NOTE *Parasitology*

Epidemiological Survey of *Theileria* Parasite Infection of Cattle in Northeast China by Allele-Specific PCR

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ABSTRACT. An epidemiological survey on a *Theileria* parasite infection of cattle in Northeast China was carried out using allele-specific PCR and DNA sequence analysis of the major piroplasm surface protein (MPSP) gene. The results showed that 14 of 104 blood samples were positive for *Theileria* by PCR. Among the positive cases, co-infection with various combinations of C- and I-type parasites was detected in 12 samples; no B- and Thai-type parasites were detected by allele-specific PCR. Phylogenetic analysis based on the MPSP gene sequences revealed that *Theileria* parasites with the MPSP types 1, 2, and 4 were distributed in Northeast China.
KEY WORDS: major piroplasm surface protein, PCR, *Theileria*.

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Theileria parasites transmitted by ticks, which are widespread among cattle all over the world, occasionally cause anemia and icterus [1, 5, 8, 14, 16]. *T. orientalis* is a member of the relatively *Theileria* group, which is often referred to as *Theileria sergenti/buffeli/orientalis* [7, 12]. The major piroplasm surface protein (MPSP) is a conserved protein that is abundantly expressed in the intraerythrocytic stage of all *Theileria* species [1, 13]. The nucleotide sequences of MPSP genes were used in epidemiological and phylogenetic research [1, 4–9, 12, 17]. Four allelic MPSP gene types were determined, namely, the C-type, I-type, B-type, and Thai-type, which were originally designated from the 32 kDa protein (p32) of a Japanese *T. orientalis* Chitose isolate, the 33 kDa protein (p33) of a Japanese *T. orientalis* Ikeda isolate, the 34 kDa protein (p34) of Australian *T. buffeli* (Warwick), and Thai *T. sp.* (Kamphaeng Saen), respectively [5, 9, 13]. Kubota *et al*. [10] developed allele-specific PCR to differentiate parasite populations. Using this allele-specific PCR method, the distribution of *T. orientalis* parasites within isolates in Western China was tested, and the results showed that the major populations of the parasites distributed were the C-, I-, and B-types [4]. However, the distribution of *Theileria* parasites in Northeast China has not been fully studied. Although local veterinarians had often detected *Theileria* infection of domestic animals through classical microscopic examination of Giemsa-stained blood smears, species identification of the parasites had not been performed. In the present study, we characterized field isolates of *Theileria* parasites from cattle in Northeast China using allele-specific PCR and DNA sequence analysis of the MPSP gene.

A total of 104 blood samples were collected in Northeast China in May 2010; 56 beef cattle blood samples were collected from two farms in Hunchun, and 48 dairy cattle blood samples were collected from a farm in Dunhua. Beef cattle in the farms were pastured with approximately 1.8 cattle per hectare, while dairy cattle were maintained indoor-housing with approximately 1.2 cattle per 10 m^2. No apparent clinical signs were observed on all sampled cattle by macroscopic examination. The Hunchun and Dunhua areas, located in Jilin Province of Northeast China, are near Russia and North Korea (Fig. 1). The climate is monsoon oceanic,

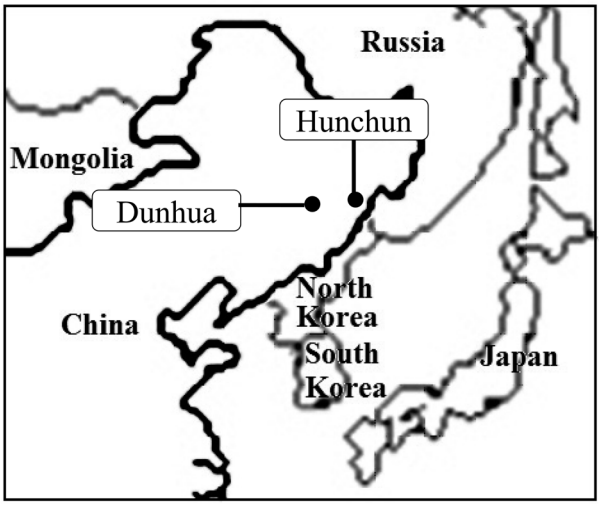

Fig. 1. Regions for collecting samples in China.

