Molecular diagnosis and epidemiology of bovine piroplasmosis

2013

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牛ピロプラズマ症の分子生物学的診断の開発と疫学調査に関する研究

平成２５年

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畜産衛生学専攻　博士後期課程

Contents

Abbreviation

AMA-1	: apical membrane antigen-1
A. bovis	: *Anaplasma bovis*
A. centrale	: *Anaplasma centrale*
A. marginale	: *Anaplasma marginale*
A. phagocytophilum	: *Anaplasma phagocytophilum*
B. bigemina	: *Babesia bigemina*
B. bovis	: *Babesia bovis*
B. divergens	: *Babesia divergens*
B. major	: *Babesia major*
B. ovata	: *Babesia ovata*
CI	: confidence interval
DNA	: deoxyribonucleic acid
ELISA	: enzyme-linked immunosorbent assay
Hb	: hemoglobin
HCT	: Hematocrit
ICT	: Immunochromatographic test
LAMP	: loop-mediated isothermal amplification
MPSP	: major piroplasms surface protein
MSA	: merozoite surface antigen
MSA-1	: merozoite surface antigen-1
MSA-2a1	: merozoite surface antigen-2a1
MSA-2a2	: merozoite surface antigen-2a2
MSA-2b	: merozoite surface antigen-2b
MSA-2a/b	: merozoite surface antigen-2a/b
MSA-2c	: merozoite surface antigen-2c
nPCR	: nested polymerase chain reaction
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
RAP-1	: rhoptry-associated protein-1
RBC	: red blood cells
T. annulata	: *Theileria annulata*
T. brucei	: *Trypanosoma brucei*
T. b. gambiense	: *Trypanosoma brucei gambiense*

T. evansi : *Trypanosoma evansi*
T. theileri : *Trypanosoma theileri*
T. orientalis : *Theileria orientalis*
T. parva : *Theileria parva*

Unit abbreviation

bp : base pair
^{o}C : degree Celsius
dl :deciliter
fg : femtogram
g : gram
µg : microgram
µl : microliter
ml : milliliter
µM : micromolar
mM : millimolar
min : minute
ng : nanogram
nM : nanomolar
pg : picogram
sec : second

General introduction

1. Bovine piroplasmosis

The livestock sector plays a crucial role in fulfilling the protein nutrient requirements of the human population by providing the milk and meat products. Especially, the farmers in the developing countries enjoy numbers of benefits from livestock farming. In addition to the direct income from the livestock products, this sector provides the farmers with the nutrients to the family members and manure and draft power for other agricultural activities. Therefore, livestock sector is closely associated with the living status of the farming community. Dairy farming which is one of the important components of the livestock industry mainly consists of cattle and water buffaloes. However, the farming operations are severely constrained by several causes such low productivity, high production costs, and low farm-gate price. Non-infectious causes such as low producing breeds, poor feeding and breeding managements, and metabolic disorders are often associated with the low productivity of cattle. On the other hand, infectious diseases also cause huge economical losses in the farming operation. In addition to the direct production losses incurred due to the infectious diseases, the indirect effects such treatment costs and poor reproductive performances might also be attributed to the economical losses.

Among the infectious diseases, piroplasmosis caused by different species of *Babesia* and *Theileria* parasites has a worldwide distribution. The livestock industry suffers enormous economical losses due to piroplasmosis. Disease outbreaks of bovine piroplasmosis have been commonly observed most especially in the tropical and subtropical regions of the world (52). The agents that casuse bovine babesiosis include *Babesia bovis*, *B. bigemina*, *B. divergens*, *B. ovata*, and *B. major*, while the bovine theileriosis is caused by *Theileria parva*, *T. annulata*, and *T. orientalis* (13, 54).

2. Diagnosis

Clinical signs: The acute infections of bovine piroplasmosis in susceptible cattle can be manifested with several clinical signs. While the *B. bovis*, *B. bigemina* and *B. divergens* are considered to be the virulent *Babesia* parasites of cattle, *B. ovata* and *B. major* are known as benign parasites (13). Similarly, while *T. annulata* and *T. parva*, the lymphoproliferative *Theileria* parasites, cause severe disease in cattle (54), *T. orientalis* is known to be relatively benign (60). Therefore, clinical signs are often associated with the infections of these virulent species. The major sign of piroplasmosis is usually the anemia, although anemia was not mainly observed with some strains of *T. parva* (21, 88). In addition, although several other clinical signs, including fever, loss of appetite,

and reduced productivity, can be observed during the acute infections with the virulent parasites, those are often non-specific. Therefore, the infection type may not be judged based on the clinical signs.

Microscopy: Microscopic examination of Giemsa-stained smears for the presence of the parasites have been considered to be the gold standard technique. Thin blood smears of infected hosts can be used to detect the intraerythrocytic stages of *Babesia* and *Theileria* parasites. However, the use of this technique is limited by its poor sensitivity and specificity (15). The epidemiological surveys often target the carrier animals in which the parasitemia might well below the detection limit of the microscopy (2). Furthermore, morphological differentiation between different species of *Babesia* as well as *Theileria* parasites can also be a difficult task (15).

Enzyme-linked immunosorbent assay (ELISA): In the past, several ELISA methods using recombinant antigens have been developed and evaluated for the detection of specific antibodies against *Babesia* and *Theileria* species (14). These assays were found to be highly sensitive and specific. The surveys based on these ELISA methods would provide the information about the exposure level of the parasites in the surveyed area, and therefore, assist the veterinary authorities to formulate appropriate control measures. However, the ELISA might not reflect the actual number of animals

that are currently infected with the parasites, as this technique detects not only the animals which are currently harboring the parasites but also the animals which have had past exposures.

Polymerase chain reaction (PCR): PCR assays were proven to be superior in sensitivity and specificity over the microscopy for the detection of the parasites (2). Several parasite-specific PCR assays have been developed and evaluated in the past (1, 22, 23). These assays were effectively used for epidemiological surveys to determine the prevalence of *Babesia* and *Theileria* parasites among the livestock populations of many countries. The commonly used *B. bigemina*-specific nested PCR assay was based on an unclassified DNA fragment of the parasites (*Spe*I-*Ava*I nPCR) (22).

However, the assay has never been tested for specificity against *B. ovata*, a benign *Babesia* parasite that resembles *B. bigemina* morphologically and phylogenetically (44, 51). Therefore, the *Spe*I-*Ava*I nPCR assay should be evaluated for specificity against DNA extracted from *B. ovata*, and a novel *B. bigemina*-specific nPCR assay must be developed if the former lacks the specificity.

On the other hand, there are no PCR assays available for the specific detection of *B. ovata*. The unavailability of PCR assay for *B. ovata* might be one of the stumbling blocks for research progress on this parasite. For example, although the coinfection with

B. *ovata* and *T*. *orientalis* is very common in Japan, as both the parasites are transmitted by the same tick vector, *Haemaphysalis longicornis*, the effect of this coinfection on the health of the cattle has not been evaluated yet (56).

Other diagnostic methods: In addition to the above mentioned diagnostic assays, other diagnostic assays have also been made available. The recently developed loop-mediated isothermal amplification (LAMP) method might be useful in detecting the parasite DNA without the use of any sophisticated instruments (58). Immunochromatographic tests (ICT) for the detection of parasite specific antibodies have been considered as a tool that can be used to detect the infection within a short period of time under field conditions (47).

3. Genetic diversity

Hemoprotozoan parasites use the antigenic polymorphisms to escape from the host's immune responses (17). In *B*. *bovis*, the merozoite surface antigens (MSA), which are known as Glycosylphosphatidylinositol (GPI)-anchored proteins, have been shown to be highly polymorphic among different field isolates (10, 50). The previous studies have suggested that the genetic diversity of gene encoding these MSAs might be linked to the lack of antigenic cross reactivity between MSAs (10, 50). Live attenuated vaccines have

been used in several countries to immunize the cattle populations against bovine babesiosis caused by *B*. *bovis* (64). In Australia, outbreaks of babesiosis due to *B*. *bovis* were observed among vaccinated herds. The Australian researchers believed that the antigenic variations between the vaccine strain and the field isolated could have triggered the outbreaks (12). Interestingly, the genetic make-up of the *MSAs* of outbreak isolates was different from those of particular vaccine strain that was used to immunize the cattle (10, 50). Therefore, the scientists concluded that the genetic diversity of *MSAs* might enable the parasite to escape from the immune pressure, and therefore outbreaks were inevitable. The studies have also shown that the *MSA* genes of vaccine stains and outbreak isolates formed separate clades in the phylogenetic trees (50). Therefore, the genes encoding the MSAs were considered to be good markers by which the parasite populations can be divided into different genotypes (4, 29). These observations have also hinted that the genetic diversity of *MSAs* should essentially be investigated in *B*. *bovis*-endemic countries prior to the introduction of immune control strategies. In addition, as MSAs were considered as candidate antigens for sub-unit vaccine, it is of paramount important to analyze the population structure of *B*. *bovis* to select suitable genotypes of MSAs to be included in such sub-unit vaccines (57, 73).

Similar to the MSAs of *B*. *bovis*, the genetic diversity of a *B*. *bigemina*

GPI-anchored protein, gp45, has been investigated previously. The genetic diversity of *gp45* shows a different pattern (24). The gene that encodes gp45 might be present in the *B*. *bigemina* genome and expressed. In some cases, the gene might be present but not expressed. In contrast, in some of the isolates, the gene was completely absent. Therefore, a comprehensive study, including the sequencing and expression analyses, is of paramount important to investigate the genetic diversity of *B*. *bigemina* based on *gp45*.

Among *Theileria* parasites, *T*. *orientalis* is known as a benign parasite but the outbreaks and economical losses due to this parasite have been reported previously (6, 53). Therefore, control of this parasite is essential to boost the cattle farming industry in the *T*. *orientalis*-endemic countries. The gene encoding major piroplasm surface protein (MPSP) is a polymorphic gene of *T*. *orientalis*. Interestingly, when the *MPSP* gene sequences were phylogenetically analyzed, the parasite populations were found to be clustered into different genotypes. Based on the phylogram, 11 genotypes, types 1 – 8, N1, N2, and N3 have been detected so far (38, 45). According to the past investigations, these genotypes might lack antigenic cross reactivity among them (60, 90). In addition, the virulence of *T*. *orientalis* might be related to its genotypes. For example, the type 2 was observed to be associated with more clinical cases that type 1 (20). Moreover, type

7 was involved in several severe clinical theileriosis in India (6). Therefore, the understanding of the genotypes of *T. orientalis* in endemic countries would not only allow the investigators to identify the genotypes of MPSP to be used as vaccine but also to find out the clinical significance of this parasite in a particular country.

Sri Lanka is an agriculture country known to be endemic for bovine babesiosis and theileriosis (69). These disease conditions are considered to be partly responsible for the production losses experienced in the dairy farming. The live attenuated vaccine belongs to Australian K-vaccine strain of *B. bovis* was introduced into the country in early 90's (87). Although, the vaccine has been used over two decades, the compatibility of vaccine to the field isolates has never been studied. Similarly, although *T. orientalis* was reported in the cattle populations of the country, no attempts were made to analyze its genetic diversity. Therefore, it is very important to analyze the genetic diversity of *B. bovis* in Sri lanka based on the *MSAs*, and to compare the findings with the sequencing data of K-strain. In addition, the genetic diversity of *T. orientalis* should also be investigated based on *MPSP* gene to predict the clinical relevance of this parasite in Sri Lanka.

4. Objectives of the present study

Bovine piroplasmosis caused by various species of *Babesia* and *Theileria* parasites results in huge economical losses in the endemic countries. Therefore, epidemiological studies targeting the parasites that induce babesiosis and theileriosis in cattle should be undertaken to generate the baseline data to design control strategies against them. However, unavailability of highly specific PCR assays might be a stumbling block for epidemiological surveys. The commonly used *B. bigemina*-specific *Spe*I-*Ava*I nPCR has never been tested against *B. ovata* although both parasites are similar in their morphologies. Therefore, the present study focused on the development of highly specific novel nPCR assay based on the *B. bigemina apical membrane-1* (*AMA-1*) gene. In addition, as there were no PCR assays available for the specific detection of *B. ovata*, the present study was involved in the development of an *AMA-1* gene-based *B. ovata*- specific PCR assay.

On the other hand, although the genetic diversity of hemoprotozoan parasites of cattle should be investigated to design suitable immune control strategies, such investigations have never been conducted in Sri Lanka. Therefore, the present study also focuses on the analyses of genetic diversities of *B. bovis* and *T. orientalis* that were detected from Sri Lankan cattle. The above described important issues were addressed

in the following studies.

i) Genetic detection of *Babesia bigemina* from Mongolian cattle using a*pical membrane antigen-1* gene-based PCR assay (Chapter 1)

ii) PCR detection of *Babesia ovata* from cattle reared in Japan and clinical significance of co-Infection with *Theileria orientalis* (Chapter 2)

iii) Genetic diversity of *Babesia bovis* in Sri Lankan cattle and its potential implications for live-vaccine usage (Chapter 3)

iv) Genetic diversity within *Theileria orientalis* parasites detected in Sri Lankan cattle (Chapter 4)

Chapter 1

Genetic detection of *Babesia bigemina* from Mongolian cattle using *apical membrane antigen-1* gene-based PCR assay

1-1. Introduction

Bovine babesiosis is an economically important infectious disease affecting the cattle industry, especially in the tropical and sub-tropical regions of world, and two species of the *Babesia* parasites, *B. bovis* and *B. bigemina*, are known to be the major etiological agents of the disease (52). They are transmitted by *Ixodid* ticks, and the lifecycle of *Babesia* parasites begins with the injection of sporozoites by the infected ticks (13). The disease in the host animals is often characterized by anemia, hemoglobinuria, fever, and jaundice (62). In addition, nervous signs and respiratory distress syndrome might be observed when the babesiosis is associated with *B. bovis* infection (21, 88). Although *B. bovis* is thought to be the most pathogenic among all of the *Babesia* parasites that affect the bovine hosts, the effect of the disease caused by *B. bigemina* also becomes serious if the disease goes untreated (16). Therefore, early and correct diagnosis is essential to initiate proper treatment for the disease.

Mongolia is an agricultural country, and an important sub-sector of the

Mongolian economy is the livestock industry. However, many infectious diseases threaten the farming activities, and enormous financial losses have been recently experienced in Mongolia. The status of tick-borne hemoprotozoan diseases among the cattle population was not clear in the past. Very recently, our studies of Mongolian cattle have indicated the presence of *B. bovis* and *Theileria orientalis* (1, 3, 4). However, no epidemiological studies of *B. bigemina* have been conducted. Therefore, I decided to survey the Mongolian cattle populations for the presence of *B. bigemina*.

The microscopic examination of Giemsa-stained thin blood smears is the gold standard technique to detect the *Babesia* parasites. However, the low sensitivity and difficulties encountered in species differentiation have limited the role of microscopy (15). Therefore, the technique might not be effectively used to conduct large-scale epidemiological studies, especially those aimed to detect the carrier animals (2). Consequently, new species-specific diagnostic techniques with higher sensitivity have been desired for the detection of *Babesia* parasites. Many PCR-based diagnostic methods with high sensitivity have been developed and evaluated for the genetic detection of *B. bigemina* (23, 31). Among them, a nested PCR (nPCR) assay based on a *Spe*I-*Ava*I restriction fragment of *B. bigemina* has been widely employed for the detection of the parasite from bovine blood samples (22, 59, 66, 81).

On the other hand, *B. ovata* is also known as one of the bovine *Babesia* parasites and has been reported in Japan, Korea, and China (9, 56, 76). Although the parasite is less pathogenic for the hosts than *B. bigemina* (56), the morphological differentiation between the two *Babesia* parasites is often difficult (15). A recent study found that *B. ovata* locates close to *B. bigemina* in the *18S RNA* gene sequence-based phylogram (51). However, none of the PCR techniques previously developed for the detection of *B. bigemina* has been evaluated against *B. ovata* for specificity.

A gene encoding the apical membrane antigen (AMA-1) of *B. bigemina* has recently been published (80). The gene sequences obtained from GenBank and Sanger sequencing indicate that the *AMA-1* genes are highly conserved, at least among Argentine, Italian, and Australian isolates of *B. bigemina*. Although the genomes of other *Babesia* parasites contain their original *AMA-1* sequences that are homologous to the *B. bigemina AMA-1*, a certain degree of sequence diversity among different species of *Babesia* parasites was also observed (79). Therefore, in the present study, a *B. bigemina*-specific nPCR method based on the *AMA-1* gene sequences was developed, and then compared with previously established *Spe*I-*Ava*I nPCR assay for specificity, as the latter one had not been evaluated for specificity against *B. ovata* (23). Subsequently, the Mongolian cattle populations were screened using nPCR assays to clarify the

presence of *B. bigemina* in Mongolia and to evaluate the field utility of the newly developed nPCR assay.

1-2. Materials and methods

DNA samples: The Argentina strain (S1A) of *B. bigemina* (34), the Texas strain of *B. bovis* (32), and the Miyake strain of *B. ovata* (56) were maintained in *in vitro* cultures using previously established continuous micro-aerophilous stationary-phase culture systems (35, 84). The bovine red blood cells (RBCs) infected with the parasites were washed in cold phosphate-buffered saline (PBS) three times; when the parasitemia reached 5%, the parasite DNAs were then extracted from the infected RBCs using a Qiagen Blood Mini Kit (Qiagen, Hilden, Germany). The extracted DNA samples were kept at -30°C until use. *Theileria orientalis* DNA was prepared from the blood of cattle experimentally infected with the parasite. *Trypanosoma brucei gambiense* maintained by an Axenic culture system (33) was kindly provided from Dr. Noboru Inoue (National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan), and the parasite DNA was also extracted as described above. Bovine DNA samples were obtained from normal blood collected from a healthy cow. Qiagen Blood Mini Kit (Qiagen) was employed to extract DNA from 200 µl of

each of 266 bovine blood samples collected from three different provinces (Khentii, Uvs, and Uvurkhangai) of Mongolia in 2010 (Table 2) (3).

