Type-specific PCR assays for *Babesia bovis* *msa-1* genotypes in Asia: revisiting the genetic diversity in Sri Lanka, Mongolia, and Vietnam

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Abstract

Babesia bovis is the most virulent *Babesia* organism, resulting in a high mortality rate in cattle. The genetic diversity of *B. bovis* merozoite surface antigens (MSAs), such as MSA-1, MSA-2b, and MSA-2c, might be linked to altered immune profiles in the host animals. The present study aimed to develop type-specific PCR assays for Asian *msa-1* genotypes, thereby re-analyzing the genetic diversity of *msa-1* in Sri Lanka, Mongolia, and Vietnam. Specific primers were designed for nine Asian *msa-1* genotypes, which had been detected based on the phylogeny constructed using *msa-1* gene sequences retrieved from the GenBank database. Specificity of the type-specific PCR assays was confirmed using plasmids containing the inserts of *msa-1* gene fragments that represent Asian genotypes. Furthermore, no amplicons were observed by these PCR assays when DNA samples of *B. bigemina*, *B. ovata*, *Theileria annulata*, *T. orientalis*, *Trypanosoma evansi*, *T. theileri*, *Anaplasma marginale*, *A. bovis*, and non-infected bovine blood were analyzed. In total, 109 *B. bovis*-positive blood DNA samples sourced from Sri Lanka (44 cattle), Mongolia (26 cattle), and Vietnam (23 cattle and 16 water buffaloes) were then screened by the type-specific PCR assays. The sequences derived from all of the PCR amplicons were phylogenetically analyzed. Out of 109 DNA samples, 23 (20 from cattle and 3 from water buffaloes) were positive for at least one genotype. In agreement with previous studies, five and four different genotypes were detected among the DNA samples from Sri Lanka and Vietnam, respectively. In contrast, four genotypes, including three novel genotypes, were detected from Mongolia. Five DNA samples were found to be co-infected with multiple genotypes. The sequences of the PCR amplicons clustered phylogenetically within the

corresponding clades. These findings indicated that the type-specific PCR assays described herein are useful for the determination of genotypic diversity of the *B. bovis* *msa-1* gene in Asia.

Keywords: Asia, *Babesia bovis*, Genetic diversity, *msa-1*, Type-specific PCR

1. Introduction

Bovine babesiosis, caused by several different species of *Babesia*, such as *B. bovis*, *B. bigemina*, *B. divergens*, *B. ovata*, and *B. major*, has a worldwide distribution (Bock et al., 2004). Among these parasitic species, *B. bovis* is known to be the most pathogenic organism, resulting in a high mortality rate in cattle (Bock et al., 2004; Brown, et al., 2006; Ristic, 1981). In addition to severe hemolytic anemia commonly linked with *Babesia* infection, *B. bovis* infection induces cerebral babesiosis in cattle, due to the sequestration of parasite-infected erythrocytes in the cerebral capillaries (Everit et al., 1986).

The known *B. bovis* merozoite surface antigens (MSAs) include at least five proteins, MSA-1, MSA-2c, MSA-2a1, MSA-2a2, and MSA-2b, with molecular weights ranging from 42 to 44 kDa (Florin-Christensen et al., 2002; Mosqueda et al., 2002a,b; Sivakumar et al., 2013b; Tattiyapong et al., 2014). The *msa-1* is a single copy gene (Suarez et al., 2000), while *msa-2* gene family consists of four tandemly arranged genes in *B. bovis* genome (Florin-Christensen et al., 2002). *B. bovis* MSAs are proposed to mediate initial attachment of the merozoites to the host erythrocytes (Suarez et al., 2000; Yokoyama et al., 2006). Therefore, MSAs are considered as candidates for the development of subunit vaccines (Brown and Palmer, 1999; Hines et al., 1992; Palmer and McElwain, 1995). However, the genetic diversity often results in antigenic variations leading to lack of immunological cross reactivity between MSAs from different *B. bovis* strains (Berens et al., 2005; Leroith et al., 2005). Therefore, the genetic diversity of genes encoding MSAs among different isolates of *B. bovis* poses a challenge when considering their use as vaccines. In addition, genetic variations within

