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Molecular Detection of *Theileria* species in Cattle from Jilin Province, China

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Abstract. Bovine theileriosis is a tick-borne disease that is hampering the development of the domestic cattle industry in northern China. This study involved a molecular survey of bovine *Theileria* species in 137 blood samples from cattle in the Jilin province of China. The DNA samples were screened by species-specific 18S rRNA PCR. Results revealed that 19.7% (27/137), 17.5% (24/137) and 10.9% (15/137) were found to be infected with *Theileria sinensis*, *Theileria orientalis*, respectively. *Mixed infection* was found in 8.8% (12/137). The overall detection rates of Baishan, Yanji, Jilin and Liaoyuan districts was 60.0%, 17.5%, 5.3% and 0%, respectively. There is little information on the detection and distribution of bovine *Theileria* species in northern China. Therefore, this study provides important data for understanding the epidemiology of *Theileria* species and designing appropriate approaches for the diagnosis and control of bovine theileriosis in northern China.

INTRODUCTION

Bovine theileriosis is an important tick-borne disease of domestic and wild bovine species, caused by *Theileria* species, that leads to serious economic losses throughout the world (Aparna *et al.*, 2013; Kundave *et al.*, 2014; Adjou Moumouni *et al.*, 2015; Jirapattharasate *et al.*, 2016; Zhou *et al.*, 2016). Previous studies have identified three bovine *Theileria* species in China: *T. annulata*, *T. orientali*s and *T. sinensis* (Liu *et al.*, 2010; Liu *et al.*, 2015). These parasites often cause similar clinical signs, including anemia, anorexia, fever, icterus and lymphadenectasis, in the infected host (Ko *et al.*, 2008).

T. annulata is considered to be the most pathogenic etiological agent of theileriosis worldwide, and it is prevalent in some regions of China (Wang *et al.*, 2014; Liu *et al.*, 2015). *T. orientalis* (also known historically as *Theileria sergenti* and *Theileria buffeli*) is the major vector-borne protozoan parasite of cattle in Australia, Japan and New Zealand (Watts *et al.*, 2016). It is also one of the most important diseases of cattle and water buffaloes in China, especially where native animals are introduced into an endemic area (Liu *et al.*, 2010; Qin *et al.*, 2016). *T. sinensis* has been previously reported in cattle and yaks in Gansu and Tibet Plateau Region, China, but the level of pathogenicity is unclear (Yin *et al.*, 2002; Qin *et al.*, 2016).

The traditional diagnosis of bovine theileriosis is based mainly on microscopic examination, which has limited sensitivity and specificity (Liu *et al.*, 2015). One of the important characteristics of bovine theileriosis is that, once an animal has recovered from the primary infection, they will remain a carrier for a long time. These animals show low levels of parasitemia, which is difficult to detect by a microscope

at this stage (Li *et al.*, 2014). Therefore, molecular techniques are more reliable in diagnosing chronic subclinical infections, and are the preferred methods for detecting theileriosis in domestic animals (Yu *et al.*, 2011; Cao *et al.*, 2013).

In this study, we used a PCR technique to detection *Theileria* species in cattle from Jilin Province, China. Although all species have been reported in some areas in China, there is limited information on the detection and distribution of these parasites in northern China, including Jilin Province. Therefore, to provide further information on these parasites, a molecular survey was conducted in four districts of Jilin province, China.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 137 blood samples from cattle were collected from four districts in Jilin province, China (Fig. 1), namely Liaoyuan (n = 18), Jilin (n = 19), Baishan (n = 20) and Yanji (n = 80), from 2014 to 2016. Approximately 10 ml of blood was collected from the jugular vein using vacutainer tubes with anticoagulant and samples were transported to the laboratory where they were stored at 4°C until used.

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol, and stored at –30°C until further use. All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Permit for animal experiment: 201109-7; DNA experiment: 1211-3).