Primer designs and establishment of new PCR assays: The *AMA-1* gene sequences of *Babesia* and *Theileria* parasites were obtained from GenBank (GenBank accession numbers, *B. bigemina*: GQ257738-GQ257740, HM543726-HM543730, AB481200, *B. bovis*: AY486101, FJ588028-FJ588028, XM001610993, *B. divergens*: EU486539, *T. parva*: XM761078, *T. annulata*: XM949044). The specifically conserved regions among the *AMA-1* gene sequences of different *Babesia* parasites were identified by multiple alignments using Multalin interface, an online software program (18), and a pair of forward (5'-tggaccaggtacatgatcaagt-3') and reverse primers (5'-aatcatcgtgctgacgacccttc-3') was designed to amplify a part of the *AMA-1* homologous gene of *B. ovata* in the present study. In addition, *B. bigemina*-specific and highly conserved regions were also identified after the multiple alignments together with the newly determined *B. ovata AMA-1* gene sequence. Based on the sequence data, two pairs of primers were designed for the primary and nested PCR assays (Table 1).

For the *AMA-1* nPCR assay, 1 μl of the template DNA sample was added to a 24 μl reaction mixture, which contained 2.5 μl of a 10 x PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 200 μM of each dNTP (Applied Biosystems), 0.8

μM of the outer forward and reverse primers (BI-AMA-FO and BI-AMA-RO), 1 unit of Taq polymerase (Applied Biosystems), and double-distilled water (DDW). The initial enzyme activation at 95˚C at 5 min was followed by 35 cycles, each of them consisting of a denaturation step at 95˚C for 30 sec, an annealing step at 68˚C for 1 min, and an extension step at 72˚C for 1 min. After the final elongation step at 72˚C for 10 min, 1 μl of the PCR products was transferred to new PCR tubes, each containing a reaction mixture with the same composition as that of the first PCR except for the outer primers, which were replaced with the inner forward and reverse primers (BI-AMA-FI and BI-AMA-RI). Then, the reaction mixtures were subjected to a second PCR amplification with similar cycle conditions as those of the first round except that the annealing temperature was decreased to 60˚C. Finally, the positive reactions were identified after gel electrophoresis and ethidium bromide staining.

Specificity and sensitivity testing: The specificity of the *AMA-1* nPCR assay was assessed against the DNAs of *B. bigemina*, *B. bovis*, *T. orientalis*, *B. ovata*, *T. brucei gambiense*, and cattle blood samples as described above. One nanogram of each DNA sample was subjected to the *AMA-1* nPCR condition. In addition, the same DNA samples were also analyzed by the previously established *Spe*I-*Ava*I nPCR assay (23), and the specificity of the *AMA-1* nPCR assay was compared with that of the *Spe*I-*Ava*I

nPCR assay. For the evaluation of sensitivity, *B. bigemina*-infected RBCs with 1% parasitemia (5 x 10^7 infected RBCs per 200 μl) were prepared from the *in vitro* culture and 10-fold serially diluted with uninfected RBCs. Then, DNAs were extracted from 200 μl of each dilution and eluted into 200 μl of DDW. The *AMA-1* nPCR assay was carried out using 1 μl of each of the DNA samples to evaluate the sensitivity.

Evaluation of field DNA samples by two nPCR assays: Field DNA samples prepared from Mongolian cattle as described above were screened for the presence of *B. bigemina* using the *AMA-1* and *Spe*I-*Ava*I nPCR assays, and the obtained results were compared between the two assays to evaluate their practical utility for genetic diagnosis.

Cloning and sequencing of PCR amplicons: Cloning and sequencing analyses were performed as described previously (4). Briefly, the PCR products amplified by both of the *AMA-1* and *Spe*I-*Ava*I nPCR methods were gel-extracted and then ligated to a cloning plasmid vector (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, USA). The plasmids were transferred to Top 10 *E. coli* competent cells (Invitrogen) and then cloned according to the manufacturer's instructions. The corresponding PCR methods were employed to select the positive clones, which contained the plasmids with the inserted PCR fragments. Finally, the plasmids were extracted from *E. coli* using a QIAprep Spin Miniprep kit (Qiagen), and the nucleotide sequences of the target DNA

fragments were determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

1-3. Results

Development of *B. bigemina AMA-1* nPCR assay: A part of the *B. ovata AMA-1* sequence (1,371 bp) was determined before designing *B. bigemina AMA-1* nPCR primers (GenBank accession no. AB634843), and the identity between *B. bigemina* and *B. ovata* gene sequences was found to be 87.1%. Although most of the nucleotide sequences in both the outer and inner forward primers aligned with the *B. ovata AMA-1* gene sequence, the two reverse primers (BI-AMA-RO and BI-AMA-RI, Table 1) were carefully designed to ensure that there was a significant difference between the primer sequences, especially at their 3' ends, and the *B. ovata AMA-1* sequence. As expected, 738-bp and 211-bp PCR products were observed for the primary and nested rounds of *AMA-1* nPCR assay, respectively, as shown in Figure 1 (Panels A and B, respectively).

Specificity and sensitivity of *B. bigemina AMA-1* nPCR assay: The *AMA-1* nPCR assay specifically identified the genomic DNA of *B. bigemina* extracted from their *in vitro* culture, however, it did not amplify any control DNAs derived from *B.*

bovis, *T. orientalis*, *B. ovata*, *T. brucei gambiense*, and cattle blood (Fig. 1A and 1B). In contrast, although a previously established *Spe*I-*Ava*I nPCR assay differentially detected the *B. bigemina* DNA, at least against control DNAs of *B. bovis*, *T. orientalis*, *T. brucei gambiense*, and cattle blood, the assay also amplified similar PCR fragments from *B. ovata* DNA in the nested as well as the primary round of PCR (Fig. 1C and 1D, respectively). The DNA cloning and sequencing analyses of PCR products that had been amplified by the *Spe*I-*Ava*I nPCR assay from the genomic DNA of *B. ovata* (Fig. 1D) revealed the presence of the homologous fragment in *B. ovata* (GenBank accession no. AB634845), and the percent identity was 63.8 between the two sequences of *B. bigemina* and *B. ovata*. The sequence of the *Spe*I-*Ava*I-like fragment of *B. ovata* was 11 bp shorter than that of *B. bigemina* (GenBank accession no. S45366).

In a sensitivity test, the lowest detection limit of the *AMA-1* nPCR assay was calculated as 5 x 10^2 *B. bigemina*-infected RBCs per 200 μl of total RBCs (2.5 infected RBCs/ μl of RBCs) (Fig. 2).

PCR detection of *B. bigemina* from Mongolian cattle populations: The field DNA samples prepared from Mongolian cattle blood were analyzed with both nPCR assays. All 7 districts analyzed in the survey included cattle populations harboring the *B. bigemina* in their blood streams (Table 2 and Fig. 3). In summary, the *AMA-1* nPCR

assay detected a total of 25 animals (9.4%) as positive for *B. bigemina*, while the previously established *Spe*I-*Ava*I nPCR assay indicated that a total of 90 blood samples (33.8%) consisted of the target DNAs.

The nucleotide sequences of all 25 Mongolian *AMA-1* nPCR products were determined, and they were identical to those of the already available *B. bigemina AMA-1* gene registered in the GenBank (GenBank accession no. AB481200). On the other hand, the product sequences prepared by the previous *Spe*I-*Ava*I nPCR assay consisted of a mixture of 4 different sequences. Of 45 sequences determined in the present study, 18 and 4 sequences were similar to those of *B. bigemina* and *B. ovata Spe*I-*Ava*I (like) fragments, respectively (Fig. 4). In contrast, the origin of the other two types was unknown. However, the two unknown types of nucleotide sequences that were 180 and 174 bp in length showed 80.0% and 85.6% identity to the *B. bigemina Spe*I-*Ava*I restriction fragment that was originally deposited in the GenBank (Accession number S45366). Fifteen of 18 field DNA samples, from which these *B. bigemina Spe*I-*Ava*I fragments were amplified, were also positive for the *AMA-1* nPCR assay. Of the 4 *B. ovata Spe*I-*Ava*I-like fragments, 3 were 158 bp in length, while the sequence of the other fragment consisted of 199 nucleotides.

1-4. Discussion

B. bigemina is widespread in many continents, posing a real threat to the livestock sector (52). The disease requires early diagnosis and veterinary intervention to minimize financial losses. In addition, precise diagnostic techniques are essential to screen the infected animals to fulfill the international trade requirement. Furthermore, highly species-specific diagnostic tools are of paramount importance, especially when a closely related species of the parasite exists. As previously reported by researchers, *B. ovata* is similar to *B. bigemina* genetically (51). Therefore, every diagnostic technique developed for *B. bigemina* infection must be evaluated for specificity, especially against *B. ovata*. The previously established *Spe*I-*Ava*I nPCR assay (23) has been extensively used for the detection of *B. bigemina* among susceptible cattle populations (59, 66). The assay was proven to be highly sensitive, as evaluated by several researchers (59). However, Guerrero et al. (2007) (31) indicated the unknown identity of the target sequence and the possible mismatch of primer BiIBN toward 3' end of the target region as the limitations of the *Spe*I-*Ava*I nPCR assay.

The *B. bigemina AMA-1* nPCR assay developed in the present study was found to be devoid of any amplification from many other blood parasites, including *B. ovata*, and, therefore, proven to be specific for *B. bigemina*. It is noteworthy that the

previously developed *Spe*I-*Ava*I nPCR assay amplified a homologous fragment derived from *B. ovata*. Although the fragment of *B. ovata* shown in Figure 1 is 11 bp shorter than that of *B. bigemina* in the sequences, it might be difficult to differentiate the two amplicons on agarose gel. The nucleotide sequences derived from nPCR products of the *Spe*I-*Ava*I nPCR assay that had been conducted using field DNA samples of Mongolian cattle also included the *Spe*I-*Ava*I-like fragment of *B. ovata*. The size of a single *B. ovata Spe*I-*Ava*I-like fragment (199 bp) out of 4 determined sequences was larger than expected (158 bp). This observation is considered to be due to the mispriming of the inner forward primer (BiAN) toward the 5' region of the *Spe*I-*Ava*I-like fragment in the *B. ovata* genome. Certainly, the *Spe*I-*Ava*I nPCR assay detected more *B. bigemina*-positive samples than the *AMA-1* nPCR assay. However, the use of the *Spe*I-*Ava*I nPCR assay is questionable for the detection of *B. bigemina* in places where *B. ovata* is present and might provide inaccurate data regarding the epidemiology of *B. bigemina*. *B. ovata* has been currently reported in Japan, Korea, and China (9, 56, 76), while the presence of the parasite has not been yet investigated among other countries. The finding suggests the presence of *B. ovata* in Mongolia, where the parasite has never been reported previously. Therefore, it is essential to use a highly specific molecular technique to detect *B. bigemina*, and I believe that the newly established *AMA-1* nPCR

might be an alternative assay. Furthermore, the specificity of any other existing *B. bigemina*-specific molecular diagnostic assays against *B. ovata* might need to be reevaluated.

It became clear that several hemoprotozoan parasites are indeed very common among Mongolian cattle populations. Our previous investigations showed the presence of *B. bovis* (9.6%) (4) and *T. orientalis* (41.7%) (3), and the present study indicated the presence of *B. bigemina* (9.4%) in the *AMA-1* nPCR assay. Although the current study also suggested the presence of *B. ovata* in Mongolia, further study about the organism would be desired to confirm the present finding. The livestock industry is a vital component of the Mongolian economy. Therefore, proper strategies should be in place to control and prevent the occurrence of hemoprotozoan infections among Mongolian cattle populations.

1-5. Summary

A new nested PCR (nPCR) assay was developed based on the *Babesia bigemina apical membrane antigen-1* (*AMA-1*) gene sequence for parasite-specific detection. The primers were designed to amplify 738-bp and 211-bp fragments of the *AMA-1* gene by primary and nested PCRs, respectively. The assay was proven to be specific for the *B. bigemina*, whereas the previously established *Spe*I-*Ava*I nPCR assay amplified not only the target fragment of *B. bigemina* but also a homologous one from *B. ovata*. The *AMA-1* nPCR assay was also evaluated using field DNA samples extracted from 266 bovine blood samples collected from Mongolia in 2010. In a comparative evaluation, 90 (33.8%) and 25 (9.4%) of the blood samples showed positive reactions for *B. bigemina* by the *Spe*I-*Ava*I nPCR and *AMA-1* nPCR assays, respectively. The sequencing analysis of the nPCR products confirmed that the *AMA-1* nPCR method had specifically detected the target *B. bigemina* DNA. However, 4 different kinds of sequences were determined among the *Spe*I-*Ava*I nPCR amplicons. Two of them were derived from *B. bigemina* and *B. ovata*, while the origins of the others were unknown. In this study, the presence of *B. bigemina* was demonstrated among Mongolian cattle populations by the current nPCR assay for the first time. The findings also indicate that the *AMA-1* nPCR assay may be a useful diagnostic tool for the specific detection of *B. bigemina*.

Table 1. Nested PCR primers designed in the current study.

PCRs	Primers*	Sequences	Positions	Sizes of amplicons (bp)
Primary	BI-AMA-FO	5'-GTATCAGCCGCCGACCTCCGTAAGT-3'	556-580	738
	BI-AMA-RO	5'-GGCGTCAGACTCCAACGGGGAACCG-3'	1269-1293	
Nested	BI-AMA-FI	5'-TACTGTGACGAGGACGGATC-3'	745-764	211
	BI-AMA-RI	5'-CCTCAAAAGCAGATTCGAGT-3'	936-955	

* Primers were designed based on the *AMA-1* gene sequence AB481200 in GenBank.

Table 2. Screening the Mongolian cattle for *B. bigemina* using *Spe*I-*Ava*I and *AMA-1* nested PCR assays.

Provinces	Districts	Sample No	*Spe*I-*Ava*I nPCR (%)	*AMA-1* nPCR (%)
Khentii	Tsenkher-mandal	42	16 (38.1)	3 (7.1)
	Dadal	42	13 (31.0)	5 (11.9)
	Jargaltkhan	55	11 (20.0)	3 (5.5)
	Binder	34	21 (61.7)	3 (8.8)
	Undurkhan	12	7 (58.3)	1 (8.3)
Uvs	Ulaangom	46	13 (28.3)	9 (19.6)
Uvurkhangai	Bogd	35	9 (25.7)	1 (2.9)
(Total)		266	90 (33.8)	25 (9.4)

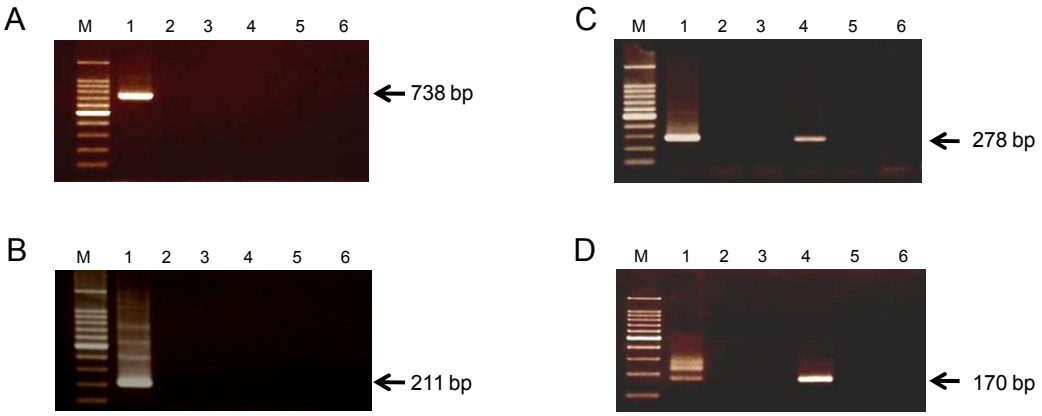

Fig. 1. Specificities of primary (A and C) and nested (B and D) PCR assays based on the *AMA-1* gene (A and B) and the *Spe*I-*Ava*I restriction fragment (C and D) of *B. bigemina*. M, 100-bp DNA ladder marker. Lanes 1-6: *B. bigemina*, *B. bovis*, *T. orientalis*, *B. ovata*, *T. brucei gambiense*, and bovine blood DNAs.

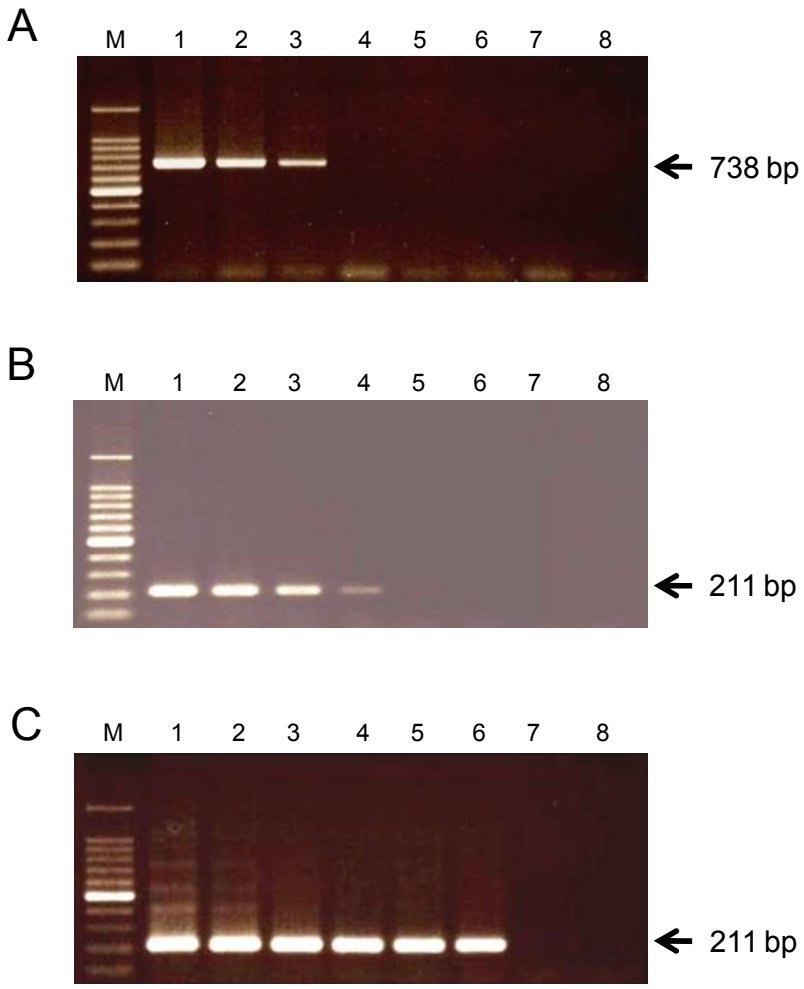

Fig. 2. Sensitivity of *AMA-1* nPCR assay. PCR assays were conducted with outer (Panel A), inner (Panel B) and nested primers (Panel C). M, 100-bp DNA ladder marker. Lanes 1 - 7, 5 x 10^7, 5 x 10^6, 5 x 10^5, 5 x 10^4, 5 x 10^3, 5 x 10^2, 5 x 10 *B. bigemina*-infected RBCs per 200 µl total RBCs. Lane 8, bovine blood DNA.