MSA genes between the vaccine strains and field isolates were thought to be responsible for disease outbreaks among vaccinated cattle populations (Berens et al., 2005; Leroith et al., 2005). Therefore, fully elucidating the genetic diversity of *MSA* genes may prove crucial in developing effective immune control strategies against *B. bovis* infection. For this reason, the genetic diversity of *MSA* genes has been investigated in several *B. bovis*-endemic countries, including Australia (Berens et al., 2005; Leroith et al., 2005), Sri Lanka (Sivakumar et al., 2013b), Mongolia (Altangerel et al., 2012), Vietnam (Sivakumar et al., 2013a; Yokoyama et al., 2015), the Philippines (Tattiyapong et al., 2014), Thailand (Nagano et al., 2013; Simking et al., 2013), Israel (Molad et al., 2014), Mexico (Borgonio et al., 2008; Genis et al., 2008, 2009), and Brazil (Nagano et al., 2013; Ramos et al., 2012).

The most common method to analyze genetic diversity is based on the sequencing of PCR-amplified target genes. However, a major problem associated with this methodology is that this technique may not detect all of the genotypes in co-infected animals. Therefore, large numbers of clones per PCR amplicon must be sequenced to detect most of the genotypes in co-infected animals (Lau et al., 2010). Type-specific PCR assays have been shown to be effective for the specific detection of different genotypes of microbes. In particular, type-specific PCR assays are now widely used for genotypic clarification of *Theileria orientalis* (Perera et al., 2015; Sivakumar et al., 2013c; Yokoyama et al., 2011, 2012). The type-specific PCR assays that eliminate the need for sequencing are particularly useful not only to determine the prevalence of each genotype, but also to detect multiple genotypes in co-infected animals.

Among the *msa* genes, *msa-1*, *msa-2b*, and *msa-2c* have been well studied in terms of their genetic diversity. Phylogenetic analyses in previous investigations

showed that both the *MSA-2b* and *MSA-2c* sequences lack geographical sub-structuring and that the *msa-1* sequences often cluster phylogenetically according to their continents of origin (Sivakumar et al., 2013b; Yokoyama et al., 2015). In the present study, therefore, we developed type-specific PCR assays for Asian *msa-1* genotypes of *B. bovis*, and then evaluated their performances using *B. bovis*-positive DNA samples that had been previously characterized for *MSA* genetic diversity in Sri Lanka (Sivakumar et al., 2013b), Mongolia (Altangerel et al., 2012), and Vietnam (Yokoyama et al., 2015).

2. Materials and Methods

2.1. Identification of *msa-1* genotypes in Asia

Babesia bovis *msa-1* gene sequences retrieved from the GenBank database were subjected to multiple alignments using a MAFFT software program (Kato et al., 2002). A maximum likelihood phylogenetic tree was constructed, based on a general time reversible substitution model hosted by the MEGA version 6.0 software (Tamura et al., 2013). The clades containing Asian sequences were identified in the phylogram.

2.2. Development and evaluation of type-specific PCR assays for the *msa-1* gene

All of the *msa-1* gene sequences used to construct the above phylogeny were aligned using a Multalin software program (<http://multalin.toulouse.inra.fr/multalin/>) (Corpet, 1988). A set of forward and reverse primers was manually designed for each Asian genotype based on the genotype-specific primer binding sites to avoid possible misamplification of non-target genotypes from Asia and other parts of world. Additionally, the regions containing insertion and deletion (INDEL) polymorphisms were omitted when designing the primers to make sure that the sizes of the PCR amplicons do not vary among the field isolates within a particular genotype.

PCR amplifications of target genes were carried out in a 10 µl reaction mixture, which included 1 µl of DNA template (2 pg of plasmid DNA or 50 – 60 ng of blood DNA), 1 µl of 10 × PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 1 µl of 2 mM dNTPs (Applied Biosystems), 0.5 µl of 10 µM forward and reverse primers (Table 1), 0.1 µl of 5 U/µl Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems), and 5.9 µl of double distilled water. The 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 30 seconds, an annealing step at the appropriate temperature (Table 1) for 1 min, and an extension step at 72°C for 1 min. After a final elongation step at 72°C for 7 min, the PCR products were resolved by agarose gel electrophoresis and then visualized under UV light. Detection of a band at the appropriate size (Table 1) was considered positive for each type-specific PCR assay.

