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Molecular prevalence of *Theileria* in sheep in Jilin province, China

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ABSTRACT

The aim of this study was to enhance our understanding of ovine theileriosis and its timely prevention in sheep. We therefore conducted an epidemiological study in four areas in Jilin province, China. We extracted DNA from 95 blood samples from sheep and analyzed them by nested polymerase chain reaction targeting the 18S RNA gene of ovine *Theileria* spp. Positive samples were further analyzed by PCR using species-specific primer sets for *T. luwenshuni*, *T. uilenbergi,* and *T. ovis*, respectively, to detect and differentiate among these three *Theileria* spp. Phylogenetic analysis of positive samples based on 18S rRNA gene sequences of *Theileria* spp. was also conducted. *Theileria* spp. was prevalent in all four investigated areas, with a positivity rate of 18.9%. *T. luwenshuni* was universally prevalent with a positivity rate of 11.6%, whereas no *T. uilenbergi* or *T. ovis* infections were detected in these regions. Phylogenetic analysis showed that the positive samples in all four regions belonged to the *T. luwenshuni* cluster. This survey confirmed the epidemiology of ovine theileriosis and provided important data to support the prevention and control of this epidemic disease in northeastern China.

Keywords: Theileriosis; China; *Theileria* spp.; sheep

Ovine and caprine theileriosis is a tick-borne hemozoon disease caused by *Theileria* spp. parasites, which infect macrophages, lymphocytes, and erythrocytes in sheep and goats (Schnittger et al., 2000; Yin et al., 2004). In chronic cases, sheep and goats generally suffer from developmental delay and reduced production of meat and wool, resulting in significant losses in the sheep industry worldwide (Li et al., 2014a; Tian et al., 2014). There have been numerous reports of ovine and caprine theileriosis in small ruminants in central and coastal areas of China, though only three *Theileria* spp. (*T. ovis, T. luwenshuni*, and *T. uilenbergi*) have been found in the reported areas (Li et al., 2010; Li et al., 2011). *T. luwenshuni* and *T.*

uilenbergi are highly pathogenic (Li et al., 2014b). A previous study using nested polymerase chain reaction (nPCR) and sequencing (Cao et al., 2013) reported that ovine theileriosis was endemic in Jilin province, with the causative agent being *T. luwenshuni*. But this report lacks the species-specific PCR assay for screening the each ovine *Theileria* spp. In the current study, we investigated the molecular prevalence of *Theileria* parasite infections in sheep in Jilin province, China, using universal nPCR and species-specific PCR to clarify the epidemic status of *Theileria* in this area.

Blood samples were collected from 95 sheep from four regions of Jilin province (Helong, Longjing, Hunchun, and Nong'an) in April 2017 (Fig. 1). All the sheep were clinically healthy and less than 1 year old. Blood was collected from the jugular vein of each sheep into a vacutainer tube containing an anti-coagulant (EDTA). Genomic DNA was extracted from whole blood samples using a genomic DNA extraction kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions, and 50 μl of each DNA solution was obtained and stored at –30°C.

Fig. 1. Sample-collection regions in Jilin province, China

The PCR primer sets used for the amplification of *Theileria* DNA are listed in Table 1. The universal primers 989/990 and TsppF1/TsppR1 were used to identify *Theileria* parasites, with the 989/990 primer acting as an outer primer and the Tspp F1/Tspp R1 primer as an inner primer in nPCR (Allsopp et al., 1993; Cao et al., 2013). The species-specific primer sets Tluw310/Tluw680, Tuil310/Tuil680, and Tssr170F/Tssr670R were used to identify *T. luwenshuni*, *T. uilenbergi*, and *T. ovis*, respectively, as described previously (Altay et al., 2005; Yin et al., 2008). The PCR reaction was performed in a 20 μl reaction mixture containing 2 μl of 10× PCR buffer, 200 μM of each dNTP, 1 unit of *Taq* DNA polymerase (Jialan Biotechnology, Beijing, China), 0.5 μM of forward and reverse primers, and 2 μl of the DNA sample for PCR or 1 μl of the PCR product for nPCR. Distilled water was used for the negative control. The PCR conditions used for each parasite species were as described previously (Allsopp et al., 1993; Altay et al., 2005; Cao et al., 2013; Yin et al., 2008). In the present study, 18.9% of the 95 samples were positive for *Theileria* spp. based on nPCR with the primers 989/990 and Tspp F1/Tspp R1. *Theileria* spp. was identified in sheep from all the investigated regions. *T. luwenshuni* was common in Jilin province, with positivity rates