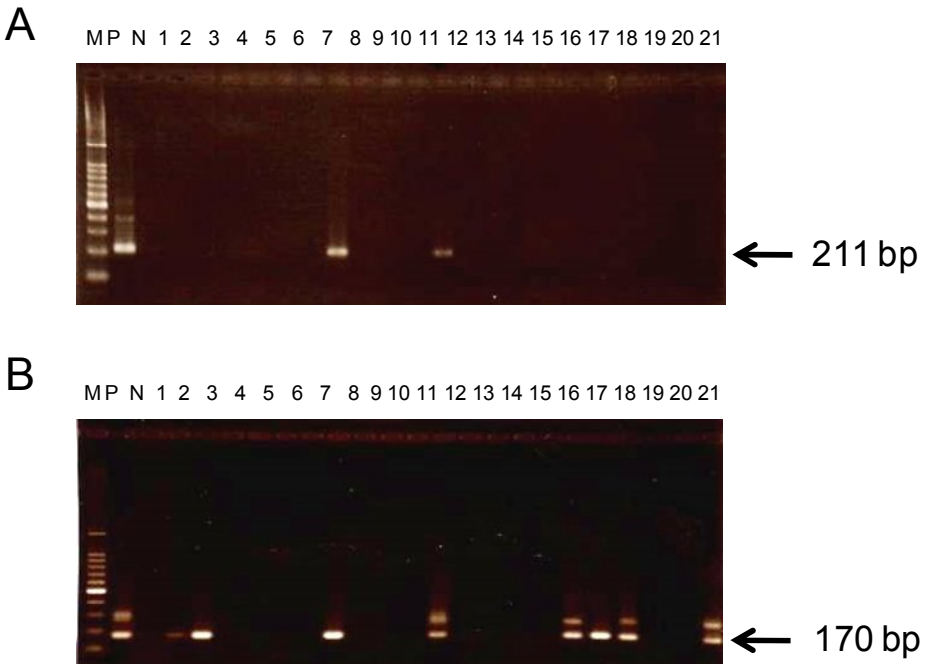

Fig. 3. PCR detection of *B. bigemina* from grazing cattle bred in Tsenker-Mandal district of Mongolia. Samples 1 - 21 were analyzed by both *AMA-1* (panel A) and *Spe*I-*Ava*I (panel B) nPCR assays.

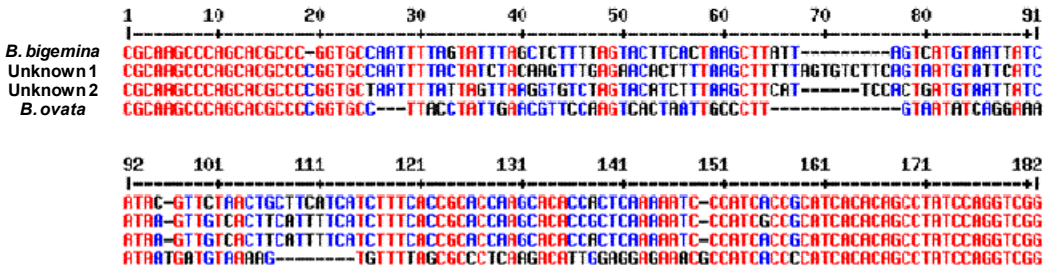

Fig. 4. Sequences detected among *Spe*I-*Ava*I nPCR amplicons derived from Mongolian cattle blood DNA. In addition to the sequences corresponding to *B. bigemina*, the sequences originated from *B. ovata* and two other unknown targets (unknown 1 and 2) were also observed.

Chapter 2

PCR detection of *Babesia ovata* from cattle reared in Japan and clinical significance of co-infection with *Theileria orientalis*

2-1. Introduction

Theileria orientalis and *Babesia ovata* are known to be two benign hemoprotozoan parasites that are endemic in Japan (7, 60, 91). *T. orientalis* is a non-lymphoproliferative *Theileria* parasite (Minami et al., 1980) that infects several host species including bovines (36), and has been reported in several countries across the globe (3, 5, 45, 46, 53, 61, 68). Although the parasite is considered to be relatively non-pathogenic, clinical signs of anemia have sometimes been observed in affected cattle (53, 61). In addition, economic losses were also reported among several Asian countries due to *T. orientalis* infections (8, 55). Although infections with *T. orientalis* can be diagnosed by demonstrating the parasites on Giemsa-stained thin blood smears under a light microscope, lack of sensitivity has limited the use of this technique. Therefore, at present, PCR methods are preferred over microscopy as they show superior sensitivity to the latter technique (78). Up to now, several PCR assays have

been developed and are available for the diagnosis of *T. orientalis* infection, based on the specific detection of the parasite's DNA in the infected cattle blood samples (61, 78).

B. ovata was first described in Japan, and later in China and Korea (9, 56, 76), and was classified into a groups of large *Babesia* parasites (56). Similar to *T. orientalis, B. ovata* is also thought to be a parasite of low pathogenesis (56). However, previous studies on splenectomized calves indicated that immuno-compromised animals might exhibit severe anemia-related clinical signs (26). As pointed out by previous researchers, the morphology among many *Babesia* parasites is similar, and therefore microscopy might not be an effective diagnostic tool for species differentiation (15). At present, many highly sensitive PCR assays are available for the specific detection of several *Babesia* species, and they are widely used for molecular, epidemiological, and clinico-pathological studies (23, 59). However, a *B. ovata*-specific PCR method is not yet established, and the unavailability of PCR technique has become one of the major stumbling blocks for studying the epidemiology and pathology of *B. ovata* infection.

Both *T. orientalis* and *B. ovata* are transmitted to the bovine host by *Haemaphysalis longicornis*, which is an Ixodid tick vector (56). Therefore, mixed infection by these two parasites is expected to be very common in endemic areas (26).

Although several researchers have focused on the clinical consequences of infections by these parasites (26, 53), the pathobiology of mixed-infections by *T. orientalis* and *B. ovata* has not been described yet.

In the current study, a novel PCR assay was developed for the specific detection of *B. ovata* in DNA samples extracted from infected bovine blood, and then, the new and previously described PCR assays were used to screen the cattle population reared in a dairy farm in Japan for the presence of *B. ovata* and *T. orientalis*, respectively. The findings were then used to discuss the status of clinical anemia among the study group in relation to the infection type.

2-2. MATERIALS AND METHODS

Sample collection: The study area, Shin-Hidaka region, is located in the Hokkaido prefecture of Japan, and known to be endemic for *T. orientalis* and *B. ovata* according to field veterinarians. Blood samples were collected from 94 randomly selected normal cattle in a dairy farm managed by Hokkaido University in the Shin-Hidaka district on the 19th of June, 2009, as described previously (61). Two milliliters of whole blood were collected from each animal into a vacutainer tube containing EDTA (NIPRO, Osaka, Japan). The blood samples were kept in ice and

immediately transported to our laboratory.

Detection of anemia: Essential anemia indicators, including red blood cell (RBC) count, hemoglobin (Hb) concentration, and the Hematocrit (HCT) value, were measured for each blood sample using a Celltac (Nihon Koden, Tokyo, Japan) automated hematological analyzer. An animal was considered to be anemic if the RBC, Hb, or HCT values were less than 5 x 10^6 RBCs/μl, 8 g/dl, or 24%, respectively (37).

DNA samples: All DNA samples were extracted using a blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and then kept at -30°C until use. The Miyake strain of *B. ovata* (56), the Argentina strain of *B. bigemina* (34), and the Texas strain of *B. bovis* (32) were maintained in *in vitro* cultures according to previously described methods (35, 84), and their DNA samples were extracted from the cultures when parasitemia reached 5%. In addition, genomic DNA samples from *Theileria annulata*, *T. orientalis*, *Trypanosoma brucei gambiense*, *T. evansi*, *T. theileri*, *Aanaplasma marginale*, *A. bovis*, *A. centrale*, *A. phagocytophilum* were also used in this study. A bovine DNA sample was also extracted from a blood sample collected from a healthy cow. Field blood samples were collected from dairy cattle in the Shin-Hidaka farm and subjected to the DNA extraction.

Development of the *B. ovata*-specific PCR method: The gene encoding

Apical membrane antigen 1 (AMA-1) of various *Babesia* and *Theileria* parasites were obtained from Genbank (Genbank accession numbers, *B. ovata*: AB634843, *B. bigemina*: GQ257738-GQ257740, HM543726-HM543730, AB481200, *B. bovis*: AY486101, FJ588028-FJ588028, XM001610993, *B. divergens*: EU486539, *T. parva*: XM761078, and *T. annulata* : XM949044), and the *B. ovata*-specific regions were identified in the sequence of the *B. ovata AMA-1* gene after a multiple alignment of these sequences (18). Subsequently, a pair of *B. ovata*-specific forward (5'-GATACGAGGCTGTCGGTAGC-3') and reverse (5'-AGTATAGGTGAGCATCAGTG-3') PCR primers was designed based on the specific regions, and the suitable composition of reaction mixture and thermal conditions were experimentally determined (designated as *B. ovata AMA-1*-PCR assay). Briefly, 1 μl of template DNA solution (approximately 6 μg/μl) was added to 25 μl of a reaction mixture that contained 2.5 μl of 10 x PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 200 μM of each dNTP (Applied Biosystems), 0.8 μM of each primer, 1 unit of Taq polymerase (Applied Biosystems), and double distilled water (DDW), before being loaded on a Veriti Thermal Cycler (Applied Biosystems). Optimized cycle conditions were employed for the PCR amplification. Briefly, after the initial step of enzyme activation at 95°C for 5 min, cycle conditions were set as a

denaturing step at 95°C for 30 sec, an annealing step at 56°C for 1 min, and an extension step at 72°C for 1 min. After the final elongation at 72°C for 10 min, 8 µl of the PCR product was mixed with 2 µl of 5 x loading buffer, and then subjected to gel electrophoresis. Finally, the positive reactions were visualized under UV light.

Ten nanograms per microliter of *B. ovata* genomic DNA solution was subjected to 10-fold serial dilutions, and 1 µl of each dilution was amplified by the *AMA-1*-PCR assay as described above. Cycle numbers were adjusted at 35, 40, 45, and 50 to evaluate the maximum sensitivity of the PCR technique. The specificity of the *B. ovata AMA-1*-PCR assay was also evaluated using several control DNA samples extracted from genomic DNA samples from *B. ovata*, *B. bigemina*, *B. bovis*, *Theileria annulata*, *T. orientalis*, *Trypanosoma brucei gambiense*, *T. evansi*, *T. theileri*, *Aanaplasma marginale*, *A. bovis*, *A. centrale*, *A. phagocytophilum* and normal bovine blood.

PCR detections of *B. ovata* and *T. orientalis* from field blood samples: All DNA samples that had been extracted from field blood samples of grazing cattle in a Shin-Hidaka farm were screened for the presence of *T. orientalis* and *B. ovata* by the respective PCR assays. A previously described *MPSP* gene-based PCR assay with a set of forward (5'-CTTTGCCTAGGATACTTCCT-3') and reverse (5'-ACGGCAAGTGGTGAGAACT-3') primers (*T. orientalis MPSP*-PCR assay) was

employed for the specific detection of *T. orientalis* (61). The same DNA samples were also analyzed using the *B. ovata AMA-1*-PCR assay developed in the present study.

Cloning and sequencing of PCR products: The amplified DNA products of the *B. ovata AMA-1*-PCR assay were cloned and then sequenced as described previously (61). Briefly, the PCR products were inserted into a PCR 2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA), and the ligated plasmids were transferred to TOP 10 *E. coli* competent cells (Invitrogen), and then cloned on X-gal (Wako, Osaka, Japan)-containing LB agar plates (Invitrogen). Clones were selected and then cultured in LB broth (Invitrogen). After the confirmation of the presence of inserted DNA fragments using the *B. ovata AMA-1*-PCR assay, the plasmids were extracted from cultures (QIAprep Spin Miniprep kit, Qiagen) for the determination of the nucleotide sequence of the inserted DNA fragment.

Statistical analysis: JMP statistical software (version 5.1., SAS institute Inc., Cary, NC, USA) was used to calculate the means, confidence intervals, and *P* values of each blood parameter.

2-3. Results

PCR detections of *B. ovata* and *T. orientalis*: A new PCR method was

developed in the current study for the diagnostic detection of *B. ovata* and it was found to amplify a 504-bp DNA fragment derived from the target *AMA-1* gene of *B. ovata* as shown in Figure 1. The PCR technique clearly showed a high specificity, at least against *B. bigemina*, *B. bovis*, *Theileria annulata*, *T. orientalis*, *Trypanosoma brucei gambiense*, *T. evansi*, *T. theileri*, *Aanaplasma marginale*, *A. bovis*, *A. centrale*, *A. phagocytophilum,,* and normal bovine blood DNA samples (Fig. 5A). Additionally, the high sensitivity of the PCR technique was also demonstrated when the PCR cycle number was set as 45. Under these conditions, the detection limit of the *B. ovata AMA-1*-PCR assay was shown to be 100 fg/μl of the DNA template concentration (Fig. 5B).

When a previously developed *T. orientalis*-specific *MPSP*-PCR assay was employed with the *AMA-1*-PCR assay against field blood samples, 14 of 94 animals (14.9%) surveyed in the present study were found to harbor both *T. orientalis* and *B. ovata* in their blood circulations, while 33 (35.1%) and 9 (9.6%) animals were infected only with *T. orientalis* and *B. ovata,* respectively (Fig. 6, Table 3). In contrast, 38 (40.4%) animals were diagnosed as non-infected with the PCR assays for both parasites. Sequencing analyses confirmed that the sequences of *B. ovata*-specific PCR amplicons were similar to the sequence of the *B. ovata AMA-1* gene registered in Genbank (Accession no: AB634843).

Hematological detection of anemia in the surveyed field cattle: All non-infected animals (n=38) and the animals that were infected only with *B. ovata* (n=9) were not anemic, according to the blood parameters measured in the current study (RBC count, Hb concentration, and HCT value) (Table 3). Twelve of 47 animals (25.5%) that were positive for the *T. orientalis* infection had abnormally low values for at least one anemia indicator, and out of these 12 animals, 6 cattle were co-infected with *B. ovata* (Table 4). Among the anemic animals, 3 animals exhibited hematologically low values for all three blood parameters, and 2 of these animals were found to be co-infected with both parasites (Table 4). Mean values for RBC count, Hb concentration, and HCT value of all categorized animals were above the lower values in the normal ranges of these three parameters (5 x 10^6/μl, 8 g/dl, and 24%, respectively) (Table 5). However, statistically significant differences were observed among the different categories of animals, based on their infection types ($P < 0.01$). The mean values of all three parameters of non-infected animals and the animals that were infected only with *B. ovata* did not significantly differ from each other. The mean RBC count of the animals (n=33) infected only with *T. orientalis* (5.90 x 10^6/μl) was lower than that of non-infected animals (7.29 x 10^6/μl), while the RBC count of co-infected animals (n=14) (5.64 x 10^6/μl) was similar to the solely *T. orientalis*-infected animals. In

contrast, a significant reduction in the mean Hb concentration was observed only in the co-infected animals (10.3 g/dl), when compared to that of the non-infected animals (11.6 g/dl), whereas *T. orientalis* infection alone did not alter the mean Hb concentration (11.2 g/dl). Similar observation was also made in the mean HCT value, in which only the co-infected animals (27.8%) showed a lower value than non-infected animals (32.0%) (Table 5).

2-4. Discussion

The present study was focused on the PCR detection of *T. orientalis* and *B. ovata* in an endemic area of Japan. Subsequently, the occurrence of anemia was also examined among the different groups of animals based on their infection types. Fifty percent of the grazing cattle included in the current study were found to be positive for *T. orientalis* infection. This finding is in agreement with a previous study that had been conducted in the same district (57.8%) (61). In contrast, *B. ovata*-specific DNA was detected in 24.5% animals, and 14.9% of total blood samples were shown to be positive for both of these parasites. Because *T. orientalis* and *B. ovata* are transmitted by the same vector in Japan, such a mixed infection may be common among cattle populations in endemic areas (26). This phenomenon is further confirmed in this study, as 29.8% of

T. orientalis- and 60.9% of *B. ovata*-positive animals were co-infected with both of the parasites.

All of the non-infected animals showed the normal values for three hematological parameters assessed in the present study, and therefore, other agents that could have contributed to the anemia among the tested animals can be ruled out. The anemic animals were either infected with *T. orientalis* alone or with both of the parasites. Previous studies have indicated that the *B. ovata* infection might not result in anemia in the endemic areas, unless the infected animals are immuno-compromised (26). Therefore, none of the animals that were only infected with *B. ovata* were also anemic. However, the findings of the present study suggested that the *B. ovata* infection might be of clinical significance when the animals are co-infected with *T. orientalis*. Out of 33 animals that were positive only for *T. orientalis*, 6 of them were anemic (18.2%), whereas 6 anemic cattle were detected among 14 animals co-infected with both of the parasites (42.9%), although the difference in the mean values of RBC counts between solely *T. orientalis* infected and co-infected cattle was not significant. The animals infected only with *T. orientalis* had the same mean Hb and HCT values as non-infected animals. In contrast, co-infected animals showed relatively lower values for the mean Hb and HCT values, and the differences were statistically significant. Based on these

findings, it can be suggested that *B. ovata* might be a risk factor for the induction of clinical anemia when the animals are co-infected with *T. orientalis*, although the parasite is unlikely to be the sole agent causing anemia in the cattle population in the endemic regions. *B. ovata* induces an intra-vascular hemolysis, like the other *Babesia* parasites, and this was evident from the hemoglobinuria that had been detected when splenectomized cattle were infected with the parasite (26). On the other hand, the anemia induced by *T. orientalis* is suggested to be due to erythrophagocytosis (75). Therefore, it can be assumed that the combination of these two different mechanisms of anemia could have potentiated the serious development of clinical anemia among the animals that were co-infected with *B. ovata* and *T. orientalis*.

