Molecular Epidemiological Survey of Benign *Theileria* Parasites of Cattle in Japan: Detection of a New Type of Major Piroplasm Surface Protein Gene

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**ABSTRACT.** Benign *Theileria* species of cattle are found in most parts of the world. The major piroplasm surface protein (MPSP), a conserved protein in all *Theileria* species, has been used as a maker for epidemiological and phylogenetical studies of benign *Theileria* species. Parasites with Ikeda- or Chitose-type MPSP genes are dominant in Japan, but we report here mixed infection cases of *Theileria* parasites with an additional MPSP type parasite infecting cattle in Abashiri District, Hokkaido. The MPSP gene sequence found in the additional type was closely related to MPSP genes of *Theileria* parasites found in Southeast Asian countries, including Thailand (Narathiwat) and Indonesia (Java). *Theileria* parasites from the blood sample were also distinguishable from the Ikeda or Chitose type parasites by the small subunit (SSU) rRNA gene sequence analysis, and they are grouped into the SSU rRNA types C/D found in Korea, North America, and Spain. The present finding of mixed infections of cattle with three different types of *Theileria* makes epidemiological feature of bovine theileriosis in Japan more complex. We have designed a set of primers specific to this MPSP type in order to conduct further epidemiological study.

**KEY WORDS:** bovine theileriosis, major piroplasm surface protein, small subunit ribosomal RNA, *Theileria orientalis*.

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*Theileria* parasites are tick-transmitted intracellular protozoa that belong to the phylum Apicomplexa [20]. *Theileria orientalis* is the causative agent of bovine piroplasmosis in Japan and Korea and this disease is reported as one of the most important hemoprotozoan diseases of cattle in these countries [3, 22]. For decades, there have been difficulties in the classification of the *T. buffeli/orientalis/sergenti* group of parasites due to the unreliability of distinguishing these *Theileria* parasites by morphological criterion [2, 8, 27]. Recently, the classification of *Theileria* species has been done on the basis of nucleotide or amino acid sequence of the major piroplasm surface protein (MPSP) gene [6, 11, 14, 17] and small subunit ribosomal RNA (SSU rRNA) gene [4, 11, 14]. The MPSP is abundantly expressed in the intraerythrocytic stage and conserved among the *Theileria* species [15, 23]. Phylogenetic analysis based on nucleotide sequences of SSU rRNA gene has proven useful for classification of many microorganisms, including *Theileria* parasites [1]. Comparative studies on the SSU rRNA genes have indicated that the distribution of *T. orientalis* and related parasites in cattle may be worldwide [4, 5, 10, 11, 14], but the presence of these parasites except the clinical cases in Japan and Korea may have been overlooked because of their low pathogenicity.

Kubota *et al.* [18] recognized two major types of MPSP genes, Chitose and Ikeda types in field isolates from Japan. It was demonstrated that these isolates often consisted of two parasite populations as was distinguished by MPSP allele-specific PCR [18, 19, 21]. Kim *et al.* [17] distinguished 6 different MPSP types in parasites from Korea and Japan. Two representative stocks, Fukushima and Ikeda, in Japan bear different SSU rRNA gene which correspond to SSU rRNA types A and B [4, 5, 14].

During a recent epidemiological survey of blood samples collected from Abashiri District in Hokkaido, we found an additional MPSP type of *Theileria* sp., which is very close to MPSP types distributed in South Asian countries. We designed primer specific to this MPSP type and conducted further epidemiological studies.

**MATERIALS AND METHODS**

**Field blood samples and DNA preparation:** Blood samples were collected from 20 field cases of bovine piroplasmosis in Abashiri District, Hokkaido, Japan. Diagnosis was done on the basis of clinical signs and light microscopic examination of Giemsa stained blood smears. Parasite DNA was extracted as described previously [25] with some modifications. Briefly, blood samples were suspended in 0.1% NaCl solution to lyse erythrocytes and centrifuged. The resultant pellet containing released piroplasms and erythrocyte debris was suspended in DNA extraction buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA) and then treated with 0.1% sodium dodecyl sulfate and proteinase K (Invitrogen, Carlsbad, CA, U.S.A.: 100 µg/ml) at 55°C for 2 hr. DNA was extracted with phenol-chloroform and precipitated with ethanol, and then dissolved in a Tris-EDTA (TE) buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA). Purified DNA was then used as a template for polymerase chain reaction (PCR) amplification.
Amplification of MPSP and SSU rRNA genes and nucleotide analysis: To determine the MPSP types of field isolates, allele-specific PCR analysis was carried out as previously described [18]. Primer pairs used to amplify the MPSP genes are listed in Table 1. A pair of primers comprising of the forward primer (Ts-U) and reverse primer (Ts-R) were used for universal amplification of any *T. orientalis* MPSP type gene [25]. On the other hand, Ts-I (Ikeda type-specific forward primer) or Ts-C (Chitose type-specific forward primer) in combination with Ts-R as a reverse primer was used for specific amplification of Ikeda and Chitose MPSP genes, respectively [18]. For the PCR amplification of full-length SSU rRNA genes, forward primer A (5’-AACCTGGTTGATCCTGCCAGT-3’) and reverse primer B (5’-CTAGGAAGACGTCCAAGTGGAATG-3’) were used [4]. PCR products of the MPSP or SSU rRNA genes were cloned into a pGEM T vector (Promega, Fitchburg, WI, U.S.A.) and the complete nucleotide sequences were determined by using an ABI 3100 automated sequencer (Applied Biosystem, Foster City, CA, U.S.A.) with the big dye chain termination kit (Applied Biosystem). The nucleotide sequences were applied to a Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) database for the homology analysis against other MPSP gene types registered in the GenBank. Phylogenetic analysis of the MPSP and SSU rRNA genes were performed by using a Neighbour-joining program in the MacVector program (Oxford Molecular Ltd., Cambridge, UK).