of 2.9%–38.5% in Helong, Longjing, Nong'an, and Hunchun regions (Table 2). This was lower than the 88.0%–100% positivity previously reported in central China (Li et al., 2014b). However, all 95 sheep blood samples were negative for *T. uilenbergi* and *T. ovis*, which was consistent with a previous report (Li et al., 2014b). The variation in positivity rates in different parts of China may be related to differences in the tick vectors. *T. luwenshuni* can be transmitted by *Haemaphysalis qinghaiensis* and *H. longicornis* (Li et al., 2007; Yin et al., 2002), but we only found *H. longicornis* in Jilin province, with no evidence of *H. qinghaiensis* (Liu et al., 2016; Wei et al., 2016). Furthermore, the positivity rate in the current study was also lower than in a previous epidemiological survey of *Theileria* spp. in Jilin province (Cao et al., 2013). This may have been due to differences in the timing of blood collection. Theileriosis is a tick-bone disease and is therefore affected by the number of ticks in the environment. In the current experiment, the blood samples were collected in April before the tick numbers were at their peak.

Table 1. Primer sets used for the amplification of *Theileria* spp. 18S rRNA

Target	Assay	Sequences (5'-3')	Fragment (bp)	Annealing temperature	Reference
Theileria spp.	PCR	989: AGTTTCTGACCTATCAG 990: TTGCCTTAAACTTCCTTG	1033	50	Allsopp et al., 1993
	nPCR	Tspp F1: GAAACGGCTACCACATCT Tspp R1: AGTTTCCCCGTGTTGAGT	778	55	Cao et al., 2013
T. luwenshuni	PCR	Tluw310: GGTAGGGTATTGGCCTACTGA Tluw680: TCATCCGGATAATACAAGT	380	57	
T. uilenbergi	PCR	Tuil310: GGTAGGGTATTGGCCTACCGG Tuil680: ACACTCGGAAAATGCAAGCA	380	55	Altay et al., 2005; Yin et al., 2008
T. ovis	PCR	TSsr170F: TCGAGACCTTCGGGT TSsr670R: TCCGGACATTGTAAAACAAA	520	60	

Table 2. Regional prevalence of different *Theileria* spp.

Regions	*Theileria* spp.	*T. luwenshuni*	*T. uilenbergi*	*T. ovis*
Helong	2.9%（1/34）	2.9%（1/34）	0	0
Longjing	17.9%（5/28）	14.3%（4/28）	0	0
Hunchun	38.5%（5/13）	38.5%（5/13）	0	0
Nong'an	35.0%（7/20）	5.0%（1/20）	0	0
Total	18.9%（18/95）	11.6%（11/95）	0	0

The positivity rates of *Theileria* spp. overall and of *T. luwenshuni* were the same in Helong and Hunchu. However, the positivity rates of *Theileria* spp. in Longjing and Nong'an regions were higher than the positivity rates of *T. luwenshuni*, suggesting that the nPCR may have been more sensitive than the conventional PCR, or that other *Theileria* spp. were present but not detected. Previous reports have indicated that *T. luwenshuni*, *T. uilenbergi*, and *T. ovis* represent the main *Theileria* spp. in sheep in China (Li et al., 2011) and we therefore targeted these three species in the current study. However, further studies are needed to assess the presence and prevalence of other *Theileria* spp. in Longjing and Nong'an in the future.