T. orientalis-related clinical disease and outbreaks have been reported in many regions around the world (6, 53, 61). However, involvement of *B. ovata* infection in these circumstances has not yet been addressed. One possibility is that investigators have relied largely on light microscopy for the detection of *B. ovata* and therefore, could have failed to detect the parasites in blood smears because the parasitemia of *B. ovata* is often very low in the infected animals (7). In the current study, I successfully developed a new PCR assay that was highly specific for at least 4 other blood parasites and sensitive enough to detect 100 fg of *B. ovata* genomic DNA. The *AMA-1*-PCR assay

might be a useful tool that can be employed to study the epidemiology of *B. ovata* in order to understand the clinical significance of the parasite under various circumstances. However, this method must be evaluated using many samples from different geographical locations to be certain of the practical utility of this technique as a universal diagnostic tool.

Although the effect of *T. orientalis* and *B. ovata* on the anemia status of the susceptible cattle population was described in this report, further studies using a large number of samples are necessary to generalize the current findings. In addition, animal experiments to study the effect of co-infection with both the parasites on the well being of susceptible animals are also of paramount importance.

2-5. Summary

In the current study, 94 cattle in a dairy farm located at Shin-Hidaka district in Hokkaido, Japan, were surveyed for *Theileria orientalis* and *Babesia ovata* using previously established and newly developed PCR assays, respectively. When blood samples collected from all the animals were analyzed to measure the red blood cell (RBC) count, hemoglobin concentration, and hematocrit value, anemia was found in a proportion of animals that were infected with both of the parasites (6 out of 14) and infected only with *T. orientalis* (6 out of 33). In contrast, the 9 animals that were positive only for *B. ovata* and the 38 animals that were non-infected, were not anemic. Additionally, animals infected only with *B. ovata* showed mean values similar to those of non-infected animals. Mean RBC counts of *T. orientalis*-infected and co-infected animals were lower than those of non-infected animals, while no significant difference was observed in the RBC counts between the *T. orientalis*-infected and co-infected animals. In contrast, only the co-infected animals showed lower mean values for hemoglobin concentration and the hematocrit value when compared to those of non-infected animals. These findings indicate that, while *B. ovata* might not cause anemia in endemic areas as a sole agent, the parasite may contribute to the clinical development of anemia when co-infected with *T. orientalis*.

Table 3. Summary of diagnostic results in 94 field grazing cattle from *T. orientalis MPSP*-PCR and *B. ovata AMA-1*-PCR assays.

Infection type*	Positive animals (%)	Anemic animals	Anemia rate (%)
Non-infected	38 (40.4)	0	0
*T. orientalis***	33 (35.1)	6	18.2
*B. ovata***	09 (9.6)	0	0
T. orientalis & *B. ovata*	14 (14.9)	6	42.9

*Animals are categorized into 4 groups, which consist of non-infected, infected only with *T. orientalis*, infected only with *B. ovata,* and co-infected animals, based on the infection types.

** Animals infected with a single parasite

Table 4. Hematological values observed in the anemic animals.

Infection type	Animal ID	RBC count (x 10^6/μl)	Hb conc. (g/dl)	HCT value (%)
T. orientalis	189	**4.93**	9.4	25.5
(n = 33)	204	**4.48**	9.6	24.9
	208	**4.11**	9.2	**23.7**
	219	**4.3**	8.9	**23.5**
	320	**3.81**	9.1	25.2
	506	**4.96**	**7.9**	**21.6**
T. orientalis & *B. ovata*	111	**4.84**	9	**23.4**
(n =14)	117	**4.79**	10.9	28.3
	129	**4.41**	9.9	25.6
	230	**4.95**	9.5	24.7
	515	**3.41**	**7.1**	**19.9**
	660	**4.97**	**7**	**19.8**

Anemia values are highlighted in boldface.

Note that all the anemia samples had abnormally low values for the RBC count.

Table 5. Mean values of RBC count, Hb and HCT observed for different categories of animals.

Infection type	Mean value ± Confidence interval		
	RBC count ($x\ 10^6/\mu l$)	**Hb conc.** (g/dl)	**HCT value** (%)
Non-infected	7.29 ± 0.29	11.6 ± 0.31	32.0 ± 0.91
T. orientalis	5.90 ± 0.35 *	11.2 ± 0.49	30.1 ± 1.39
B. ovata	7.24 ± 0.63	11.4 ± 0.44	31.7 ± 1.45
T. orientalis & *B. ovata*	5.64 ± 0.56 *	10.3 ± 0.88 *	27.8 ± 2.33 *

* Statistically significant reduction in the mean values as compared to those of non-infected animals ($P < 0.01$).

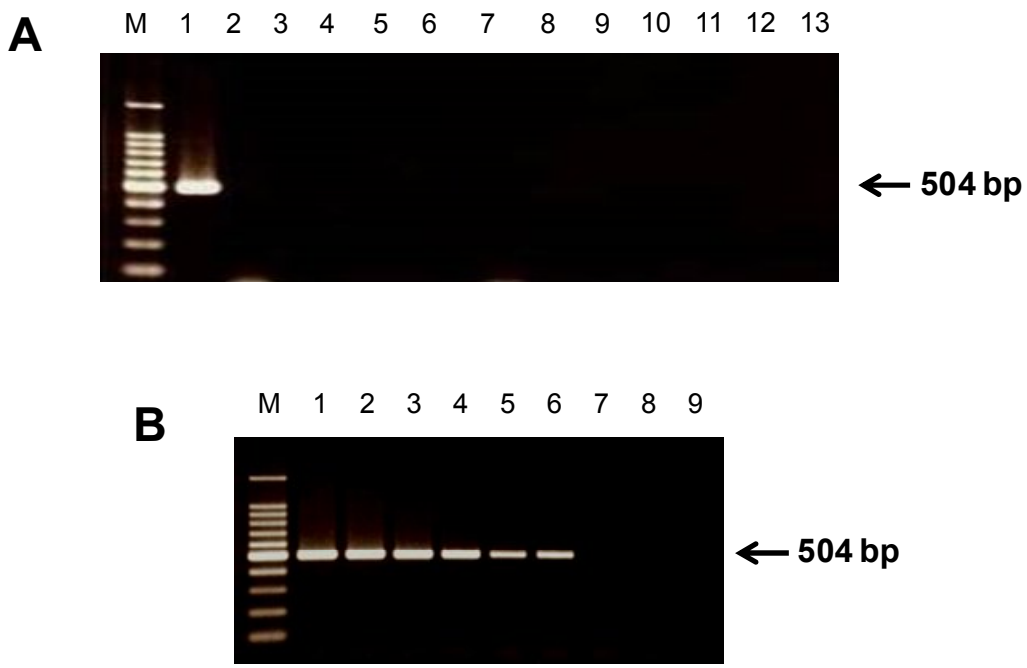

Fig. 5. *Babesia ovata*-specific *AMA-1*-PCR assay development. A new PCR method was developed for the diagnostic detection of *B. ovata,* based on the *B. ovata AMA-1* gene. Panel A: PCR specificity. Lanes 1-13 represent genomic DNA samples from *B. ovata*, *B. bigemina*, *B. bovis*, *Theileria annulata*, *T. orientalis*, *Trypanosoma brucei gambiense*, *T. evansi*, *T. theileri*, *Aanaplasma marginale*, *A. bovis*, *A. centrale*, *A. phagocytophilum,* and normal bovine blood, respectively. Panel B: PCR sensitivity. 10-fold serial dilutions of *B. ovata* genomic DNA extracted from *in vitro* culture. Lanes 1-8 represent 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 100 fg/μl, 10 fg/μl, and 1 fg/μl, respectively. An uninfected bovine DNA sample was included as a negative control (lane 9). M, 100-bp DNA marker ladder appears in both panels.

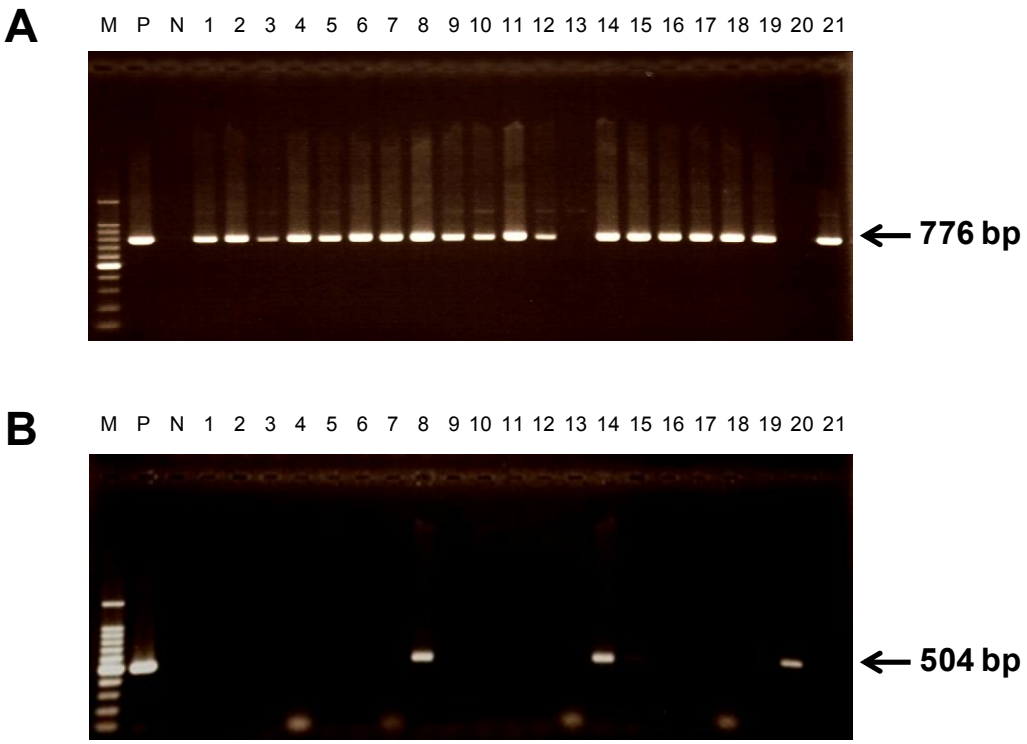

Fig. 6. DNA samples extracted from the blood of cattle bred on a Shin-Hidaka farm that were PCR screened for *T. orientalis* (Panel A) and *B. ovata* (Panel B). Lane M, 100-bp DNA ladder marker, P, Positive control (*T. orientalis* or *B. ovata* DNA), N, Negative control (Normal bovine blood DNA). Lanes 1-21 indicate the sample numbers of field blood DNAs.

Chapter 3

Genetic diversity of *Babesia bovis* in Sri Lankan cattle and its potential implications for live-vaccine usage

3-1. Introduction

Hemoprotozoan parasitic infections in cattle are considered to be a major cause of economic losses to cattle farmers worldwide. Among them, bovine babesiosis is widely distributed in most tropical and sub-tropical regions of the world, where the transmission vectors of the *Babesia* parasites, the Ixodid ticks, are present (72). Although several species of *Babesia* parasites cause bovine babesiosis, *B*. *bovis* and *B*. *bigemina* are responsible for the most severe disease manifestations in the infected cattle (52). In particular, infection with *B*. *bovis* is often fatal in cattle, because in addition to the anemia seen with other *Babesia* spp., *B*. *bovis* infection is associated with neurological and respiratory disorders (21, 62). Therefore, control of *B*. *bovis* infection is an important issue for veterinary authorities in *Babesia*-endemic countries.

The control strategies for *B*. *bovis* include tick control, chemotherapy, and vaccination (13). Tick control methods are often challenged by the rapid development of acaricide resistance in the insects (30). In addition, climatic change and the movement

of ticks to non-endemic areas are also relevant to effective tick control (77). Early diagnosis should be followed by a suitable treatment regime to obtain a better prognosis for the affected animals; nevertheless, the disease can be fatal when untreated (85). Although several studies have focused on the development of a sub-unit vaccine (28, 74), only live attenuated vaccines are currently available for prevention of clinical babesiosis caused by *B*. *bovis*.

Live attenuated vaccines are currently used in several countries, including Australia, Argentina, Brazil, Uruguay, South Africa, Israel, Colombia, Sri Lanka, Zimbabwe, and Malawi (64). Australia has a long history of using these vaccines against *B*. *bovis* infection. Initially, the K-strain of *B. bovis* was used to immunize cattle (11); however, the numbers of clinical babesiosis cases were increasingly reported in the vaccinated cattle (11). Therefore, the K-strain was replaced with the T-strain, and later with the Dixie strain. The vaccine strain was changed in the light of field evidence reporting vaccination failure (11). According to the Australian investigators, the immune-resistant populations of *B*. *bovis*, which could have been present as a minority during the initial stages of vaccination, may have increased in number and caused disease outbreaks in vaccinated animals (12).

Studies conducted in the post-genomic era have found that the merozoite

surface antigens (MSAs) of outbreak isolates and vaccine strains were genetically diverse. In addition, poor immunological cross-reactivity between outbreak isolates and vaccine strains has also observed (10, 50). Therefore, it has been speculated that the genetic variation observed in the *MSAs* could have been responsible for the disease outbreaks among the vaccinated animals.

In Sri Lanka, our recent study confirmed that *B*. *bovis* is still endemic, and that about 14% of the surveyed cattle were positive for the parasite (69). A live attenuated vaccine of the Australian K-strain has been used to immunize cattle since the early 1990s in Sri Lanka (87). Although the vaccine has been used for over 20 years, the compatibility between field isolates and the K-vaccine strain has not been studied yet. Hence, in the present study, gene sequences encoding MSAs were determined using *B. bovis*-positive field samples from Sri Lanka, and the findings were then compared with the corresponding gene sequences of the K-strain.

3-2. Materials and methods

DNA samples: Three hundred and sixteen blood samples collected in Sri Lanka (May–June, 2011) from cattle reared in four provinces (69). When the DNA extracted from the blood samples was screened using a *B*. *bovis*-specific nested PCR

assay, 44 were judged to be positive for *B. bovis* infection (69); these were used for the present study.

PCR amplification of target genes: Examination of multiple sequence alignments of genes available in GenBank enabled us to design PCR primers to amplify *MSA-1*, *MSA-2c*, *MSA-2a1*, *MSA-2a2*, *MSA-2b*, *AMA-1*, and *12D3* genes (Table 6). With the exception of the *MSA-1* gene, all of the other primers were designed to amplify the full-length sequences of the genes. For all *MSA* gene sequences, a common reverse primer (MSA R) was used based on the conserved nature of the GPI-anchor region of these genes. In addition, for the amplification of a partial *MSA-1* gene sequence, two types of forward primers were designed. One (MSA-1 F1) was targeted against some of the Australian sequences (GenBank accession: DQ028741, DQ028743, DQ028746, and DQ028747), while another (MSA-1 F2) was designed for the remaining sequences that were retrieved from GenBank. A single forward primer (MSA-2a1/2a2/2b F) was used to amplify *MSA-2a1*, *MSA-2a2*, and *MSA-2b*, because the 5' ends of these genes were, in common with the 3' ends, also highly conserved. PCR amplification of the target genes was carried out using a 30 µl reaction mixture, which included 2 µl of a *B. bovis*-positive DNA sample, 3 µl of 10 x PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 200 µM of each dNTP (Applied Biosystems), 1 unit of Taq polymerase

(Applied Biosystems), 1 μM of the forward and reverse primers, and double distilled-water. PCR cycling conditions were as follows: an initial enzyme activation step at 95°C for 5 min was followed by 45 cycles, each consisting of a denaturing step at 95°C for 1 min, an annealing step at 52°C for 1 min, and an extension step at 72°C for 2 min. After a final elongation step at 72°C for 10 min, the PCR products were subjected to agarose gel-electrophoresis, ethidium bromide staining, and were visualized under UV light. *MSA-2b* amplicons were differentiated from *MSA-2a1* and *MSA-2a2* PCR amplicons, which appear as single bands according to their sizes (i.e, *MSA-2b* is shorter than *MSA-2a1*/*MSA-2a2*).

Cloning and sequencing: PCR products were cloned and sequenced as described previously (71). Briefly, PCR amplicons were gel-extracted (Alquick Gel Extraction Kit, Qiagen, Hilden, Germany), ligated to a PCR 2.1 plasmid vector (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, USA), transformed into *Escherichia coli* (TOP 10, Invitrogen), and then plated onto LB agar plates (Invitrogen). Three clones were picked per sample and the sequences of the inserts were determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems). For *MSA-2a1* and *MSA-2a2* genes, five clones were sequenced per amplicon.

Phylogenetic analyses: Gene sequences were analyzed using GENETYX 7.0

software (GENETYX, Tokyo, Japan) and the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/). In addition to these sequences, the *MSA-1*, *MSA-2c*, *MSA-2a1*, *MSA-2a2*, and *MSA-2b* gene sequences already deposited in GenBank were used to construct the phylogenetic trees. *MSA-2a1* and *MSA-2a2* sequences were used to construct a single phylogram. Australian *MSA-2a*/*b* sequences were used for both of the *MSA-2a1*/*MSA-2a2* and *MSA-2b* gene-based phylogenetic tress. The MAFFT software program (available online) was used to construct the phylogenetic trees (41).

