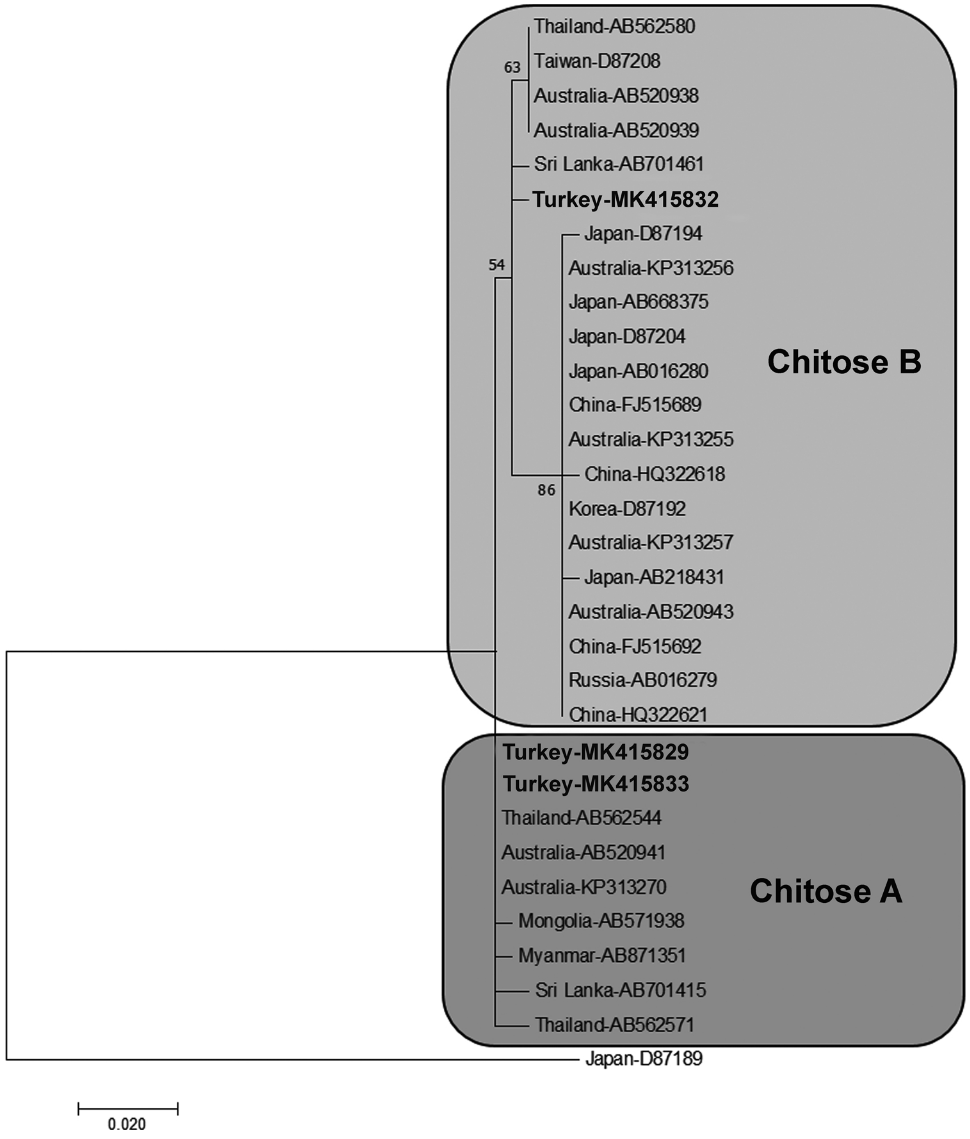

**Fig. 2.** Phylogenetic analysis by maximum likelihood using Chitose genotypes MPSP gene sequences. The evolutionary history was inferred using the Kimura 2-parameter model. The tree is shows the phylogenetic relationship of Chitose genotypes determined in this study (bold letters) with other chitose genotypes. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 38 nucleotide sequences. All positions containing gaps and missing data were eliminated.

**Table 1**
*Theileria orientalis* investigated in field collected blood samples of cattle from five provinces in Turkey.

| | No. of positive samples for *T. orientalis* genotypes | | | |
|---|---|---|---|---|
| Province | No. of positive samples | Type1 | Type3 | Type1-Type3 |
| Tokat | 15 | 2 | 12 | 1 |
| Yozgat | 18 | – | 11 | 7 |
| Elazığ | 12 | – | 4 | 8 |
| Kırklareli | 18 | 5 | 2 | 11 |
| Σ | 63 | 7 (%11.1) | 29 (%46) | 27 (%17.5) |

## Acknowledgement

This work was supported financially by a grant (VF1633) from the Unit of Scientific Research Projects (BAP), Firat University.

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