

## Molecular Detection of *Theileria* Species in Sheep from Northern China

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**ABSTRACT.** Ovine theileriosis is a tick-borne disease that restricts the development of small ruminant husbandry in northern China. In this study, we report on a molecular epidemiological survey of ovine *Theileria* spp. in 198 blood samples taken from sheep in northern China. The DNA samples were screened by a nested polymerase chain reaction (PCR) targeting the 18S rRNA gene of ovine *Theileria* spp. The prevalence of ovine *Theileria* spp. in Yanji, Nongan, Longjing, Toudao and Jinchang was 80%, 40%, 37%, 24% and 32%, respectively. The sequencing analyses approved the present of the *T. orientalis* and/or *T. luwenshuni* in these regions. Taken together, we have demonstrated a high incidence of *Theileria* spp. in northern China that calls for the need to design effective control programs for ovine theileriosis.

**KEY WORDS:** China, ovine *Theileria* spp., sheep, *Theileria luwenshuni*.

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Ovine theileriosis is an important tick-borne disease of sheep in the tropical and subtropical regions of the world caused by *Theileria* parasites. Among ovine *Theileria* species, *Theileria lestoquardi*, *T. luwenshuni* and *T. uilenbergi* are considered highly pathogenic [19], and other species, *T. ovis*, *T. recondita* and *T. separata*, are considered to be benign and cause subclinical infection in small ruminants [2, 5]. Recently, five ovine *Theileria* species have been observed in China, including *T. luwenshuni*, *T. ovis*, *T. separata*, *T. lestoquardi* and *T. uilenbergi* [19]. *T. luwenshuni* and *T. uilenbergi*, the newly identified *Theileria* spp. (China 1) and *Theileria* spp. (China 2), represent a major constraint to the development of sheep and goat husbandry in northwestern China [17, 18]. Hence, an epidemiological survey of ovine theileriosis is necessary to control the disease in these areas. The microscopic examination as gold standard for diagnosis of *Theileria* infection remains the convenient technique for day-to-day diagnosis of clinical cases in the laboratory, but for the detection of carrier animal containing low parasitemia. Recently, polymerase chain reaction (PCR) has been developed for detecting *T. ovis* and *T. annulata* infection with high sensitivity and specificity [3, 11].

The 18S small subunit ribosomal RNA (18S rRNA) gene has been shown to be an effective marker for investigation in *Theileria* spp [1]. The 18S rRNA genes have been successfully applied to identify and classify several previously unknown *Theileria* spp., especially the benign *Theileria* spp. which infects ruminants [14, 18]. Thus, the nested PCR

based on the 18S rRNA gene is an alternative molecular diagnostic tool that enables detection of mixed infections of ovine *Theileria* spp. [17]. The 18S rRNA gene could be a reliable molecular marker for studies on epidemiological surveys of ovine *Theileria* spp. In this study, we examined 198 ovine DNA samples originating from northern China using a nested PCR based on the 18S rRNA gene. Furthermore, the sequence analyses of the 18S rRNA gene were used to differentiate the ovine *Theileria* spp. and determined the phylogenetic status of the parasites.

The fieldly collected blood samples of the asymptomatic sheep (n=198) were obtained from 5 geographical areas in 2 northern provinces of China, namely, Yanji (15 samples), Nongan (20 samples), Longjing (54 samples), Toudao (37 samples) and Jinchang (72 samples) as shown in Fig. 1.



Fig. 1. Regions for collecting samples in northern China.

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Table 1. The results of nested PCR method showing positive results for ovine *Theileria* spp. in sheep in northern China

Province	Region	Total number of collected samples	Number of positive samples (%)	Isolated number of <i>T. orientalis</i>	Isolated number of <i>T. luwenshuni</i>
Jilin	Yanji	15	12 (80)	1	5
	Nongan	20	8 (40)	6	0
	Longjing	54	20 (37)	4	2
	Toudao	37	9 (24)	4	2
Gansu	Jinchang	72	23 (32)	3	3

The age of the sheep was more than 1 year and reared under open system management. Blood sampling was performed during the active tick season in May 2011. The climate in northern China has warm summers and cold winters, and the main incidence of theileriosis occurred from March to May, which was closely related to the temperature in spring [6]. Approximately 5 ml of blood was collected from either the caudal or jugular vein using vacutainer tubes with EDTA and transported to a laboratory for further analyses. Genomic DNA extraction was performed using a QIAamp<sup>®</sup> DNA Blood (Qiagen, Hilden, Germany) on the basis of the manufacturer's instructions. A volume of 200  $\mu$ l of whole blood was used for DNA extraction, and 100  $\mu$ l eluted DNA samples (approximately 6  $\mu$ g/ $\mu$ l) were obtained and stored at  $-30^{\circ}\text{C}$  for subsequent PCR analysis. A nested PCR was used to detect ovine *Theileria* spp. DNAs. The outer primer, F1 (5'-GAAACGGCTACCACATCT-3') and R1 (5'-AGTTTCCCCGTGTTGAGT-3'), amplified a 778-bp fragment from the 18S rRNA gene of the conserved sequence of *Theileria* spp., while the inner primer, F2 (5'-TTA-AACCTCTTCCAGAGT-3') and R2 (5'-TCAGCCTTGC-GACCATAC-3'), amplified a 581-bp fragment.