Molecular detection of *Theileria* species

To detect *Theileria* species, previously described PCR assays were employed to screen all of the DNA samples (Table 1). All the amplification reactions were performed in a 20 µl reaction mixture, which contained

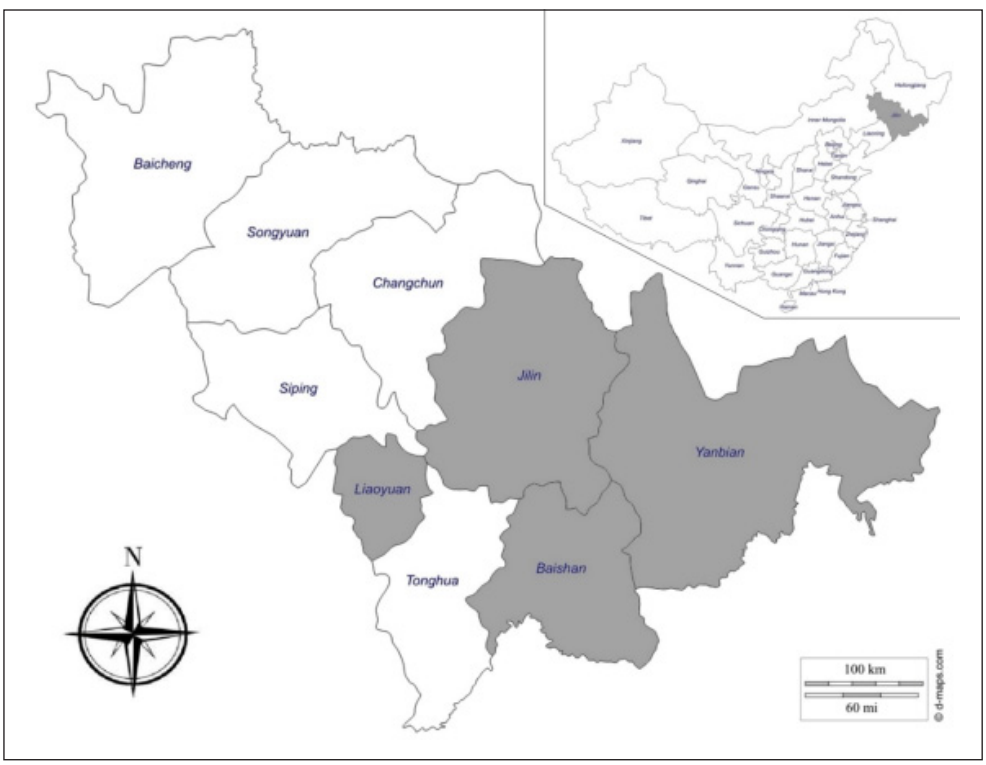

Figure 1. Map of Jilin province in China, showing locations where the blood samples were collected from cattle.

Table 1. Diagnostic PCR primers used in the present study

Pathogen	Target gene	Assay	Oligonucleotide sequences(5'→3')	Product size (bp)	Annealing temperature (°C)	Reference
Theileria spp.	18S rRNA	PCR	GAAACGGCTACCACATCT AGTTTCCCCGTGTTGAGT	778	55	Cao *et al.* (2013)
		nPCR	TTAAACCTCTTCCAGAGT TCAGCCTTGCGACCATAC	581	55	
T. annulata	Tams-1	PCR	GTAACCTTTAAAAACGT GTTACGAACATGGGTTT	721	55	Martín-Sánchez *et al.* (1999)
		nPCR	CACCTCAAAACATACCCC TGACCCACTTATCGTCC	453	60	
T. orientalis	MPSP	PCR	CTTTGCCTAGGATACTTCCT ACGGCAAGTGGTGAGAACT	776	58	Ota *et al.* (2009)
T. sinensis	MPSP	PCR	CACTGCTATGTTGTCCAAGAGATATT AATGCGCCTAAAGATAGTAGAAAAC	887	56	Liu *et al.* (2010)

4 µl of template DNA (30–150 ng/µl), 10 pmol of each primer, 250 µM concentration of each deoxynucleotide triphosphate, 2 µl of 10× Ex *Taq* buffer, and 1 unit of Ex *Taq* DNA polymerase (Takara Bio, Kyoto, Japan). Amplifications were performed using the MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) under the conditions described in Table 1. Double distilled deionized water was used as a negative control in each PCR reaction. The PCR products were checked by electrophoresis in 1.0% agarose gels. The resultant amplicons were purified with a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instruction.