Estimation of the percentage similarity scores among the deduced amino acid sequences: The gene sequences generated in the present study were translated into amino acid sequences using the Transeq program (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The similarity scores among the amino acid sequences determined were calculated using the online program EMBOSS NEEDLE (http://emboss.bioinformatics.nl/cgi-bin/emboss/needle). Sequence similarity scores between the Sri Lankan and K-strain MSAs were also determined.

3-3. Results

Although all of the 44 DNA samples that tested *B. bovis*-positive were subjected to PCR amplification to isolate and clone the six genes, visible bands were

obtained from only 23 of the samples (Table 7). Low numbers of sequences were obtained for *MSA-2a2* (n=3), *AMA-1* (n=6), and *MSA-1* (n=8) genes, while the highest numbers of sequences were determined for *MSA-2c* and *MSA-2b* genes (both, n=18). Among the *MSA-1* sequences, 7 (AB787574 - AB787580) of 8 were amplified using the MSA-1 F1 primer, while the PCR products from the MSA-1 F2 primer resulted in a single sequence (AB787581) only. For the *MSA-2a1* and *MSA-2b* sequences, two variants were obtained from one (P81; AB787604 and AB787605) and three (P31; AB787614 and AB787615, P75; AB787620 and AB787621, and AK29; AB787629 and AB787630) *B*. *bovis*-positive DNA samples, but identical sequences were obtained from different clones for all of the other amplicons (Table 7). With the exception of the *MSA-2a2*, *AMA-1*, and *12D3* gene sequences, which were of equal lengths (936, 1,818, and 1,038 bp, respectively), size differences were often observed for all of the other gene sequences (i.e., *MSA-1*: 853–936, *MSA-2c*: 783–798, *MSA-2a1*: 933–1,008, and *MSA-2b*: 789–828 bp). Additionally, with the exception of two shorter sequences (AB787590 and AB787593, 783 bp), all of the other *MSA-2c* sequences were of similar size (798 bp).

In the phylogenetic analysis, *MSA-1* sequences split into a total of fourteen clades of which the Sri Lankan sequences were seen in five different clades (clades 1, 4,

5, 6, and 7) (Fig. 7). Importantly, four of these five clades (clades 1, 4, 5, and 6) only comprised the Sri Lankan sequence(s), and formed a major branch with isolates from the Australian outbreaks, including a sequence from the K-vaccine-outbreak isolate (DQ028743 in clade 2). Additionally, a single Sri Lankan sequence (AB787581) clustered with many sequences derived from several other countries to form clade 7. Similarity values among the deduced amino acid sequences of MSA-1 from Sri Lanka were determined as 36.8–100%, while those between the Sri Lankan and K-strain (DQ028736) MSA-1 sequences were 40.0–61.8% (Fig. 8).

MSA-2c gene sequences clustered into three clades in the phylogenetic tree, while the Sri Lankan sequences were found in two different clades (clades 1 and 3) (Fig. 9). Out of the 18 Sri Lankan sequences, 16 were located in clade 3, in which a majority of the sequences derived from several other countries were found. In contrast, two shorter Sri Lankan *MSA-2c* sequences (AB787590 and AB787593) appeared in clade 1, together with a similarly shorter sequence from an Australian K-vaccine-outbreak isolate (DQ173962). The similarity values among the Sri Lankan MSA-2c amino acid sequences were determined as 68.7–100%, whereas those between the Sri Lankan and K-strain (DQ173970) were 69.8 –93.2% (Fig. 10).

In the *MSA-2a1*/*MSA-2a2*-based phylogram, the sequences can be seen to

cluster into thirteen clades (Fig. 11). While the Sri Lankan *MSA-2a2* sequences appear in a single clade (clade 2), the *MSA-2a1* sequences are dispersed across four different clades (clades 6, 8, 10, and 12). Out of 11 *MSA-2a1* sequences, 6 clustered together with several Argentine *MSA-2a1* sequences in clade 6, with a total of three and one of the sequences forming clades 8 and 12, respectively. Furthermore, another Sri Lankan sequence (AB787605) clustered in clade 10, together with those from the USA (XM_001608901) and Mexico (AY052538). Similarity values among the deduced MSA-2a1 and MSA-2a2 amino acid sequences were 80.3–100 and 100%, respectively. In addition, the K-strain MSA-2a/b (DQ173954) amino acid sequence shared 64.5–69.8 and 69.3% similarity with the Sri Lankan MSA-2a1 and MSA-2a2 sequences, respectively (Fig. 12). MSA-2a1 has previously been reported to contain two repeat sequences of 24 amino acids in length between positions 212 and 259 (25), but the two MSA-2a1 sequences (AB787600 and AB787602) from Sri Lanka analyzed here had three repeat sequences between amino acid positions 212 and 283 (data not shown).

In the *MSA-2b* phylogram, all of the sequences fell into seventeen clades, while the Sri Lankan sequences were detected in nine of the clades (clades 2, 3, 4, 7, 9, 10, 12, 13, and 14), seven of which were formed only by the Sri Lankan sequence(s) (clades 2, 3, 4, 7, 9, 10, and 14) (Fig. 13). Among these seven clades, four (clades 2, 3, 4, and 7)

were located closely to the clusters of Australian sequences (clades 5, 6, and 8). The sequence similarity scores among the deduced MSA-2b amino acid sequences from Sri Lanka were 68.3–100%, and those between the MSA-2b sequences of the Sri Lankan isolates and K-strain (DQ173954 in clade 1) were 70.5–80.3% (Fig. 14).

Although high genetic diversity was observed among the *MSAs* genes, the *AMA-1* and *12D3* genes were highly conserved among the Sri Lankan isolates as well as those from elsewhere. The similarities among the Sri Lankan AMA-1 and 12D3 amino acid sequences were 99.8–100 and 99.7–100%, respectively (data not shown).

3-4. Discussion

The genetic diversity observed among *B. bovis MSA* genes suggests the presence of antigenically different populations of *B. bovis* worldwide (10, 50). Antigenically diverse parasites could evade protective immune responses that may have developed in cattle during past exposure to *B. bovis* parasites (17). Therefore, genetic diversity in *B. bovis* should be investigated in order to establish proper immune control strategies in *B. bovis*-endemic countries. In the present study, genetic variation was analyzed among Sri Lankan *B. bovis* isolates, and the findings were compared with the sequence data from the currently used vaccine strain (K-strain). Our findings show that

the Sri Lankan *B. bovis* isolates were genetically diverse, and that conservation between the Sri Lankan and K-strain *MSA* sequences was poor.

With the exception *MSA-2a2*, which formed a monophyletic clade in the phylogram, the sequences of the other *MSA* genes appeared in multiple clades in the respective phylogenetic trees. In addition, the similarity values of the deduced amino acid sequences were very low among the Sri Lankan populations. The *MSA-2c* gene is considered to be relatively conserved among *MSAs* (4). However, a previous study, in which a shorter sequence was detected in an Australia isolate, hinted that *MSA-2c* might also be highly polymorphic (10). Detection of two shorter sequences in the present study provides further confirmation that *MSA-2c* sequences are highly diverse. Furthermore, two MSA-2a1 sequences with three repeat regions were detected, indicating the high sequence diversity of this gene. Taken together, our findings clearly show that the *B. bovis* isolates from Sri Lanka are genetically diverse.

The studies conducted in Australia suggest that virulent *B. bovis* parasites that differ antigenically from the vaccine strains might trigger disease outbreaks in vaccinated animals (50). Thus, if the Sri Lankan isolates differ in virulence and antigenically to the K-strain, disease outbreaks could be inevitable in vaccinated cattle. Crucially, although the Australian outbreak isolates were genetically divergent from

their respective vaccine strains, it has not yet been established whether antigenically divergent *B*. *bovis* virulent strains could cause clinical babesiosis in vaccinated animals. However, in a previous study, the vaccines failed to protect the vaccinated animals when experimentally challenged with outbreak isolates obtained from cattle immunized with the same vaccines (12). This suggests that, regardless of other animal factors, antigenic differences in the parasites could be one of the prime causes of vaccine failures.

In the present study, low similarity scores were observed when the deduced amino acid sequences of Sri Lankan *MSAs* were compared with those of the K-strain. These findings are comparable to those obtained in previous studies in which the K-strain MSA-1 and MSA-2a/b amino acid sequences were compared with those of the outbreak isolates obtained from cattle previously immunized with the K-strain (10, 50). In the phylogenetic analyses, none of the Sri Lankan *MSA* family sequences were found in the clades where the K-strain sequences were located. Interestingly, some of the Sri Lankan sequences were observed together with or closer to the Australian K-vaccine-outbreak isolates (F-series isolates). These data collectively suggest that genetic conservation is poor between the K-vaccine strain and Sri Lankan field isolates. Consequently, further studies in Sri Lanka are now essential to analyze the antigenic variation between a larger Sri Lankan isolate dataset and the K-strain.

In conclusion, the present study has shown that *B*. *bovis* parasites in Sri Lankan cattle populations are genetically diverse, and that the isolates are genetically divergent from the currently used K-strain vaccine. The findings highlight the importance of conducting further studies to determine the level of protection of the K-strain against different field isolates of *B*. *bovis* in Sri Lanka.

3-5. Summary

Babesia bovis, the causative agent of severe bovine babesiosis, is endemic in Sri Lanka. The live attenuated vaccine (K-strain), which was introduced in the early 1990s, has been used to immunize cattle populations in endemic areas of the country. The present study was undertaken to determine the genetic diversity in *B. bovis* isolates from Sri Lankan cattle, and to compare the gene sequences obtained from such isolates against those of the K-strain. Forty-four bovine blood samples isolated from different geographical regions of Sri Lanka and judged to be *B. bovis*-positive by PCR screening were used to amplify the genes encoding merozoite surface antigens (MSAs: MSA-1, MSA-2c, MSA-2a1, MSA-2a2, and MSA-2b), 12D3, and AMA-1 from parasite DNA. Although the *AMA-1* and *12D3* gene sequences were highly conserved among the Sri Lankan isolates, the *MSA* gene sequences from the same isolates were highly diverse. Sri Lankan *MSA-1*, *MSA-2c*, *MSA-2a1*, *MSA-2a2,* and *MSA-2b* sequences clustered within 5, 2, 4, 1, and 9 different clades in the gene phylograms, respectively, while the minimum similarity values among the deduced amino acid sequences of these genes were 36.8, 68.7, 80.3, 100, and 68.3%, respectively. In the phylograms, none of the Sri Lankan sequences fell within clades containing the respective K-strain sequences. Additionally, the similarity values for MSA-1 and MSA-2c amino acid sequences were

40–61.8 and 90.9–93.2% between the Sri Lankan isolates and the K-strain, respectively, while the K-strain MSA-2a/b sequence shared 64.5–69.8, 69.3, and 70.5–80.3% similarity with the Sri Lankan MSA-2a1, MSA-2a2, and MSA-2b amino acid sequences, respectively. The present study has shown that genetic diversity among Sri Lankan *B. bovis* isolates is very high, and importantly, from a vaccine efficacy perspective that the field isolates diverged genetically from the K-strain.

Table 6. PCR primers used to amplify target genes in *B. bovis*.

Primers	Oligonucleotide sequences (5' - 3')
MSA-1 F1 [a]	AGTACTTACCTTTTTAATGACAGCCG
MSA-1 F2 [a]	TGGCAATTACATCGGCGGGTG
MSA-2c F	ATGGTGTCTTTTAACATAATAACC
MSA-2a1/2a2/2b F [b]	ATGATCGGGAAAATCTTCTTGTTAA
MSA R [c]	TTAAAATGCAGAGAGAACGAAGTAGC
12D3 F	ATGTTGGCTACACGTTTTGTTTTTAGTC
12D3 R	AAGCTCCTGCCTTTCGCTCG
AMA-1 F	ATGCAGTTACATTACAAAATGCAG
AMA-1 R	TTAGTTGATTTTAGAGAGTTTTAC

[a] Two different types of forward primers were used to amplify the *MSA-1* gene sequences.

[b] A single forward primer was used to amplify *MSA-2a1*, *MSA-2a2*, and *MSA-2b* gene sequences simultaneously.

[c] A common reverse primer was used to amplify all five *MSA* genes.

Table 7. GenBank accession numbers of the gene sequences obtained in the present study.

District	Isolate	GenBank accession numbers						
		MSA-1	*MSA-2c*	*MSA-2a 1*	*MSA-2a2*	*MSA-2b*	*AMA-1*	*12D3*
Nuwara Eliya	H4		AB787582					
	H25	AB787574						AB787638
Polonnaruwa	P31	AB787575	AB787583	AB787600	AB787611	AB787614, AB787615[a]		AB787639
	P32			AB787601		AB787616	AB787632	AB787640
	P53	AB787576	AB787584					AB787641
	P58	AB787577	AB787585		AB787612	AB787617	AB787633	AB787642
	P61		AB787586	AB787602	AB787613	AB787618		AB787643
	P65					AB787619		
	P70		AB787587					AB787644
	P75		AB787588	AB787603		AB787620, AB787621[a]		AB787645
	P81		AB787589	AB787604, AB787605[a]		AB787622		
Ampara	AT7					AB787623		AB787646
	AT9		AB787590	AB787606		AB787624	AB787634	AB787647
	AT21		AB787591					
	AM1		AB787592					AB787648
	AM2		AB787593			AB787625		AB787649
	AM13		AB787594			AB787626	AB787635	
	AM14							
	AM16		AB787595					AB787650
	AM21	AB787578	AB787596	AB787607		AB787627		
	AM24		AB787597	AB787608		AB787628		AB787651
	AK29	AB787579	AB787598	AB787609		AB787629, AB787630[a]	AB787636	AB787652
Jaffna	J6	AB787580, AB787581[b]	AB787599	AB787610		AB787631	AB787637	AB787653
Total		**8**	**18**	**11**	**3**	**18**	**6**	**16**

[a] Two different sequences were determined for this *B. bovis*-positive DNA sample.

[b] The sequence amplified by the MSA-1 F2 primer.

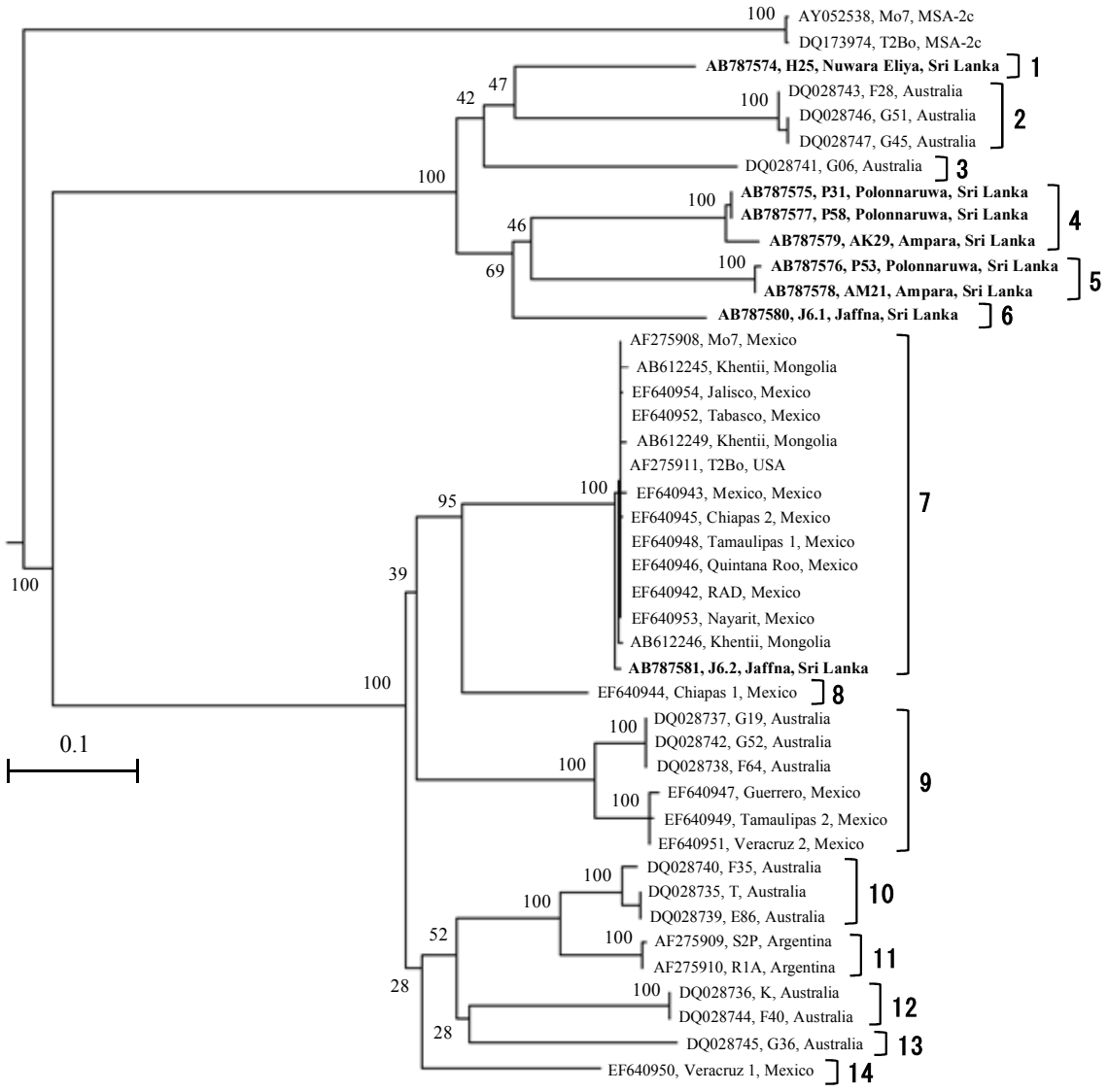

Fig. 7. Phylogenetic analysis of *MSA-1* gene sequences. Boldface type letters indicate the sequences determined in the present study. Note that the Sri Lankan sequences fall into five different clades.