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>Ts-U 5’-CACGCTATGTTGTCCAAGAG-3’</td>
<td>Ts-R 5’-TGTGAGACTCAATGCGCCTA-3’</td>
</tr>
<tr>
<td>Ts-I 5’-AAGGATCCGTCTCTGCTACCGGC-3’</td>
<td></td>
</tr>
<tr>
<td>Ts-C 5’-GGGATCCATGCTCTGCTGCAAAT-3’</td>
<td></td>
</tr>
<tr>
<td>A16 5’-CTCTGCAACTGCGCA-3’</td>
<td></td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
</tr>
<tr>
<td>Ts-R 5’-TGTGAGACTCAATGCGCCTA-3’</td>
<td></td>
</tr>
</tbody>
</table>

*Primer sets Specificity*

- Ts-U and Ts-R For PCR amplification of MPSP gene [18]
- Ts-I and Ts-R Specific for Ikeda type *T. orientalis* [16]
- Ts-C and Ts-R Specific for Chitose type *T. orientalis* [21]
- Ts-A16 and Ts-R Specific for Abashiri type *T. orientalis* [This report]

Underline: BamHI restriction site.

Fig. 1. PCR analysis of *Theileria* parasites isolated in Abashiri using primers amplifying MPSP genes.

RESULTS AND DISCUSSION

By PCR examination of 20 blood samples, 14 samples showed positive reaction with Ts-U/Ts-R primer pair, and both of the MPSP types were detected in 6 samples (Fig. 1), indicating a mix-infection with the Chitose and Ikeda types of *T. orientalis*. Out of 14 samples, three samples were positive with Ts-I/Ts-R (Sample No. 8, 9, 19), and 4 with Ts-C/Ts-R (No. 6, 11, 14, 20). However, sample No. 16 was positive with a combination of Ts-U and Ts-R primers, but negative with the I- or C-type specific primer pair (Fig. 1). This result suggested the presence of unknown MPSP type(s) of *Theileria* in this sample. In order to characterize MPSP allele of the parasite in this blood sample, the product of 875 bp amplified with Ts-U/Ts-R primers was cloned into a plasmid vector. A total of 10 clones were subjected to nucleotide sequencing to minimize amplification errors caused by Taq polymerase. The sequences of 2 clones were identical to Chitose-type MPSP gene of *T. orientalis* (D12691), indicating the mixed infection of Chitose type, while those of other 8 clones did not match to any MPSP sequences reported [11, 17]. However, a BLAST search on GenBank revealed that the sequences from No. 16 sample showed a high similarity to those of MPSP genes of the Narathiwat (Accession No. AB081329; score 1094, E-value 0.0) and Java (AF102500; 930, 0.0) isolates of unclassified benign *Theileria* species in Thailand and Indonesia, respectively. MPSP gene sequences of *Theileria* sp. listed in Table 2 were used for a phylogenetic study using the neighbor
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Phylogenetic analysis revealed that the amino acid sequences from the two clones were closely related to those of the Narathiwat and Java isolates, and this group was clearly different from those of Chitose and Ikeda types (Fig. 2).

In order to detect this MPSP type specifically, we designed a new primer pair specific for the Abashiri isolate, designated as A16 (Table 1). This primer set was able to specifically amplify the Abashiri-type from No. 16 calf as shown in Fig. 3 (panel A). Blood samples collected on different dates showed different population composition, as the first sample (Fig. 3, panel B-a) contained only Abashiri-type, but in the second sample collected 4 months later (Fig. 3, panel B-b), Chitose type was also detected. In order to determine the geographical distribution of this MPSP type in Japan and its presence in retrospective samples, we ana-
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analyzed 60 DNA samples including the ones used in the study by Kubota et al. [18], and found positive reaction with the sample from Kumamoto Prefecture (Fig. 3, panel C). The nucleotide sequence of this PCR product was identical to that of Abashiri-type. This result indicated that this type had been widely distributed in Japan a decade back, but presence of this type might have been overlooked because of lack of specific primers specifically amplifying this MPSP type.