We also cloned one random nPCR-positive sample based on TsppF1/TsppR1 from each geographic region into the pMD19-T vector (Takara Biotechnology, Dalian, China) and selected three clones for sequencing by BGI (Beijing, China). The obtained sequences were deposited in GenBank (accession numbers MG574948–MG574951), and the sequence similarities were analyzed using the GenBank nucleotide BLAST program and Lasergene software. The partial *Theileria* spp. 18S rRNA gene sequences from the Jilin province isolates shared 99.4%–99.5% nucleotide sequence identities with those of *T. luwenshuni* (accession numbers LC326005, KU518032, JF719831, KC414096, AY262117, KU356908). Phylogenetic analysis of the sequenced *Theileria* spp. 18S rRNA gene was performed by the neighbor-joining program using CLUSTAL X software, and the bootstrap probabilities of each node were calculated with 1,000 replications. The results showed that all of the newly identified *Theileria* spp. was in the same cluster, and classified into the same clade as *T. luwenshuni* with a previous published *Theileria* spp. sequence (KC414096) in Jilin province, China (Fig. 2).

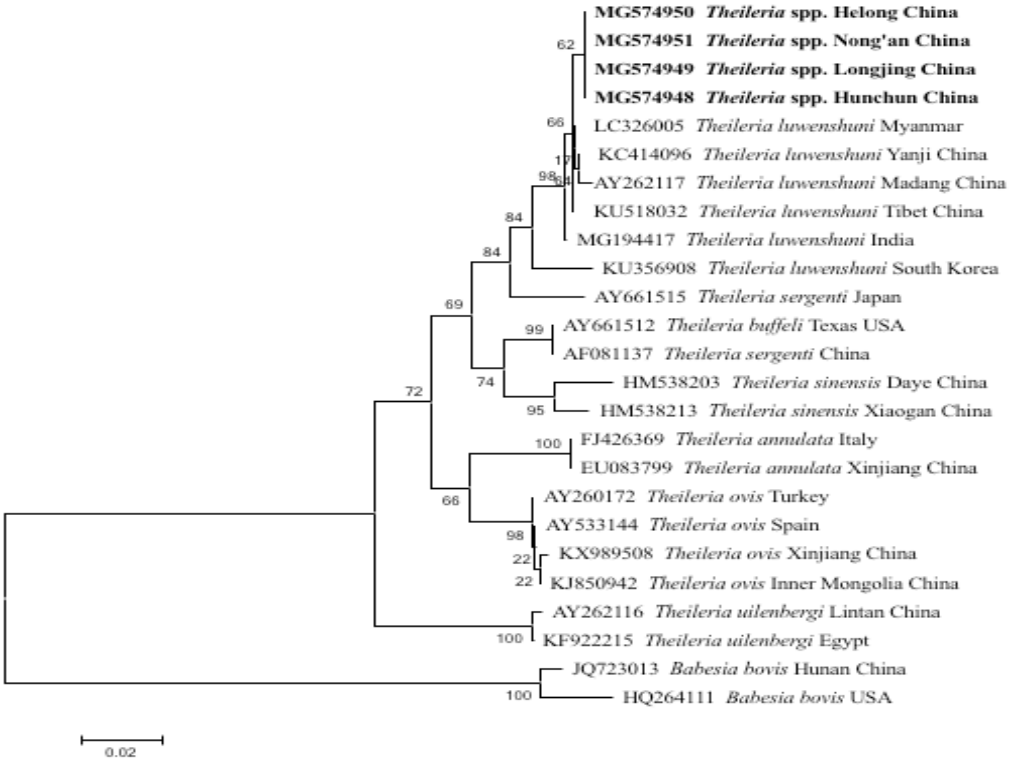

Fig. 2. Phylogenetic analysis of *Theileria* spp. 18S rRNA gene sequences

Ovine and caprine theileriosis is currently common in many provinces in China (Cao et al., 2013; Ge et al., 2012; Li et al., 2014b). Cao et al. (Cao et al., 2013) previously investigated the molecular prevalence of *Theileria* spp. in Jilin province directly using nPCR with universal primers for *Theileria* spp. In the current study, *T. luwenshuni*, *T. uilenbergi* and *T. ovis* infections were evaluated in sheep in Jilin province, China. *T. luwenshuni* was identified in all four investigated regions, while neither *T. uilenbergi* nor *T. ovis* were detected in these areas.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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