Clade		**1**	**4**			**5**		**6**	**7**	**12**
Acc. No.		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**K**
AB787574	**1**	100	63.7	63.7	62.4	60.8	60.9	59.8	38.6	**40.0**
AB787575	**2**		100	100	84.8	64.5	64.8	70.2	37.1	46.8
AB787577	**3**			100	84.8	64.2	64.4	69.8	37.1	46.7
AB787579	**4**				100	65.5	65.5	67.0	**36.8**	41.6
AB787576	**5**					100	97.5	66.0	40.5	43.0
AB787578	**6**						100	67.9	41.0	42.9
AB787580	**7**							100	38.9	42.9
AB787581	**8**								100	61.8
DQ028736	**K**									100

Fig. 8. Similarity analyses of MSA-1 amino acid sequences. The deduced MSA-1 amino acid sequences from Sri Lanka, together with that of K-strain (K), were analyzed to determine their percentage similarities. The lowest similarity among the Sri Lankan isolates was 36.8%, while the minimum similarity score shared with the K-strain was 40.0%.

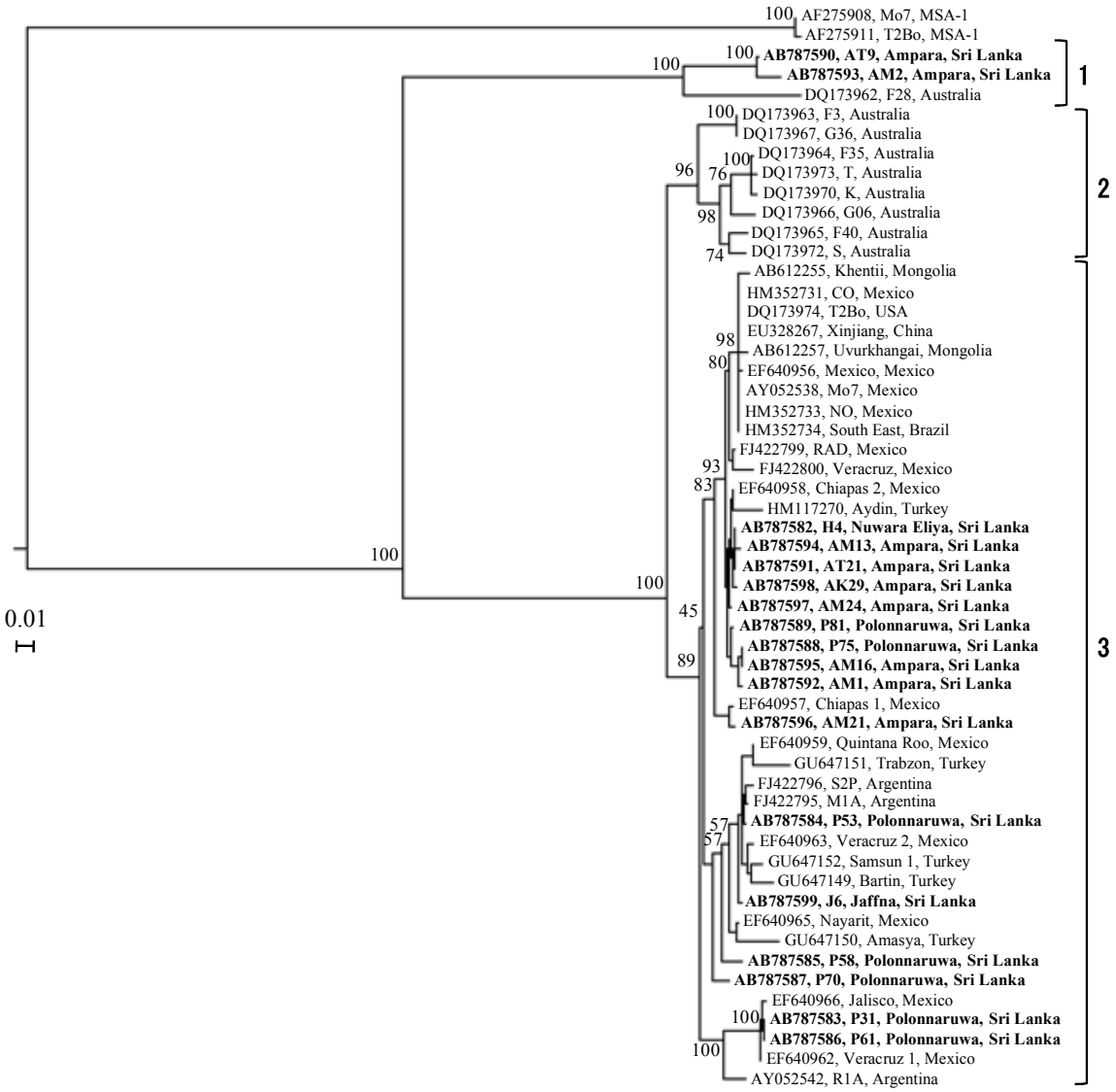

Fig. 9. Phylogenetic analysis of *MSA-2c* gene sequences. Boldface type letters indicate the sequences determined in the present study. Note that the Sri Lankan sequences fall into two different clades.

Clade		1		3															2
Acc. No.		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**K**
AB787590	**1**	100	99.2	69.8	69.8	69.8	69.8	69.8	69.1	69.8	69.8	69.8	69.1	**68.7**	**68.7**	**68.7**	69.1	69.1	69.1	**69.8**
AB787593	**2**		100	69.8	69.8	69.8	69.8	69.8	69.1	69.8	69.8	69.8	69.1	**68.7**	**68.7**	**68.7**	69.1	69.1	69.1	**69.8**
AB787582	**3**			100	99.2	100	99.6	99.6	99.2	99.2	99.2	98.9	97.7	95.1	95.5	96.2	98.1	96.2	96.2	92.8
AB787594	**4**				100	99.2	98.9	98.9	98.5	98.5	98.5	98.1	97.0	94.3	94.7	95.5	97.4	95.5	95.5	92.8
AB787591	**5**					100	99.6	99.6	99.2	99.2	99.2	98.9	97.7	95.1	95.5	96.2	98.1	96.2	96.2	92.8
AB787598	**6**						100	99.2	98.9	98.9	98.9	98.5	97.4	95.5	95.8	95.8	97.7	96.6	96.6	93.2
AB787597	**7**							100	99.6	99.6	99.6	99.2	98.1	95.5	95.8	96.6	98.5	96.6	96.6	93.2
AB787589	**8**								100	99.2	99.2	98.9	97.7	95.1	95.5	97.0	98.1	96.2	96.2	92.8
AB787588	**9**									100	100	99.6	97.7	95.1	95.5	96.2	98.1	97.0	97.0	92.8
AB787595	**10**										100	99.6	97.7	95.1	95.5	96.2	98.1	97.0	97.0	92.8
AB787592	**11**											100	97.4	94.7	95.1	95.8	97.7	96.6	96.6	92.5
AB787596	**12**												100	97.4	97.7	97.0	97.4	94.7	94.7	91.3
AB787584	**13**													100	99.6	97.4	96.2	93.6	93.6	90.9
AB787599	**14**														100	97.7	96.6	94.0	94.0	91.3
AB787585	**15**															100	96.6	94.0	94.0	91.7
AB787587	**16**																100	95.8	95.8	92.5
AB787583	**17**																	100	100	91.7
AB787586	**18**																		100	91.7
DQ173970	**K**																			100

Fig. 10. Similarity analyses of MSA-2c amino acid sequences. The deduced MSA-2c amino acid sequences from Sri Lanka, together with that of K-strain (K), were analyzed to determine their percentage similarity scores. The lowest score among the Sri Lankan isolates was 68.7%, while the minimum similarity shared with the K-strain was 69.8%.

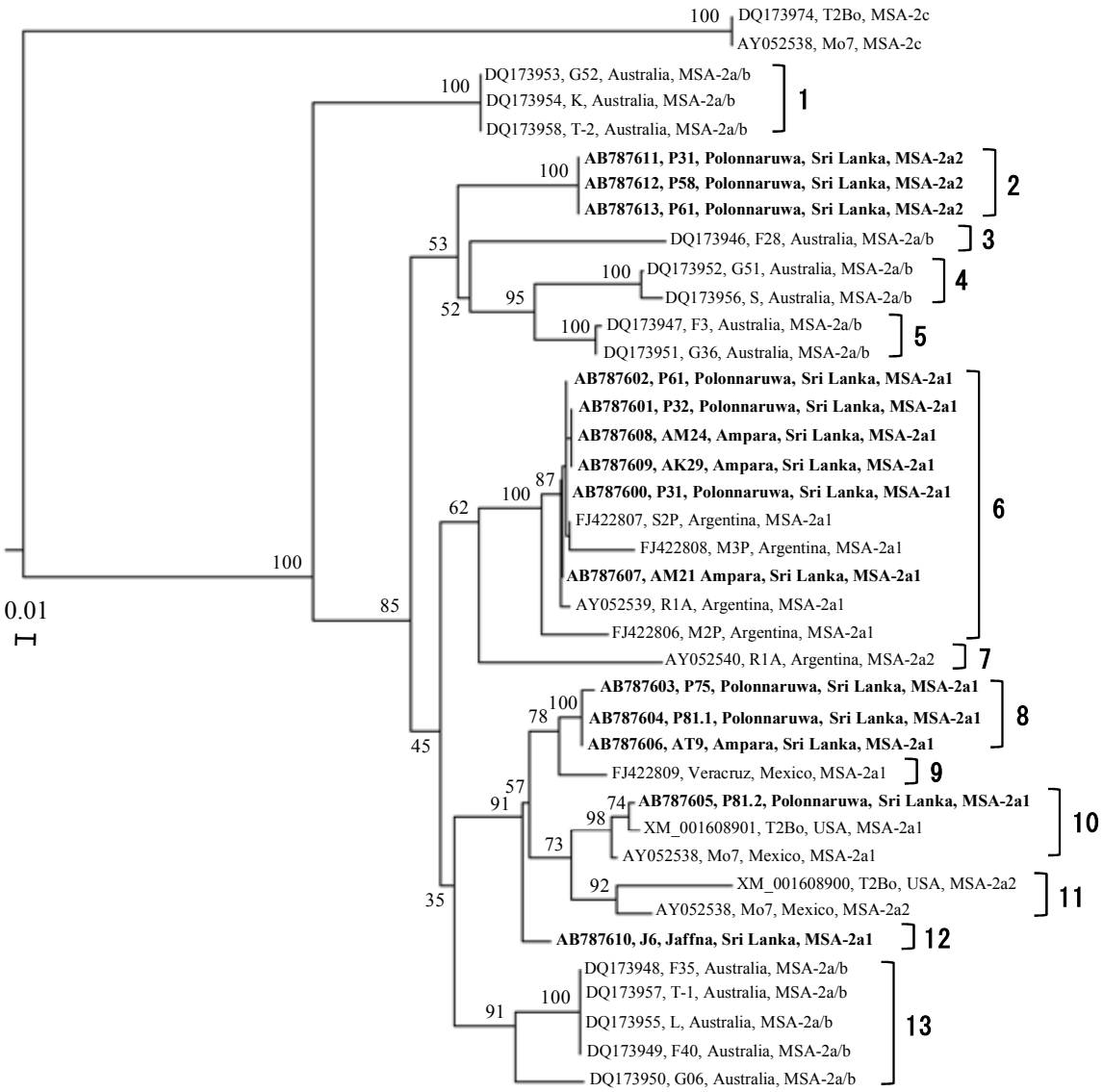

Fig. 11. Phylogenetic analysis of *MSA-2a1* and *MSA-2a2* gene sequences. Boldface type letters indicate the sequences determined in the present study. Note that the Sri Lankan *MSA-2a1* sequences fall into four different clades, while the *MSA-2a2* sequences formed a single clade.

Gene		MSA-2a1											MSA-2a2			MSA-2a/b
Clade		6						8			10	12	2			1
Acc. No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	K
AB787602	**1**	100	91.9	91.9	91.6	100	91.9	80.3	80.3	80.6	81.8	85.1	81.2	81.2	81.2	**64.5**
AB787601	**2**		100	100	99.7	91.9	98.7	85.9	85.9	85.9	87.7	91.3	87.5	87.5	87.5	69.8
AB787608	**3**			100	99.7	91.9	98.7	85.9	85.9	85.9	87.7	91.3	87.5	87.5	87.5	69.8
AB787609	**4**				100	91.6	98.4	85.5	85.5	85.5	87.4	91.0	87.1	87.1	87.1	69.8
AB787600	**5**					100	91.9	**80.3**	**80.3**	80.6	81.8	85.1	81.2	81.2	81.2	**64.5**
AB787607	**6**						100	86.2	86.2	86.5	87.7	91.3	88.1	88.1	88.1	69.5
AB787603	**7**							100	100	99.7	91.5	94.2	86.5	86.5	86.5	68.7
AB787604	**8**								100	99.7	91.5	94.2	86.5	86.5	86.5	69.0
AB787606	**9**									100	91.8	94.5	86.8	86.8	86.8	69.0
AB787605	**10**										100	96.2	87.1	87.1	87.1	67.9
AB787610	**11**											100	90.4	90.4	90.4	68.7
AB787611	**12**												100	100	100	**69.3**
AB787612	**13**													100	100	**69.3**
AB787613	**14**														100	**69.3**
DQ173954	**K**															100

Fig. 12. Similarity analyses of MSA-2a1 and MSA-2a2 amino acid sequences. Deduced MSA-2a1 and MSA-2a2 amino acid sequences from Sri Lanka, together with the MSA-2a/b sequence of the K-strain (K), were analyzed to determine their percentage similarity scores. The lowest score among the Sri Lankan isolates for MSA-2a1 amino acid sequences was 80.3%, while the MSA-2a2 sequences were identical to each other. The minimum similarity scores shared by MSA-2a1 and MSA-2a2 with the K-strain MSA-2a/b were 64.5 and 69.3%, respectively.

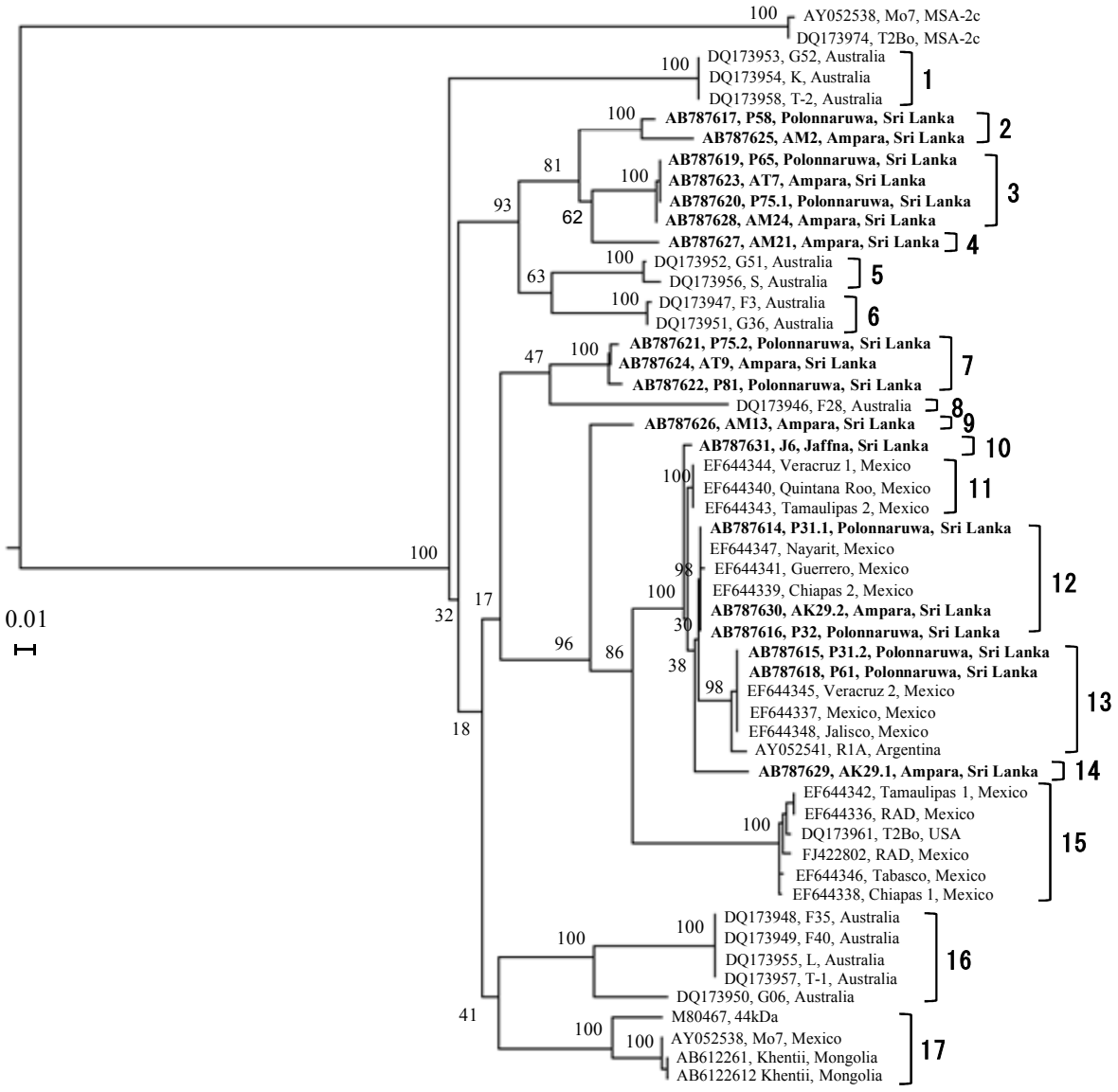

Fig. 13. Phylogenetic analysis of *MSA-2b* gene sequences. Boldface type letters indicate the sequences determined in the present study. Note that the Sri Lankan sequences fall into nine different clades.