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Table 1. Oligonucleotide sequences and primer sets used for the amplification of the MPSP genes of benign *Theileria* parasites

Primer	Sequences (5'–3')
Sense primers	
Ts-U	CACGCTATGTTGTCCAAGAG
Ts-I	AAGGATCCGTCTCTGCTACCGCCGC
Ts-C	GCGGATCCTCATCGTCTCTGCAACT
Ts-B	GCGGATCCGCTCTGCAACCGCAGAG
p33/34-F	TATGTTGTCCAAGAGATCGT
Antisense primers	
Thai 3'-510	CGACGAAGTCATAGAGGCAC
Ts-R	TGTGAGACTCAATGCGCCTA
p33/34-R	TGAGACTCAGTGTCGCCTAGA
Primer sets	Specificity
Ts-U and Ts-R	for PCR amplification of MPSP p32 of *T. orientalis* except Thai-type
p33/34-F and -R	for PCR amplification of MPSP p33/34 of *T. orientalis* and *T. buffeli*
Ts-I and Ts-R	for allele-specific PCR amplification of *T. orientalis* (Ikeda) defined as I-type
Ts-C and Ts-R	for allele-specific PCR amplification of *T. orientalis* (Chitose) defined as C-type
Ts-B and Ts-R	for allele-specific PCR amplification of *T. buffeli* (Warwick) defined as B -type
Ts-U and Thai 3'-510	for allele-specific PCR amplification of *T. sp.* (Kamphaeng Saen) defined as Thai-type

with warm summers and cold winters. The average temperature is 20.4°C (Hunchun) or 19.8°C (Dunhua) in summer (July) and –11.8°C (Hunchun) or –17.4°C (Dunhua) in winter (January). The vector tick species, including *Haemaphysalis longicornis*, an effective vector for *T. orientalis*, are active in the areas from spring to autumn [11].

Genomic DNA was extracted from 100 μl of whole blood samples using a QIAamp® DNA Blood Mini Kit (Qiagen, Germany), and 100 μl of DNA solutions was obtained and stored at –30°C. The PCR primer sets used in this study for the amplification of parasite DNA are listed in Table 1. The 1st universal primer set comprising p33/34-F and p33/34-R for the amplification of genes encoding MPSP p33/p34 of *T. orientalis* and *T. buffeli* [6] and the 2nd universal primer set comprising Ts-U and Ts-R for the amplification of the gene encoding MPSP p32-34 of *T. orientalis* were used [1, 10]. A combination of Ts-U and Thai 3'-510 primers was used for the amplification of the Thai-type parasites, which were not amplified by the Ts-U and Ts-R primers [5]. The other three sets of specific primers were used for allele-specific PCR to differentiate parasite populations within the *T. orientalis* group. The three allele-specific primers, Ts-I, Ts-C, or Ts-B, in combination with the Ts-R primer, were used to amplify the MPSP genes of the I-, C-, and B-types of *T. orientalis*, respectively [9, 10]. PCR amplification was performed in a 20 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer, 1 unit of *Taq* DNA polymerase (NEB, U.S.A.), and 2 μl of DNA template. All PCRs were performed using a myCycler Thermal Cycler (Bio-Rad Laboratories, U.S.A.) followed by 35 cycles. The PCR was performed as follows: initial denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were subjected to electrophoresis on 1.5% agarose gels, stained with ethidium bromide (Sigma-Aldrich, U.S.A.), and then visualized under an ultraviolet light. Amplified gene products encoding MPSP p32-34 were cloned into a pGEM-T Easy vector (Promega, U.S.A.), and the complete nucleotide sequences were determined from both strands using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystem, U.S.A.) with the BigDye® Terminator Cycle sequencing kit (Applied Biosystem, U.S.A.). Phylogenetic analysis of MPSP gene sequences were performed by the neighbor-joining program using CLUSTAL X [3], and the bootstrap probabilities of each node were calculated with 1,000 replications. The representative sequences obtained in the present study were registered in the GenBank database (National Center for Biotechnology Information, U.S.A.), as shown in Fig. 2.

Among 104 blood samples, *Theileria* parasites were identified by PCR using MPSP p32–34 or p33/34 genes-specific primers in 14 blood samples from two farms in which the beef cattle were pastured (Table 2). No products were observed upon PCR with the primer set for the Thai-type MPSP in all cases. Allele-specific PCR revealed that 14 and 12 field blood samples contained C- and I-type MPSP, respectively, whereas B-type parasites could not be detected in this survey (Table 2). Moreover, about eighty-five percent (12/14) of the infected cattle displayed C- and I-type mixed infections. In contrast, *Theileria* parasites had been negative in dairy cattle that had been raised in captivity. The present study revealed that the *Theileria* species in Northeast China were mixed parasite populations. The infection with such a mixed population bearing different MPSP types might disturb the host immune surveillance system as suggested by Iwasaki *et al*. [2].