Cloning and sequencing

The purified PCR products were cloned into the pGEM-T easy vector (Promega Corporation, Madison, WI, USA) and subsequently transformed into *Escherichia coli* DH5α competent cells. The transformed cells were plated immediately on pre-warmed LB agar plates supplemented with ampicillin (50 µg/ml). The positive clones were confirmed by colony PCR using gene specific primers. For each sample, three clones of a given gene were sequenced at least twice with a BigDye v3.1 Terminator Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the pUC/M13 universal primers.

Phylogenetic analysis

The eight major piroplasm surface protein (MPSP) genes of *T. orientalis* and *T. sinensis* isolates from Jilin province in China and those from other regions of the world were submitted to phylogenetic analyses using MEGA version 6.0 (Tamura *et al.*, 2013). The maximum likelihood distance method and Tamura 3 parameter with Gamma distribution (T92 + G) model were used to construct the phylogenetic tree. Bootstrap analysis with 1000 replications was used to estimate the confidence levels of the branching patterns of the trees.

Statistical analysis

The detection and confidence intervals (95%) for each species were calculated using the OpenEpi program (http://www.openepi.com/Proportion/Proportion.htm).

RESULTS

Detection of single and co-infections

The rates of infection with *Theileria* species, as detected by PCR, are presented in Table 2. The PCR result revealed that 27/137 (19.7%) of the blood samples from cattle were infected with *Theileria* species: 17.5% (24/137) with *T. sinensis* and 10.9% (15/137) with *T. orientalis*. *T. annulata* infection was not found. *Mixed infection* with *T. sinensis* and *T. orientalis* was found in 8.8% (12/137). Among the districts surveyed, the highest level of infection was found in Baishan (60.0%), followed by Yanji (17.5%) and Jilin (5.3%). *Theileria* infection was not found in Liaoyuan in this study.

Comparative analysis

The partial MPSP gene (776 bp) of *T. orientalis* obtained in this study (GenBank accession nos. KX375396–KX375399) showed 89.1%–99.5% nucleotide identity and shared 87.6%–99.6% amino acid identity with a previously published sequence from Fujian, China (AB571970). In addition, the complete MPSP gene (873 bp) of *T. sinensis* (KX375400–KX375403) showed 99.5%–99.9% nucleotide identity and shared 99.3%–99.7% amino acid identity with a previously published sequence from Dingxi in Gansu province, China (GQ180193).

Phylogenetic analysis

The phylogenetic analysis revealed that the *T. orientalis* and *T. sinensis* MPSP genes isolated in this study were classified into three clades: type 2, type 6 and type 7 (Fig. 2). The MPSP genes of *T. orientalis* isolated from Yanji (KX375398 and KX375399) were located in type 2 and were closely related to sequences previously reported from Brazil (AB581627), Egypt (AB917303) and Fujian province in China (AB571970). In addition, the MPSP gene sequences of *T. orientalis* obtained from Baishan (KX375397) and Jilin (KX375396) in this study were closely related with those in type 7 previously isolated from India (EU700057), Indonesia (AF102500), Thailand (KT460098) and Mongolia (AB571887). Type 6 was formed from nine *T. sinensis* MPSP gene isolates, including four isolates obtained in this study, from Jilin (KX375400), Baishan (KX375401) and Yanji (KX375402 and KX375403), which were closely related to sequences previously isolated from China (D50305, GQ180193, KU518038 and AF236093) as well as an isolate from the USA (AB010702).

DISCUSSION

Bovine *Theileria* species are usually found as co-infections in field samples and cause important health and management problems which lead to reduced production of milk and meat (Liu *et al.*, 2015). To limitations of clinical management and diagnosis, prevention and control of bovine theileriosis are difficult to achieve worldwide Additionally, the parasites are carried for a long time by individuals with low levels of parasitemia, which is difficult to detect by microscopy (Li *et al.*, 2014). Therefore, it has been suggested that the bovine theileriosis should be periodically screened by PCR method (Mohammad Al-Saeed *et al.*, 2010; Yu *et al.*, 2011; Sivakumar *et al.*, 2012).