Clade		2		3				4	7			9	10	12			13		14	1
Acc. No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	K
AB787617	1	100	95.1	92.5	92.5	92.9	92.5	91.7	83.5	83.8	83.8	77.4	78.9	77.8	77.4	78.2	68.9	68.9	82.0	78.5
AB787625	2		100	89.5	89.5	89.5	89.1	88.7	82.9	83.3	83.3	76.4	76.9	76.8	76.4	77.2	**68.3**	**68.3**	81.0	75.9
AB787619	3			100	100	99.6	99.2	93.6	82.7	83.1	83.1	77.4	79.7	78.2	77.8	78.6	70.1	70.1	80.5	78.8
AB787623	4				100	99.6	99.2	93.6	82.7	83.1	83.1	77.4	79.7	78.2	77.8	78.6	70.1	70.1	80.5	78.8
AB787620	5					100	99.6	93.2	83.1	83.5	83.5	77.8	79.7	78.6	78.2	78.9	69.6	69.6	80.8	78.5
AB787628	6						100	92.9	82.7	83.1	83.1	77.4	79.3	78.2	77.8	78.6	69.3	69.3	80.5	78.1
AB787627	7							100	82.3	82.7	82.7	78.9	80.5	78.2	78.6	78.6	70.4	70.4	80.1	79.6
AB787621	8								100	99.6	99.6	89.3	84.8	86.6	86.3	86.3	76.0	76.0	87.4	78.5
AB787624	9									100	100	89.7	85.2	87.0	86.6	86.6	76.3	76.3	87.8	78.8
AB787622	10										100	89.7	85.6	87.4	87.0	87.0	76.7	76.7	88.2	78.8
AB787626	11											100	93.9	95.4	95.8	95.0	85.3	85.3	88.5	74.8
AB787631	12												100	97.0	97.3	96.6	87.5	87.5	90.9	75.9
AB787614	13													100	99.6	99.6	88.2	88.2	92.0	75.2
AB787630	14														100	99.2	88.5	88.5	92.4	74.8
AB787616	15															100	87.8	87.8	91.6	75.2
AB787615	16																100	100	81.4	**70.5**
AB787618	17																	100	81.4	**70.5**
AB787629	18																		100	80.3
DQ173954	K																			100

Fig. 14. Similarity analyses of MSA-2b amino acid sequences. Deduced MSA-2b amino acid sequences from Sri Lanka, together with the MSA-2a/b sequence of the K-strain (K), were analyzed to determine their percentage similarities. The lowest similarity score among Sri Lankan isolates was 68.3%, while the minimum similarity score shared with the K-strain was 70.5%.

Chapter 4

Genetic diversity within *Theileria orientalis* parasites detected in Sri Lankan cattle

4-1. Introduction

Hemoprotozoan parasites that infect cattle are of worldwide clinical and economic importance (82, 86). Many different species of *Babesia*, *Theileria*, and *Trypanosoma* parasites are known to have a detrimental impact on cattle health (52). Among such parasites, a group of non-lymphoproliferative *Theileria* parasites, which include *T. orientalis*, *T. sergenti*, and *T. buffeli*, is generally considered to have low pathogenicity in cattle (55). Some authors have suggested that these 3 parasites are the same, while others are in the opinion that they must be considered as different species (27, 39, 83). We used a common name *T. orientalis* to describe this benign *Theileria* group. Although the parasite is considered benign, *T. orientalis* disease outbreaks that have caused substantial economic losses have been reported in different regions of world (6, 53). *T. orientalis* parasite populations can be divided into well-defined genotypes, some of which lack antigenic cross reactivity between each other (60, 90). Hence, susceptible cattle can be repeatedly infected with different *T. orientalis*

genotypes.

Initial studies divided *T. orientalis* parasites into four types, namely the Ikeda, Chitose, Thai, and Buffeli types (39, 63) using limited numbers of gene sequences of major piroplasm surface protein (MPSP). However, later studies that generated more numbers of *MPSP* sequences have speculated that genetic differences might exist within the above genotypes. Therefore, Kim et al. (1998) (48) proposed a different approach, by which six genotypes designated types 1 to 6 were identified based on *MPSP* gene sequences. Subsequently, five more genotypes, i.e., types 7, 8, N1, N2, and N3, have been added to the list of previously described genotypes (38, 45). The latter classification method have been employed to analyze the *T. orientalis* population structure in different countries (3, 5, 45, 91).

Sri Lanka is an agriculturally rich country, and the livestock industry has been identified as a potentially important sector for future development. If dairy cattle milk production can be improved, the large sums of foreign currency that are currently being spent on importing milk products could be diverted to issues of national importance. However, the spread of infectious diseases in Sri Lankan cattle is one of the major obstacles hindering milk production in dairy herds.

Our recent PCR-based study conducted among Sri Lankan cattle populations

indicated that *T. orientalis* was the predominant parasite and over 50% of the animals surveyed were infected with this parasite (69). In one of the sampling locations, the prevalence of *T. orientalis* was recorded as 98.8%, indicating that almost all of the cattle populations in that area were infected with this pathogen. Different virulence characteristics have been reported for *T. orientalis* genotypes (40). For instance, a recent study has indicated that more clinical cases of *T. orientalis* were associated with type 2 (Ikeda type) than type 1 (Chitose type) (20). The involvement of type 7 in clinical theileriosis was also previously observed in India (6). Therefore, the detection of *T. orientalis* genotypes is important to determine the clinical significance of this pathogen in Sri Lanka. In addition, better understanding about genetic diversity in *T. orientalis* parasites is an important prerequisite for devising effective immune control strategies in future, as the previous studies showed that sub-unit vaccines based on MPSP could be effectively used for the control of clinical theileriosis (60). The present study, therefore, aimed to determine the number of *T. orientalis* genotypes circulating among Sri Lankan cattle. Although the nucleotide sequences of the *MPSP* genes collected from the *T. orientalis*-positive blood DNA samples may provide preliminary information on the genotypes, the actual prevalence of such genotypes cannot be estimated accurately, as the mixed infections with different genotypes are commonly observed. Therefore,

parasite genotype-specific PCR assays were developed and employed to determine the prevalence of each genotype detected by DNA sequence analysis.

4-2. Materials and methods

DNA samples: In Sri Lanka, 316 blood samples were collected from cattle populations bred in four different districts namely the Nuwara Eliya (n = 83), Polonnaruwa (n = 84), Ampara (n = 88), and Jaffna (n = 61) in May – June 2011 using EDTA-coated Vacutainer tubes (NIPRO, Osaka, Japan). All the sampled animals were clinically normal and over one year of age. Subsequently, DNA samples were prepared from whole blood as described in our previous report (69) using a DNA extraction kit (Qiagen, Hilden, Germany). Among these DNA samples, 169 (Nuwara Eliya; 82, Polonnaruwa; 26, Ampara; 38, and Jaffna; 23) were positive for *T. orientalis* (69) and used in the present study.

PCR amplification, cloning, and sequencing: A previously described *MPSP*-PCR assay was employed to amplify a part of the hyper-variable region of the *MPSP* gene (776 bp) from randomly selected *T. orientalis*-positive DNA samples obtained from each district (Table 8), using a set of forward (5'-CTTTGCCTAGGATACTTCCT-3') and reverse

(5'-ACGGCAAGTGGTGAGAACT-3') primers (61). After agarose gel electrophoresis, *T. orientalis*-specific PCR products were purified (Alquick Gel Extraction Kit, Qiagen), ligated to a PCR 2.1 plasmid vector (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, USA), transformed into Top 10 *E. coli* competent cells (TOP 10, Invitrogen), and then plated onto Luria Broth (LB) agar plates (Invitrogen) that contained X-gal (Wako, Osaka, Japan). After incubation overnight at 37˚C, individual white colonies were picked and cultured in LB broth (Invitrogen). Plasmid DNA was extracted from the bacterial cultures (QIAprep Spin Miniprep kit, Qiagen), and the nucleotide sequences of the inserts were determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Construction of phylogenetic trees: The *MPSP* gene sequences generated in the present study (n=71), together with 6 other Sri Lankan sequences that had been determined in our previous investigation (AB690866 – AB690871) (69), were analyzed using GENETYX 7.0 software (GENETYX, Tokyo, Japan). Selected numbers of Sri Lankan *MPSP* gene sequences (n=37) together with those reported from other countries (n=47) were used to construct a neighbor-joining phylogenetic tree using the MAFFT program (41). Bootstrap values were calculated using the same software.

Genotype-specific PCR assays: The specificities of the previously described

(91) and newly developed genotype-specific PCR assays were evaluated using DNAs derived from field samples and plasmids containing *MPSP* gene fragments derived from *T. orientalis*-positive Sri Lankan cattle. The primers for the genotype-specific PCR assays developed in the present study were carefully designed using specific and conserved regions of *MPSP* sequences as described previously (91). Composition of PCR reaction mixtures and the cycling conditions of all the genotype-specific PCR assays were essentially described by Yokoyama et al. (2011) (91). Briefly, 1 µl of DNA sample was added to 9 µl of reaction mix that contained 1 µl 10 x PCR buffer (Ex Taq buffer, Takara, Tokyo, Japan), 200 µM of each dNTPs (Takara), 0.1 µM of forward and reverse primers (Table 9), 0.5 units of Taq polymerase (Ex Taq DNA polymerase, Takara) and 6.95 µl of double distilled water. An initial enzyme activation step at 94˚C for 5 min was followed by 35 cycles each of which included a denaturing step at 94˚C for 30 sec, an annealing step for 45 sec at different temperatures shown in Table 9, and an extension step at 72˚C for 1 min. The final elongation at 72˚C for 7 min was followed by gel electrophoresis, ethidium bromide staining, and visualization of PCR amplicons under UV light. All of the *T. orientalis*-positive DNA samples were screened using the genotype-specific PCR assays. Finally, the prevalence of each genotype among the Sri Lankan cattle was calculated based on the total numbers of samples

collected.

4-3. Results

The *MPSP* sequences (n=71) isolated from 71 *T. orientalis*-positive DNA samples in the present study have been registered with GenBank (AB701408 – AB701478). The sequence analyses revealed the presence of four major genotypes, consisting of types 1, 3, 5, and 7 of *T. orientalis* (Fig. 15 and Table 8). Among them, types 5 (n=31) and 7 (n=22) were identified as the most frequently occurring genotypes, accounting for 43.7% and 31.0% of the total sequences, respectively. Inspection of the phylogenetic tree showed that most of the Sri Lankan *T. orientalis MPSP* genes within a particular cluster were located close to each other (Fig. 15). In addition, the sequences of the Sri Lankan types 1 and 3 *MPSP* fragments are most similar to the Mongolian (AB571938) and Thai (AB562545) sequences, and those from Vietnam (AB560821), respectively (Fig. 16A). In contrast, while type 5 shared greater sequence identity with the Mongolian (AB602385), Thai (AB562557), and Vietnamese (AB560828) sequences, type 7 was closer to the type 7 sequence from Thailand (AB081329). Among the type 5-specific sequences, four gene fragments, which were amplified from the DNA samples from Nuwara Eliya (AB701416, AB701424, and AB701436) and Polonnaruwa

(AB701448) districts, showed a 3 nucleotide deletion at the same location in the gene. This 3 nucleotide deletion resulted in the loss of a single amino acid (Aspartic acid), when compared to the other type 5 sequences (Fig. 16B).

The sequence specificities of the PCR genotyping assays used here were confirmed before they were employed to screen the Sri Lankan *T. orientalis*-positive DNA samples. The genotype-specific PCR assays distinguished all of the cloned plasmids containing the inserts of types 1, 3, 5, and 7 *MPSP* gene fragments prepared in the present study (Fig. 17). The results of the genotype-specific PCR assays indicated that among the 169 *T. orientalis*-positive DNA samples, 48, 55, 101, and 112 were positive for genotypes 1, 3, 5, and 7, respectively; suggesting that the prevalences of the respective genotypes were 15.2, 17.4, 32.0, and 35.4% among cattle populations in Sri Lanka. On a per district basis, type 7 was the most common genotype in Ampara and Jaffna, while type 5 was the most common in Nuwara Eliya and Polonnaruwa (Fig. 18). Mixed infections with different genotypes were commonly observed, and 31.3% of the animals were infected with more than one genotype (Data not shown). Among the animals that had mixed infections, 45.4, 35.4, and 19.2% were infected with two, three, and four genotypes, respectively. Furthermore, 67.5, 17.8, 30.7, and 16% of the animal populations in Nuwara Eliya, Polonnaruwa, Ampara, and Jaffna, respectively, were

infected with multiple genotypes.

4-4. Discussion

Several hemoprotozoan pathogens use antigenic polymorphism as an effective mean of escaping host immune responses (43). In particular, their surface proteins are readily recognized by the host's defense system, and such proteins often show remarkable variations among field isolates (10, 42). MPSP, which is a surface protein of *T. orientalis*, is also a polymorphic antigen (49). Interestingly, *T. orientalis* isolates from different geographical locations can be divided into a total of eleven genotypes based on their *MPSP* gene sequences (3, 5, 61, 91). The present study has determined the prevalence of different *T. orientalis* genotypes circulating among Sri Lankan cattle using *MPSP* gene sequences and genotype-specific PCR assays.

The phylogenetic analysis results indicate the presence of at least four genotypes, consisting of types 1, 3, 5, and 7, in the Sri Lankan cattle. The Sri Lankan *MPSP* sequences were found to be closely related to those from Vietnam, Mongolia, Thailand, and Japan. This contrasts with the type N3 sequence, which has been observed among Vietnamese, Mongolian, and Thai cattle populations (3, 5, 45), but was not detected in the Sri Lankan cattle. However, the possible presence of other genotypes,

including type N3, cannot be ruled out, as some of the genotypes which infected the minor proportions of cattle populations could have been left undetected by sequencing analyses. Among the type 5 sequences, 4 sequences had a 3 nucleotide base deletion resulting in the absence of a single aspartic acid, a residue that is present in the other type 5 sequences. We have previously reported a 3 nucleotide insertion in a *MPSP* sequence (AB560833) that had been isolated from a sheep in Vietnam (45). Although it was not clear why or how the sequence deletion or insertion occurred, one hypothesis is that some of the type 5 sequences might have been originated from the parasites that were transmitted to cattle from non-cattle reservoir hosts. However, larger numbers of *MPSP* sequences of *T. orientalis* parasites which were isolated from such non-cattle hosts should be analyzed to confirm our assumption.

The results of the genotype-specific PCR assays revealed that type 7 was most common in the northern (Jaffna) and eastern (Ampara) districts of Sri Lanka. In the late 1980s, during the time of conflict, Indian bred animals were brought to the northern and eastern regions of Sri Lanka without following proper quarantine procedures (65). It is noteworthy that type 7 was detected in southern India; this genotype is similar to the Thai type 7 sequences (6). With this background in mind, the presence of type 7 as the predominant genotype in the northern and eastern Sri Lanka might be explained. The

type 2 genotype, which is considered to be highly pathogenic compared to the other genotypes (40), was not detected in the present study. On the other hand, involvement of the type 7 genotype was reported in severe clinical cases of *T. orientalis* in southern India (6). Since 35.4% of cattle in our study were infected with genotype 7, it appears likely that Sri Lankan cattle populations are at risk of developing *T. orientalis* infections that require clinical treatment.

The results of the genotype-specific PCR assays have revealed that mixed infections with different *T. orientalis* genotypes are very common in Sri Lanka. Genotype-specific immune responses were observed with some of the genotypes (60, 90), and therefore, the immunity induced by such a genotype might not protect cattle from subsequent infection with unrelated genotypes. This might explain the high prevalence of *T. orientalis* parasites among Sri Lankan cattle. However, extensive studies on the genotype-specific immunity are essential to determine whether all the genotypes are antigenically distinguishable from each other. In conclusion, the present study should raise awareness of the importance of considering *T. orientalis* a potential pathogen of Sri Lankan cattle. Hence, proper management strategies should be made available for the prevention and control of disease outbreaks that could have a detrimental impact on cattle farming.

4-5. Summary

In the present study, the genetic diversity of *Theileria orientalis* parasites circulating among Sri Lankan cattle was investigated. The analysis of the gene sequences encoding major piroplasm surface protein (MPSP) amplified from *T. orientalis*-positive DNA samples (from bovine blood) revealed the presence of four parasite genotypes. The genotypes consisted of types 1, 3, 5, and 7. Phylogenetic analysis indicated that the Sri Lankan *MPSP* sequences were closely related to those reported from Vietnam (types 3 and 5), Mongolia (types 1 and 5), Thailand (types 1, 5, and 7), and Japan (type 7). Subsequently, genotype-specific PCR assays determined that the most common genotype was type 7, followed by types 5, 3, and 1. Genotype 7 has been reported to be involved in disease outbreaks in India. Therefore, preventive and control measures are essential to avoid potential economic losses due to *T. orientalis* infection in Sri Lanka. This is the first report that describes the genetic diversity of *T. orientalis* circulating among Sri Lankan cattle.

Table 8. *Theileria orientalis* genotypes detected by analysis of *MPSP* sequences determined from parasite-positive DNA samples.

District	No. of *MPSP* gene sequences	Genotypes			
		1	**3**	**5**	**7**
Nuwara Eliya	36	4	4	21	7
Polonnaruwa	13	0	4	7	2
Ampara	13	1	3	3	6
Jaffna	09	1	1	0	7
Total	**71**	**6**	**12**	**31**	**22**

Table 9. List of primers used for the detection of types 1, 3, 5, and 7 of *T. orientalis*.

Type	Primer *	Primer sequence (5' – 3')	Annealing temperature (°C)	Size of amplicon (bp)	Reference
1	F	TTGCCTAGGATACTTCCTCATCG	64	559	Yokoyama et al., 2011
	R	TGCGGTGTATTTGGCCTTC			
3	F	CCCTTCAAGGTTAAGAGT	58	287	Present study
	R	ACGGCAAGTGGTGAGAACT			Ota et al., 2009
5	F	CAGTCAATGCAACAAAACCCGA	56	424	Yokoyama et al., 2011
	R	CTTTTTAGGATCACCGACATCCAG			
7	F	GGAAAAGAAAGACCTCGATGTG	65	232	Present study
	R	GTCACCCGTGTAGAAGTAGTCC			

***** F, forward primer, R, reverse primer

Fig. 15. Phylogenetic tree. The phylogenetic tree was constructed using *T. orientalis MPSP* gene sequences from Sri Lanka (boldface letters) and other countries. Bootstrap values are provided at the beginning of each branch.