For the specificity testing, plasmids containing inserts of the *msa-1* gene sequences that represent Asian genotypes and DNA samples of several bovine hemoprotozoan parasites were used in the PCR assays. Briefly, plasmids (n = 32) containing the inserts of *msa-1* gene fragments that had been amplified from *B. bovis*-positive DNA samples from Sri Lanka (AB787574 – AB787581) (Sivakumar et al., 2013b), Mongolia (AB612244 – AB612248, AB612250, and AB612251) (Altangerel et al., 2012), Vietnam (LC004298 – LC004306) (Yokoyama et al., 2015), and the Philippines (AB819770 – AB819777) (Tattiyapong et al., 2014), were used for specificity testing. In addition, DNA samples of *B. bigemina*, *B. ovata*, *Theileria annulata*, *T. orientalis*, *Trypanosoma evansi*, *T. theileri*, *Anaplasma marginale*, *A. bovis*, and non-infected bovine blood (Sivakumar et al., 2012b) were also used to analyze the specificity of each type-specific PCR assay. In total, 109 *B. bovis*-positive blood DNA samples from Sri Lanka (44 cattle) (Sivakumar et al., 2013b), Mongolia (26 cattle) (Altangerel et al., 2012), and Vietnam (23 cattle and 16 water buffalo) (Yokoyama et al., 2015) extracted using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany) were then screened by the type-specific PCR assays.

2.3. Cloning, sequencing, and phylogenetic analyses

All of the PCR amplicons derived from field DNA samples were cloned into a plasmid vector (PCR 2.1- TOPO, Invitrogen, Carlsbad, CA, USA), and two clones per PCR amplicon were sequenced, as described previously (Sivakumar et al., 2013b).

2.4. Bioinformatic analyses

The determined sequences were analyzed by the basic local alignment tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The identity scores among the sequences were determined using an EMBOSS NEEDLE (<http://emboss.bioinformatics.nl/cgi-bin/emboss/needle>). The newly determined sequences and the *msa-1* sequences that represent the Asian genotypes were used to construct a maximum likelihood phylogeny, using a general time reversible substitution model hosted by the MEGA 6.0 software.

3. Results

3.1. Development of type-specific PCR assays for the detection of B. bovis msa-1 genotypes

The phylogeny of *msa-1* gene sequences revealed 18 clades (Fig. 1). Except for the sequences in clade C, which included sequences from Asia, America, and Africa, all of the other *msa-1* sequences showed apparent geographical sub-structuring. These sequences could be classified into Asian (AS1 – AS8), Australian (AU1 – AU6), and American (AM1 – AM3) genotypes.

Primer sets were designed for nine *msa-1* genotypes (C and AS1 – AS8) that contained Asian sequences as described in Table 1. Specificity analyses demonstrated that the type-specific PCR assays were highly specific for the respective genotypes as shown in Figure 2. In addition, no PCR amplicons were detected when DNA samples of several other bovine blood pathogens were analyzed by these type-specific PCR assays (data not shown).

3.2. Genetic diversity of B. bovis msa-1 in Sri Lanka, Mongolia, and Vietnam as determined by type-specific PCR assays

Having confirmed the specificity of the PCR assays, 109 *B. bovis*-positive DNA samples from Mongolia, Vietnam, and Sri Lanka were analyzed by type-specific PCR assays. Of these 109 DNA samples, 23, including 7, 7, and 9 DNA samples from Sri Lanka, Mongolia, and Vietnam, respectively, were positive for at least one genotype (Table 2). Out of nine samples that were positive for the type-specific PCR assays in Vietnam, six and three were from cattle and water buffaloes, respectively. In agreement

with the previous studies (Sivakumar et al., 2013b; Yokoyama et al., 2015), five (C, AS1, AS4, AS5, AS7, and AS8) and four (AS1, AS4, AS6, and AS8) different genotypes were detected in the DNA samples from Sri Lanka and Vietnam, respectively. In contrast, in addition to the previously described genotype C (Altangerel et al., 2012), three novel genotypes (AS1, AS4, and AS6) were detected in the DNA samples from Mongolia. Genotypes AS2 and AS3, which contained only sequences from the Philippines, were not detected in the DNA samples analyzed in the present study. Furthermore, co-infections with different genotypes were detected in all three countries investigated. Co-infections with three different genotypes were found in Sri Lanka and Mongolia, where two individual DNA samples were detected with genotypes C, AS7, and AS8 and with genotypes C, AS1, and AS6, respectively. Furthermore, one and two DNA samples from Mongolia and Vietnam, respectively, showed co-infections with two different genotypes (C and AS6, AS1 and AS4, and AS4 and AS6, respectively).