PCR was performed in a 20  $\mu$ l total reaction volume containing 3  $\mu$ l DNA, primers (20 pg), dNTP (250  $\mu$ M of each deoxynucleotide triphosphate), a 10  $\times$  PCR buffer (100 mM Tris-HCl, 500 mM KCl and 1% Triton X-100) and *Taq* polymerase (1.25 U, Promega, Madison, WI, U.S.A.); the mixture was then subjected to cycling conditions. The amplification conditions were done as follows:  $95^{\circ}\text{C}$  for 5 min to activate the *Taq* DNA polymerase, followed by 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 1 min, annealing at  $55^{\circ}\text{C}$  for 1 min and extension at  $72^{\circ}\text{C}$  for 90 sec. A final extension was done at  $72^{\circ}\text{C}$  for 10 min. Two microliters of the PCR amplicons were used for the subsequent nPCR using the same amplification conditions described above. Following gel electrophoresis, detection of a 581-bp fragment under ultraviolet light was considered positive for *Theileria* spp. Analyses of 198 ovine blood samples obtained from five different regions of northern China revealed that 80% (12/15) in Yanji, 40% (8/20) in Nongan, 37% (20/54) in Longjing, 32% (23/72) in Jinchang and 24% (9/37) in Toudao were infected by *Theileria* spp. (Table 1). These results indicated that the incidence of ovine theileriosis was different regionally.

Furthermore, the obtained PCR products were purified using a commercial kit (Qiagen) and cloned into the

pMD18-T vector (Takara, Otsu, Japan) described in our previous study [4]. From each isolate, at least three clones were sequenced using the ABI BigDye<sup>™</sup> Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster, CA, U.S.A.), and analyzed on an ABI PRISM 377 DNA sequencer. The representative sequences obtained in this study were registered in the GenBank database, and the phylogenetic analyses of 18S rRNA gene sequences were performed by the neighbor-joining program using CLUSTAL X. Homology search and alignment of the sequenced 18S rRNA gene of both *T. luwenshuni* and *T. orientalis* fragments revealed that the gene is conserved among the isolates in the 5 regions of northern China. The partial *T. luwenshuni* 18S rRNA gene sequences of the northern China isolates shared 100% and 99.7% nucleotide sequence identity with those of *T. luwenshuni* Madang and Ningxia China isolated strains (Accession no. JF719831 and AY26118). Similarly, the partial *T. orientalis* 18S rRNA gene sequences of the northern China isolates shared 100% nucleotide sequence identities with the Australia isolate (Accession no. AB520956). Furthermore, the haplotype diversity, nucleotide diversity ( $\pi$ ), average number of pairwise differences of nucleotides ( $\kappa$ ) and minimum number of recombination events were analyzed using the DnaSP version 4 [12]. The 18S rRNA gene of ovine *Theileria* spp. showed that the nucleotide diversity per site,  $\pi$ , was 0.0293, and the average number of nucleotide differences,  $\kappa$ , was 14.034. The results revealed that the 18S rRNA gene of ovine *Theileria* spp. was conserved in the partial sequences. Additionally, statistical analysis revealed that the infection rate of theileriosis has no significant difference related with ages and sex at a level of  $P > 0.05$ .

Phylogenetic analysis of the sequenced *Theileria* spp. 18S rRNA gene revealed that *T. luwenshuni* and/or *T. orientalis* was observed among the 5 regions of northern China. As shown in Fig. 2, the isolates of Yanji, Longjing, Toudao and Jinchang were obviously separated with the other ovine *Theileria* spp. on the phylogenetic tree and formed a cluster with the *T. luwenshuni* gene sequences previously isolated from sheep in other regions of China. The 5 isolated sequences from Yanji, Nongan, Longjing, Toudao and Jinchang formed a cluster with *T. orientalis* isolated from Australia cattle. *T. orientalis* is the major species that causes bovine theileriosis and has prevalence in northern China. The 18S rRNA gene sequences of *T. orientalis* isolated from sheep or goats still remain unavailable in the GenBank database. Therefore, a further study will be required for identifying the genotype