A

Type	*MPSP* Sequences	Sri Lanka							
		1		3		5		7	
		AB701415	AB701461	AB701465	AB701432	AB690869	AB701416	AB701478	AB701451
1	Russia (AB016279)	98.4	98.6						
	Korea (D87192)	98.4	98.6						
	Japan (D12689)	98.2	98.5						
	Vietnam (AB560819)	98.5	98.5						
	Mongolia (AB571938)	**99.3**	**98.8**						
	Thailand (AB562545)	**99.3**	**98.8**						
3	Mongolia (AB571922)			95.5	95.0				
	UK (AB008369)			99.0	98.5				
	Taiwan (D87207)			99.0	98.5				
	Thailand (AB562540)			99.0	98.5				
	Australia (D87189)			99.2	98.6				
	Vietnam (AB560821)			**99.6**	**98.9**				
	Zimbabwe (AB016278)			99.3	98.6				
	Japan (AB218436)			96.2	95.5				
	China (EU584238)			96.2	95.5				
5	Japan (AB491347)					97.8	95.9		
	Korea (D87201)					97.4	95.2		
	Mongolia (AB602385)					**98.4**	96.2		
	Thailand (AB562557)					**98.4**	96.2		
	Vietnam (AB560828)					**98.4**	**96.5**		
7	Thailand (AB081329)							**99.5**	**98.9**
	India (HQ444179)							99.0	98.5
	Japan (AB218430)							98.8	**98.9**
	Mongolia (AB571935)							95.9	96.0
	Vietnam (AB560823)							96.2	96.3
	Indonesia (AF102500)							95.8	95.9

B

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AB701416  KFENLDVGDSKNAKYTAVKVYVGTD-VKIVRLDYFYTRDERFKEVYFKLVDG
AB701436  KFENLDVGDSKNAKYTAVKVYVGTD-VKIVRLDYFYTRDERFKEVYFKLVDG
AB701448  KFENLDVGDSKNAKYTAVKVYVGTD-VKIVRLDYFYTRDERFKEVYFKLVDG
AB701424  KFENLDVGDSKNAKYTAVKVYVGTD-VKIVRLDYFYTRDERFKEVYFKLVDG
AB701469  KFENLDVGDPKKAKYTAVKVYVGTDDKKIVRLDYFYTGDERFKEVYFKLVDG
AB701412  KFENLDVGDPKKAKYTAVKVYVGTDDKKIVRLDYFYTGDERFKEVYFKLVDG
AB701422  KFENLDVGDPKKAKYTAVKVYVGTDDKKIVRLDYFYTGDERFKEVYFKLVDG
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Fig. 16. The percent identities between *MPSP* gene sequences from Sri Lanka and other countries, and analysis of Sri Lankan type 5 MPSP amino acid sequences. Panel **A**: Percent identity of selected *Theileria orientalis MPSP* gene sequences for genotypes 1, 3, 5, and 7 from Sri Lanka compared with genotypes 1, 3, 5 and 7 originating from other countries. Maximum identities were indicated by boldface letters. Panel **B**: Multiple alignment of short fragments from the predicted MPSP amino acid sequences of genotype 5. An asterisk indicates the absence of an aspartic acid due to a 3 nucleotide base deletion in the type 5 *MPSP* gene sequences.

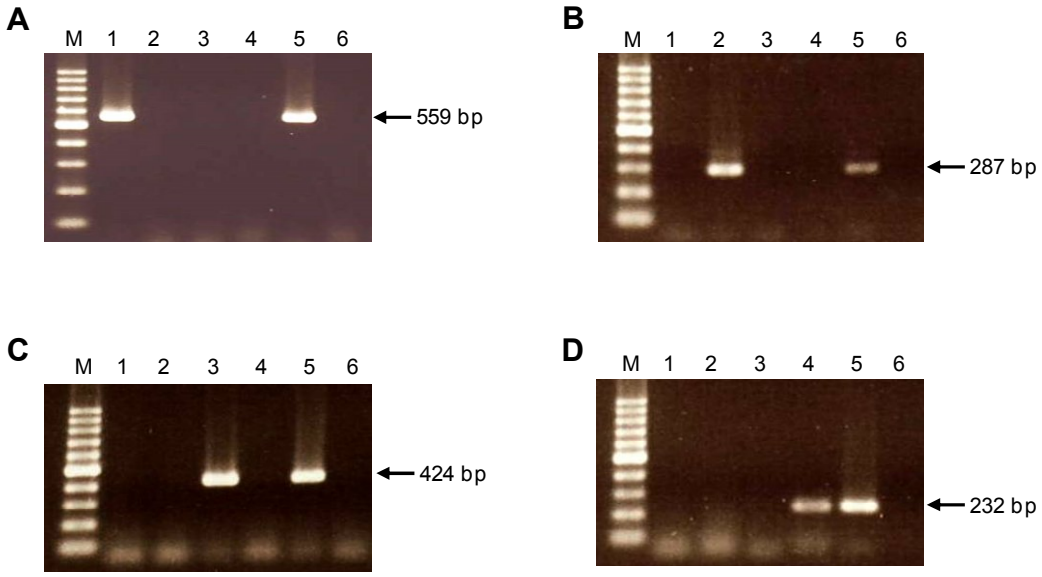

Fig. 17. Specificities of the PCR genotyping assays used for detection of type 1 (Panel **A**), type 3 (Panel **B**), type 5 (Panel **C**), and type 7 (Panel **D**) *MPSP* gene fragments from *Theileria orientalis*. M: 100-bp DNA ladder. Lanes 1 – 4, PCR 2.1 plasmid vectors containing the inserts for types 1, 3, 5, and 7 *MPSP* gene fragments amplified from Sri Lankan *T. orientalis*-positive DNA samples from bovine blood. Lane 5, DNA samples that were positive for types 1 (Panel **A**), 3 (Panel **B**), 5 (Panel **C**), and 7 (Panel **D**) as determined by DNA sequencing. An *MPSP*-PCR-negative DNA field sample was included as a negative control (Lane 6).

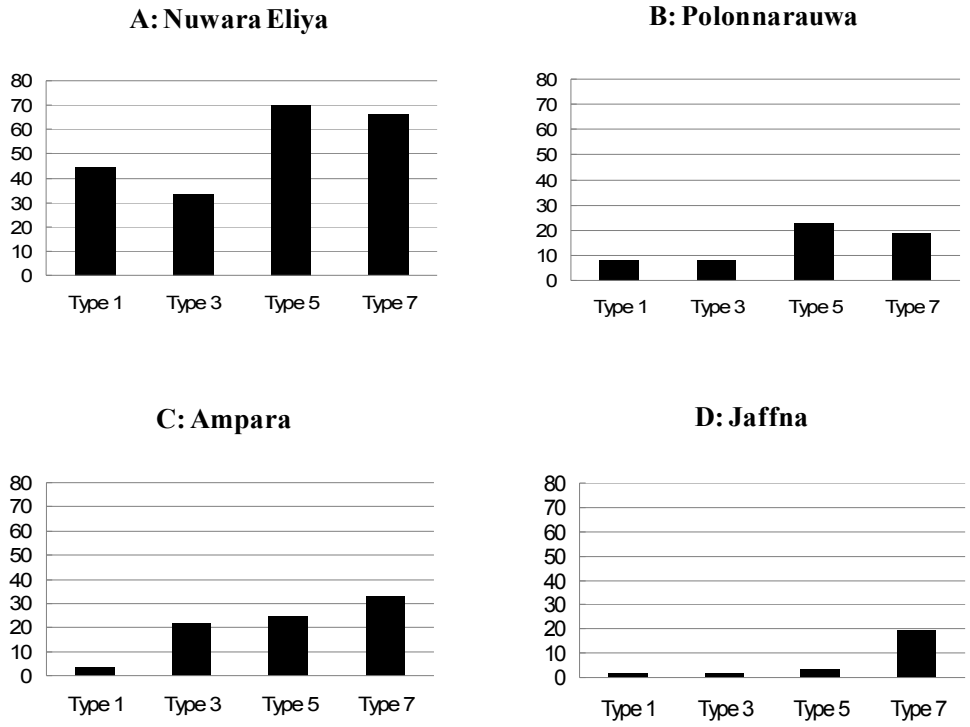

Fig. 18. Prevalence of *Theileria orientalis MPSP* genotypes 1, 3, 5, and 7 among the cattle in the Nuwara Eliya, Polonnaruwa, Ampara, and Jaffna districts of Sri Lanka as determined by genotype-specific PCR assays. The distribution values are expressed as the percentages of the total numbers of cattle (i.e., Nuwara Eliya; 83, Polonnaruwa; 84, Ampara; 88, and Jaffna; 61) that were sampled from each district.

General discussion

Bovine piroplasmosis represents a serious threat to the farming operation worldwide (52). Therefore concrete preventive and control methodologies must be formulated to bringdown the prevalance of this disease in endemic countries. The control strategies against the bovine piroplasmosis include the tick control, chemotherapy, vaccination, and the movement control (16). The tick control methods are often challenged by the rapid development of acaricide resistance (19). For the successful outcome of the infected animals, early diagnosis should be followed by the suitable treatment chioces (13). Live attenuated vaccines have been used to immunize the susceptible cattle populations against *B. bovis* and *B. bigemina* (64). However, the disease outbreaks were reported in cattle that were previously immunized with live vaccines (11). The studies showed that the antigenic differences between the vaccine strain and the virulent field isolates would be the possible cause for these outbreaks (12).

The epidemiological surveys are of very important to understand the current status of the hemoprotozoan parasites in a country. Therefore, the integrated control methods can be made available against hemoprotozoan parasites. Although the microscopy is thought to be the gold standard technique for the detection of *Babesia*

and *Theileria* parasites, this technique lacks specificity and sensititvity (15). Therefore PCR assays, which are superior in sensitivity and specificity than the microscopy, are often preferred for epidemiological surveys (2). However, the widely used *B. bigemina* specifc *Spe*I-*Ava*I nPCR has never been tested against *B. ovata* which resembles *B. bigemina* morphologically (23, 44, 51). Interestingly, when the *B. ovata* geneomic DNA was subjected *Spe*I-*Ava*I nPCR, the PCR assay has amplified a homologous fragment from *B. ovata*. Therefore, a *B. bigemina*-specific nested PCR assay based on the *AMA-1* gene was developed in the present study (67). The assay was found to be highly specific and sensitive for the detection of *B. bigemina*. Subsequently, when the Mongolian cattle were screened using both PCR assays, while the *AMA-1* nPCR detected only *B. bigemina*, the *Spe*I-*Ava*I nPCR has amplified not only the *B. bigemina* but also *B. ovata* and other unknown sequences. *AMA-1* PCR assay was later employed to screen cattle populations in Sri Lanka (69), Philippines (89), and Vietnam, and the sequencing analyses confirmed that the assay has amplified only the target gene fragment of *B. bigemina*. Therefore, the *AMA-1* PCR assay might be well suitable for epidemiological surveys of *B. bigemina*.

On the other hand, there were no specific PCR assays available in the past to detect *B. ovata*. Therefore, a PCR assay based on the gene that encodes AMA-1 was

developed in the present study. The assay showed high specificity and sensitivity (70). *B. ovata*-specific *AMA-1*-PCR was later used to screen over 2000 DNA samples prepared from cattle bred in 10 different countries which are located in Asia, Africa, and South America (92). While the specificity of the novel PCR assay was confimed, the parasite was detected from Japan, Mongolia, and Thailand. Because this is the first report of *B. ovata* in Mongolia and Thailand, as a next step, the parasite should be isolated in these countries for further analyses.

The clinical significance of the coinfection with *B. ovata* and *T. orientalis* was also evaluated in a herd of cattle reared in Hokkaido, Japan. The findings confirmed that the single infection by *B. ovata* might not induce a detectable anemia in cattle. However, the coinfection with *T. orientalis* significantly increased the chances of developing anemia in the infected cattle. The progress of *B. ovata*-related research works have been much slower than that of other virulent *Babesia* parasites. The reasons for the slower research progress might be linked to the benign nature and geographically delimited distribution of *B. ovata*. However, it is now very clear that under certain circumstances the parasite might cause severe clinical disease in cattle. Therefore, the research on *B. ovata* must be accelerated and the control methods similar to those available for other virulent *Babesia* parasites must be made available. In addition, tick vectors capable of

transmitting *B. ovata*, other than *H. longicornis*, should be identified. Furthermore, seasonal activities of such tick species must be investigated in order to have a systematic control strategy.

The genetic diversities of *B. bovis* and *T. orientalis* detected from cattle populations in Sri Lanka were also analyzed, as such studies have never been executed in this country. The genetic diversities of *MSA-1*, *MSA-2c*, *MSA-2a1*, and *MSA-2b* of *B. bovis* in Sri Lanka were found to be very high. The deduced amino acid sequences of MSAs shared low homologies with those of currently used K-vaccine strain. According to the previous studies, if the antigenicity of the virulent *B. bovis* is different from the vaccine strains, the outbreak is inevitable among vaccinated cattle (11). The investigations done in the post-genomic era found that the genes encodings the MSAs in outbreak isolates were higly diverse from the relavent vaccine strains (10, 50). Therefore, in the present study, the genetic diversity *MSAs* was compared with the respective sequence data of K-strain. The findings showed that the MSAs sequences of K-strain shared low similarities with those of field isolates. In future investigations, a comprehensive study is essential to compare the antinicity diffrences between the K-strain and Sri Lankan field isolates. In addition, experiments to evaluate the protection level of K-strain against the field isolates are of paramount important.

Alternatively, suitable *B. bovis* strains must be identified in Sri Lanka to be used as a live attenuated vaccine.

The PCR screening of Sri Lankan cattle populations showed that over 50% surveyed animals were harboring *T. orientalis* in their blood streams during the study period (69). The sequencing and phylogenetic analyses indicated the presence of 4 different genotypes of this parasite (types 1, 3, 5, and 7) (71). The studies conducted in India suggested the involvement of type 7 in several clinical cases of bovine thileriosis. In the present study, the type 7 was found as the most predominant genotype. Therefore, the Sri Lankan cattle populations are indeed in a risk of developing clinical theileriosis caused by *T. orientalis*. However, clinical cases due to *T. orientalis* have never been reported in Sri Lanka according to the field veterinarians. In Sri Lanka, the choice of treatment strategy for the diseases caused by hemoprotozoan parasites is mainly based on the clinical signs. Therefore, *T. orientalis* infections could have been misdiagnosed as other virulent *Babesia* or *Theleria* parasites. In some cases, although the blood smear examination is performed, there is a possibility that *T. orientalis* could have been judged as *T. annulata*, a virulent *Theileria* parasite which is also endemic in Sri Lanka (69). Therefore, *T. orientalis* must be considered when making a differential diagnosis of hemoprotozoan infections in this country.

In summary, the newly developed PCR assays for specific detection of *B. bigemina* and *B. ovata* might be used as universal molecular diagnostic tools. In addition, the studies conducted in Sri Lanka generated the preliminary data which can be used as a foundation to formulate better control strategies against *B. bovis* and *T. orientalis*.

Conclusions

The present studies were undertaken to develop PCR assays for the specific detection of *B. bigemina* and *B. ovata*. In addition, the clinical significance of coinfection with *B. ovata* and *T. orientalis* in cattle was also assessed in these studies. A molecular epidemiological study was conducted in Sri Lanka to determine the genetic diversity of *B. bovis* field isolates and to analyze the compatibility of currently used K-vaccine strain. Furthermore, *MPSP* genotypes of *T. orientalis* in Sri Lankan cattle were also investigated to predict the clinical relevance of this parasite. The major findings of the present studies are summarized below.

1. The newly developed *B. bigemina*-specific *AMA-1* nPCR assay might be a suitable alternative assay for the existing PCR assays that lack the specificity.
2. The *AMA-1*-PCR assay for the detection of *B. ovata* is the first of this kind. The assay was found to be highly specific, and therefore, can be effectively used for epidemiological studies and for other research works.
3. The findings demonstrated that *B. ovata* contributes to the anemia development in cattle when coinfects with *T. orientalis*. Therefore *B. ovata*

should be considered as an economically important pathogen.

4. The studies conducted among Sri Lankan cattle demonstrated that the *B. bovis* isolates are different from the currently used K-vaccine strain. Therefore, the Sri Lankan researchers must evaluate the protection offered by this strain against the virulent field isolates.
5. Four different genotypes of *T. orientalis* were identified in Sri Lanka, and these results suggested that *T. orientalis* might be associated with clinical disease in this country. Therefore, field veterinarians must be more careful when making diagnosis of a hemoprotozoan infection.

Acknowledgments

These studies were conducted in the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine. The research works were supported by a grant from the Global COE Program from the Japanese Ministry of Education, Science, Sports, Culture and Technology, and Grants-in-Aid for Scientific Research from the Japan Society for Promotion of Science (JSPS), Japan.

I am very much thankful to my supervisor Prof. Ikuo Igarashi for accepting me as doctoral course student and for guiding me throughout my studies. I would also like to thank Prof. Naoaki Yokoyama for his continuous encouragement and motivation.

My deepest thanks are expressed to Prof. Hiroshi Suzuki, Prof. Xuenan Xuan, Prof. Noboru Inoue, and Assoc. Prof. Yoshifumi Nishikawa for their constructive criticisms on my research works.

I wish to thank to Prof. Hisashi Inokuma, Dr. Kotaro Matsumoto, and Mr. Michihito Tagawa who have supported me to conduct my studies.

I would also like express my sincere thanks to Ms. Hiroko Yamamoto for her excellent technical assistance.

I am also very much thankful to the researchers and staffs of the dept. of

Animal Production and Health, Peradeniya, Sri Lanka who were much helpful to collect the blood samples and to extract DNA samples which were used in my studies.

I am also thankful to my parents, wife and son for their encouragement and supports. I dedicate this dissertation to my wife Nishani and my son Athishshan.

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