T. sinensis and *T. orientalis* have been reported previously in some regions of China. However, *T. sinensis* has only been reported in Qinghai, Gansu, Ningxia, Sichuan, Yunnan and Tibet; these regions are 1600–4200 m above sea level (Liu *et al.*, 2010; Liu *et al.*, 2015; Qin *et al.*, 2016). This is the first report of *T. sinensis* in a region of northern China with lower altitude. In this study, the PCR result revealed that 27 out of 137 cattle blood samples were infected with *T. sinensis* and *T. orientalis*. When compared with a previous report from another region, this result revealed different levels of detection (Qin *et al.*, 2016). On the other hand, *T. annulata* infection was not found in the present study, but it has been widely reported in neighboring countries and in the southern region of China (Khan *et al.*, 2013; George *et al.*, 2015; Liu *et al.*, 2015; Jirapattharasate *et al.*, 2016). The results suggest that the prevalence of different species of bovine theileriosis may be influenced by the distributions of different ticks (Yu *et al.*, 2015).

Table 2. The detection (%) and 95% CI (lower, upper intervals) of bovine *Theileria* spp. in cattle from Jilin Province, China

	Liaoyuan (n = 18)		Jilin (n = 19)		Baishan (n = 20)		Yanji (n = 80)		Total (n = 137)	
	No. positive	95% CI	No. positive	95% CI	No. positive	95% CI	No. positive	95% CI	No. positive	95% CI
Single infection										
T. orientalis	-0	0 (0–17.59)	0	0 (0–16.82)	0	0 (0–16.11)	3	3.8 (1.28–10.45)	3	2.2 (0.75–6.24)
T. sinensis	0	0 (0–17.59)	0	0 (0–16.82)	8	40.0 (21.88–61.34)	4	5.0 (1.96–12.16)	12	8.8 (5.08–14.69)
Mixed infection										
T. orientalis + *T. sinensis*	0	0 (0–17.59)	1	5.3 (0.94–24.64)	4	20.0 (8.07–41.60)	7	8.8 (4.30–16.98)	12	8.8 (5.08–14.69)
Single and mixed infections										
T. orientalis	0	0 (0–17.59)	1	5.3 (0.94–24.64)	4	20.0 (8.07–41.60)	10	12.5 (6.93–21.50)	15	10.9 (6.75–17.28)
T. sinensis	0	0 (0–17.59)	1	5.3 (0.94–24.64)	12	60.0 (38.66–78.12)	11	13.8 (7.86–22.97)	24	17.5 (12.06–24.74)
Total positive	0	0 (0–17.59)	1	5.3 (0.94–24.64)	12	60.0 (38.66–78.12)	14	17.5 (10.72–27.26)	27	19.7 (13.91–27.16)
Negative samples	18	100 (82.41–100)	18	94.7 (75.36–99.06)	8	40.0 (21.88–61.34)	66	82.5 (72.74–89.28)	110	80.3 (72.84–86.09)