Amplified gene products encoding MPSP p32–34 were cloned and sequenced. The MPSP sequence homology among the clones ranged from 86.6% to 100% at the nucleotide level and 86.9% to 100% at the amino acid level. *Theileria* parasites can be divided into 7 types (types 1–7),

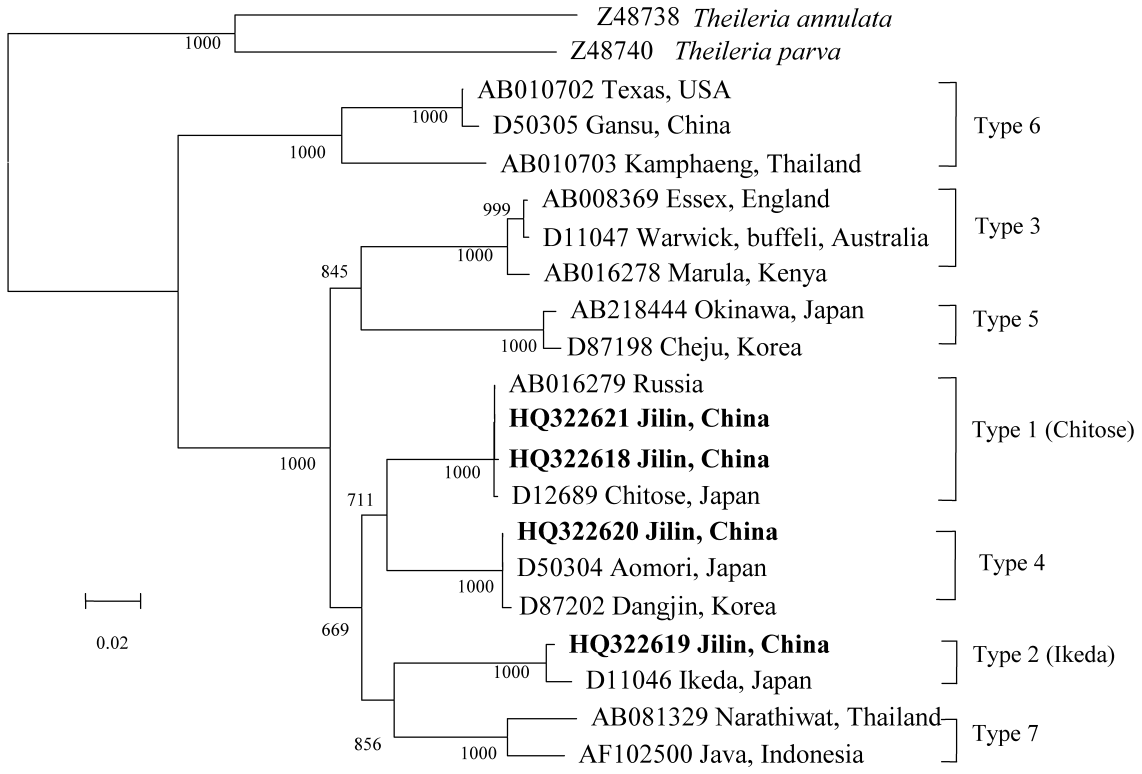

Fig. 2. A phylogenetic tree for the MPSP of *Theileria* parasites was constructed by the neighbor-joining method. The sequences of the MPSP genes determined in the present study are expressed in boldface type and refer to the GenBank accession numbers, as indicated at the end of each branch. The numbers shown at the branch nodes indicate the bootstrap values.

Table 2. Analysis of the *Theileria* parasite population in cattle by PCR

Region	Farm (No. of total samples)	No.of positive samples	Result with primer set for					
			p33/34	p32–34	Thai	C	I	B
Hunchun	Farm 1 (24)	12	+	+	–	+	+	–
	Farm 2 (32)	2	–	+	–	+	–	–
Dunhua	Farm 3 (48)	0	–	–	–	–	–	–

depending on the MPSP gene phylogenetic tree [7, 17]. Figure 2 depicts a phylogenetic tree for the MPSP sequences of 7 typical clones obtained from Northeast China isolates and reference stocks or isolates. The present epidemiological study showed that possible cases of infection with *Theileria* parasites of three different MPSP types (Types 1, 2 and 4) that had been previously categorized were identified under natural conditions in Northeast China (Fig. 2). Several reports on the prevalence of the different types of *T. orientalis* MPSP in Japan and South Korea that are near Northeast China, have been published [6, 7]. Therefore, large-scaled epidemiological studies are required to survey the distribution of different genotypes of *T. orientalis* and tick vectors of *T. orientalis* in Northeast China.

In conclusion, the present survey indicates that the *T. orientalis* infection was endemic in Northeast China. A population of *T. orientalis* with at least three types of MPSP genes is present in Northeast China. These immunogenic gene differences suggest that it is difficult to control *T. orientalis* infection. Therefore, in epidemiological investigations, it is important to provide essential information for the development of geographical distributions and diagnostic measures [12].

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