For further characterization of the parasite found in Abashiri District, we amplified SSU rRNA gene of this parasite from sample No. 16 collected on October 22, 2002 in which only Abashiri-, but not Ikeda- or Chitose-type MPSP gene was detected (Fig. 3, panel C). Three clones containing about 1,800 bp of the small subunit (SSU) rRNA gene were prepared, and their complete nucleotide sequences were determined. Chae et al. [4, 5] reported that there are at least 6 types of SSU rRNA genes (types A-E, and H) among the benign *Theileria* species in cattle (Table 2). The phylogenetic tree drawn based on SSU rRNA gene sequence (Fig. 4) revealed that clones (Abashiri 1 and 2) obtained from blood sample in Abashiri were closely related to either of types C and D SSU rRNA genes, which indicated that this particular cow had been infected with at least three different types of *Theileria* parasites. In total, four different SSU rRNA types are distributed in Abashiri District. Type C has been reported only from Korea [5] and Spain [7] so far, while type D parasites were found in Korea, U.S.A., Thailand, Japan, and Malaysia [5, 6, 14], but not in Japan.

Based on the SSU rRNA gene sequence, benign *Theileria* parasites were grouped into 3 subgroups [6]. In their report, parasites of SSU rRNA type C were located in one of these subgroups of *Theileria* sp., while Type D was allocated to a group phylogenetically separated from the above-mentioned 3 subgroups. As the SSU rRNA gene of Narathiwat and Java isolates have not been reported, we are not able to link the SSU rRNA types to MPSP type because our amplification

<table>
<thead>
<tr>
<th>SSU rRNA GeneBank Accession no.</th>
<th>GeneBank Accession no.</th>
<th>Host Species</th>
<th>Country of origin</th>
<th>Representative Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>A U07047</td>
<td>Bovine</td>
<td>South Korea, Japan, U.S.A., Kenya</td>
<td>Chitose&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>B U97048</td>
<td>Bovine</td>
<td>South Korea, Japan</td>
<td>Ikeda&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C U97051</td>
<td>Bovine</td>
<td>South Korea, Spain, Japan</td>
<td>KKN2, Abashiri-1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>D U97052</td>
<td>Bovine</td>
<td>South Korea, U.S.A., China, Malaysia, Thailand, Japan</td>
<td>USELT2&lt;sup&gt;h&lt;/sup&gt;, KCN4&lt;sup&gt;i&lt;/sup&gt;, China&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>E U97053</td>
<td>Bovine, Elk</td>
<td>South Korea, U.S.A.</td>
<td>KKN&lt;sup&gt;b&lt;/sup&gt;, USELK&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>F U97054</td>
<td>Deer, Elk</td>
<td>U.S.A., Canada</td>
<td>USWTD2&lt;sup&gt;j&lt;/sup&gt;, CNELK&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>G U97055</td>
<td>Deer, Elk</td>
<td>U.S.A., Canada</td>
<td>USWTD1&lt;sup&gt;j&lt;/sup&gt;, CNELK&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>H U97050</td>
<td>Bovine</td>
<td>South Korea</td>
<td>KKW&lt;sup&gt;b&lt;/sup&gt;</td>
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</tbody>
</table>

*Fig. 4. Phylogenetic relationship among the nucleic acid sequences of the SSU rRNA genes of benign *Theileria* sp. This phylogenetic tree was constructed using Neighbour-Joining program in the MacVector package.*
was made from blood samples from possible mixed infection with parasites of at least three SSU rRNA types. Whether the tick vector for SSU rRNA type A/B parasites can also transmit type C/D parasites remains to be determined. In Asia, *T. orientalis* group parasites are predominantly transmitted by ticks belonging to the genus *Haemaphysalis* that are commonly present in Japan [9, 12] and Thailand [26], but it is possible that this parasite is also transmitted by a broad range of tick species [24].

To date, only a mixed-infection of the Ikeda and Chitose types of *T. orientalis* had been reported from Japan [18]. The present epidemiological study indicates possible cases of mixed infection with parasites of three different MPSP types (1, 2 and 7) or four different SSU rRNA types (A, B, C and D) under natural condition. The infection with such a mixed population bearing different MPSP types might disturb the host immune surveillance system as suggested by Iwasaki [13]. Ikeda- and Chitose-type MPSP are antigenically different although their amino acid sequence homology is 86% [18]. As the same degree of difference was found between Abashiri- and Chitose- or Ikeda-types, therefore antigenic comparison of these MPSP types is essential to establish reliable serological diagnostic measures. Our present observation that type C and D are present in Japan may raise epidemiological issues on piroplasmosis in Japan, as population structures of piroplasm were turned out to be more complex. Allele-specific primer designed in this study may be a useful tool to study molecular epidemiology of bovine benign *Theileria* species in Japan.

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