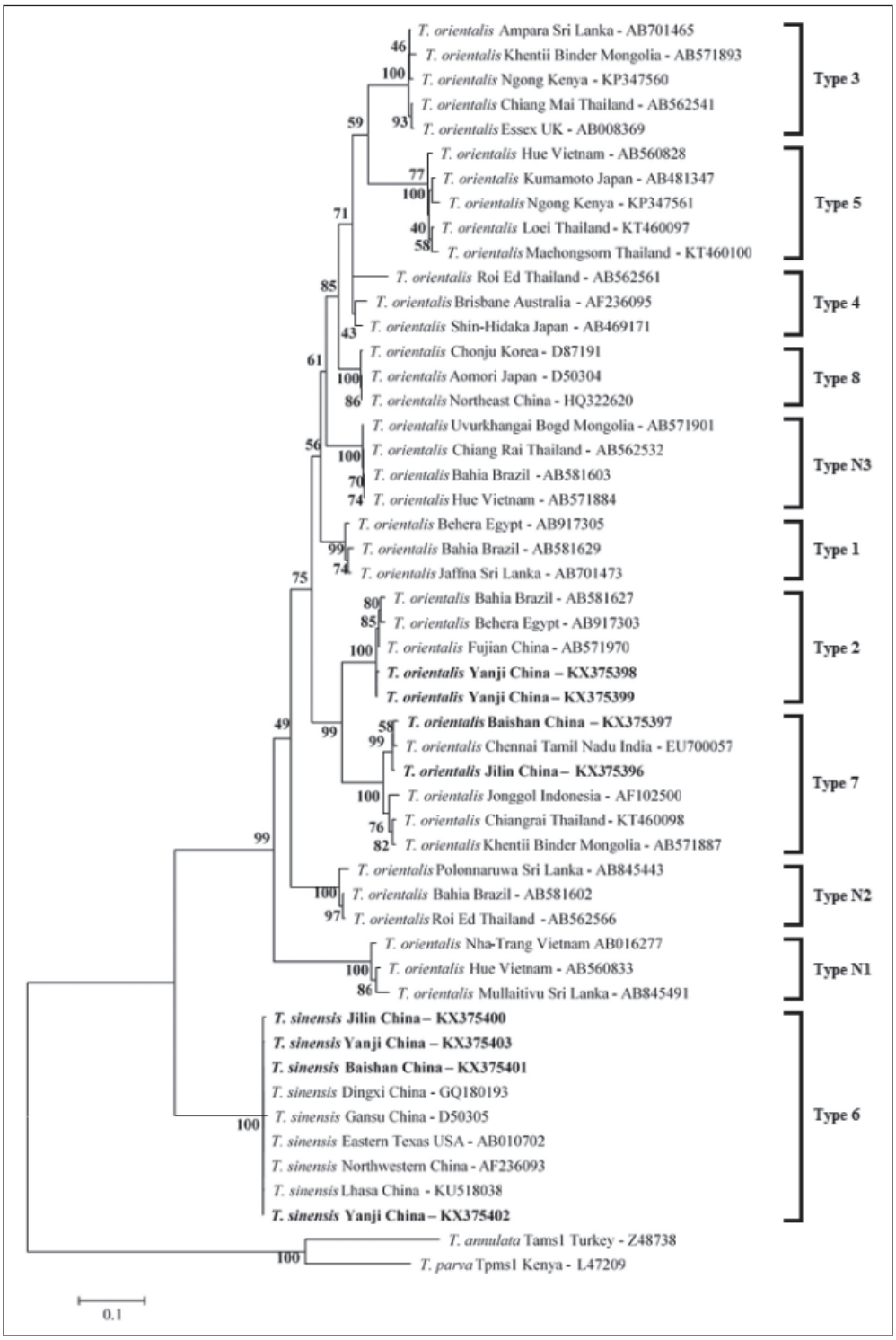

Figure 2. Phylogenetic analysis of *Theileria* species based on the MPSP gene sequences. The tree was constructed with the maximum likelihood method using the Tamura 3 parameter with Gamma distribution (T92 + G) model in the MEGA version 6.0. The sequences determined in this study are shown in bold font. Numbers at nodes represent the percentage occurrence of clades in 1000 bootstrap replications of the data. The *Tams1* gene sequence of *T. annulata* (Z48738) and the *Tpms1* gene sequence of *T. parva* (L47209) were used as outgroup.

Among the districts surveyed, the highest level of *Theileria* infection was found in Baishan (60.0%), which was obviously higher than the samples collected in Yanji (17.5%) and Jilin (5.3%). The level of *Theileria* infection in Baishan may be related to its location. Baishan is located in the Changbai mountain area, tick species and tick quantities may be higher than other districts (Geng *et al.*, 2010). *Theileria* infection was not found in the 18 cattle blood samples collected in Liaoyuan in this study. Given the limited number of samples, the reason for this result is unclear. Therefore, further investigation of samples from this region may help to clarify this perspective.

Phylogenetic analysis revealed that the *T. orientalis* and *T. sinensis* MPSP gene sequences obtained in this study were classified into three clades (type 2, type 6 and type 7). This confirmed that the MPSP gene is a polymorphic antigen and shows wide diversity among different field isolates (Sivakumar *et al.*, 2014). This is why the MPSP gene has been recognized as a useful marker for epidemiological and phylogenetic studies of *Theileria* species in many countries (Adjou Moumouni *et al.*, 2015; Jirapattharasate *et al.*, 2016; Zhou *et al.*, 2016).

In conclusion, our study involved a molecular survey of *Theileria* species in cattle from four districts of Jilin province in China. It also provided molecular evidence on the prevalence and geographical distribution of *Theileria* parasites in northern China. The findings underline the importance of introducing effective prevention and control strategies throughout the country to minimize the infection of cattle with *Theileria* parasites in China.

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