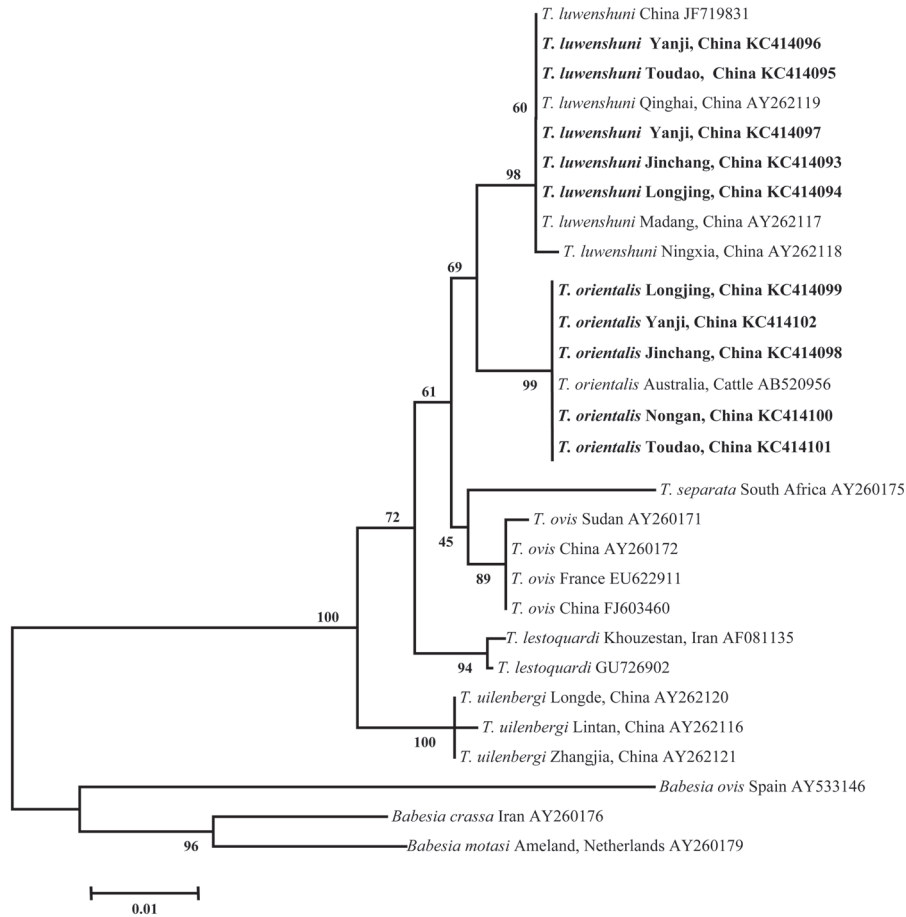


Fig. 2. Dendrogram based on the 18S rRNA gene partial sequences of *T. orientalis* and *T. luwenshuni* and previously registered sequences in the GenBank database. Phylogenetic tree showing the relationship of the genotypes identified in this study with previously registered sequences in GenBank database of *Theileria* spp.

of *T. orientalis* that causes ovine theileriosis and then understanding the geographical relationships among the genotypes. In addition, it will be difficult to exclude the other *Theileria* spp. existing in sheep in these regions.

*T. orientalis* is a member of the generally benign *Theileria* group (*T. orientalis/sergenti/buffeli*), which is locally known as *T. sergenti* in China, Japan, Korea and Russia [8, 15]. *T. sergenti* is an invalid name from a taxonomic viewpoint, since it has been used to previously describe a parasite of sheep [9, 16]. Recently, some genotypes of *T. orientalis* that affected cattle have been identified in some countries, such as China [10], Thailand [13], Vietnam [7] and Japan [20]. However, little information was available for the occurrence of *T. orientalis* in sheep and goats. In this report, molecular detection demonstrated the presence of *T. orientalis* in domestic sheep of northern China for the first time, suggesting that *T. orientalis* infection of sheep is endemic in these areas. On the other hand, *Theileria* spp. 1 (China) and *Theileria* spp. 2 (China) have been recently reported in northern China

[19]. They were discriminated from other *Theileria* spp. according to the 18S rRNA gene sequences and named *T. luwenshuni* and *T. uilenbergi*, respectively [19]. They are considered to be pathogenic parasites for small ruminants. In this study, the results confirmed that *T. luwenshuni* was detected and distributed in northern China. *T. uilenbergi* was not detected in these samples. The results indicate that *T. uilenbergi* may not be prevalent in these regions. Therefore, the specific primers for each ovine *Theileria* spp. should be used for screening more samples in other areas, which could assist with developing a meticulous distribution map of each ovine *Theileria* spp.

In conclusion, our results indicated that at least 2 species of *Theileria* currently exist in sheep in northern China. Furthermore, the present study revealed that very high proportions of sheep in the sampling populations harbor *T. luwenshuni* or *T. orientalis* in their blood stream with six districts having over 23% *Theileria* spp.-positive sheep. It suggests that *T. luwenshuni* and *T. orientalis* may be present

in these 5 regions rather than other ovine *Theileria* spp. In addition, this is the first report of *T. orientalis* infection by molecular and epidemiological studies in sheep of northern China. Moreover, mixed infections of *T. orientalis* and *T. luwenshuni* were observed in these regions, indicating that the small ruminant industry faces a real threat from *Theileria* spp. infection. Therefore, further large-scale investigations are necessary to provide essential information about the geographical distributions, host specificities and clinical pathologies of *Theileria* spp. in order to develop diagnostic and control measures of ovine theileriosis.

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