A total of 30 PCR amplicons derived from 23 DNA samples were cloned, and two clones were sequenced for each PCR amplicon. The sequencing analysis confirmed that the PCR assays indeed amplified the *msa-1* gene fragments, and that the sequences determined for the two clones were identical for a given amplicon. The lengths of the sequences were similar to those predicted for each type-specific PCR assay (Table 1). The newly generated *msa-1* sequences (Accession numbers: LC066106 – LC066135) shared high identity scores with those previously determined in the respective clades. For instance, the newly generated sequences from the amplicons of PCR assays specific for genotypes C, AS4, AS5, AS6, AS7, and AS8 (Table 2) shared 98.6 – 99.6%, 93.8 – 99.6%, 98.1 – 100%, 95.9 – 100%, 99.8 – 100%, 100%, and 100% identity scores with previously determined sequences belonging to genotypes C (AB787581), AS1

(LC004301), AS4 (LC004306), AS5 (AB787577), AS6 (LC004298), AS7 (AB787576), and AS8 (AB787580), respectively. In the phylogenetic analysis, all of the sequences determined from the amplicons of type-specific PCR assays were found in the corresponding clades, confirming the findings of the type-specific PCR assays and sequencing analyses (Fig. 3).

4. Discussion

The genetic diversity of *MSA* genes is associated with immune evasion of *B. bovis* in host animals (Berens et al., 2005; LeRoith et al., 2005). Consequently, genetic diversity in these genes has been actively investigated in several *B. bovis*-endemic countries. Although PCR-sequencing is a commonly used technique for the determination of such diversity, this method is constrained by factors, such as the inefficiency in detecting multiple genotypes in co-infected animals and the high cost of equipment and reagents. By comparison, type-specific PCR assays, which are useful for detecting different genotypes in co-infected animals, do not require any expensive reagents or equipment. Therefore, the aim of the present study was to establish type-specific PCR assays to determine the diversity of *MSA* genes.

The initial phylogenetic analysis found that all *msa-1* gene sequences, except those in clade C that contained many sequences originating from Asia, America, and Africa, could be divided into Asian, Australian, and American genotypes, allowing us to develop *msa-1* type-specific PCR assays for different geographical regions. Type-specific PCR assays were developed for nine *msa-1* genotypes that contained Asian sequences (C and AS1 to AS8).

Specificity testing demonstrated that the PCR assays were highly specific for the respective genotypes. Additionally, the PCR assays did not amplify the DNA samples of several other bovine blood pathogens, suggesting that these PCR assays can be used to analyze *msa-1* genotypic variations even when animals are co-infected with other hemoparasites. The type-specific PCR assays were then employed to screen the *B. bovis*-positive DNA samples that had previously been characterized for *msa* genetic

diversity in Sri Lanka (Sivakumar et al., 2013b), Mongolia (Altangerel et al., 2012), and Vietnam (Yokoyama et al., 2015). Among the nine genotypes analyzed, seven were detected in the DNA samples. The two *msa-1* genotypes (AS2 and AS3) that were not detected in the present study included only the sequences from the Philippines, and it is therefore reasonable to speculate that these genotypes are specific to this country. Furthermore, co-infections with different genotypes were detected in all three countries investigated. All of the previously characterized genotypes in Sri Lanka (Sivakumar et al., 2013b) and Vietnam (Yokoyama et al., 2015) were also detected by type-specific PCR assays in these countries. In addition, the distribution of four *msa-1* genotypes, including three novel genotypes, was demonstrated in Mongolia. Although a previous study that found the *msa-1* sequences isolated in Mongolia shared 98.5 – 100% identity scores and clustered within a single clade (genotype C) in the phylogeny suggested that the *msa-1* gene was conserved in this country (Altangerel et al., 2012), our present study clearly demonstrated that the diversity of *msa-1* was much higher than that previously determined in this country. These findings suggest that the type-specific PCR assays described herein are useful for the analysis of *msa-1* genotypic diversity in Asian countries. The sequences amplified by the type-specific PCR assays shared high identity scores with those already found in the respective clades, and clustered within the corresponding clades in the phylogeny. These findings further confirm that the type-specific PCR assays can detect the respective genotypes specifically and suggest that the sequences amplified by these PCR assays can be efficiently analyzed phylogenetically.

The type-specific PCR assays that we developed in the present study might have the potential to be useful tools for the development of effective immune control strategies in

Asian countries. For instance, the detection of major *msa-1* genotypes by these PCR assays might be useful in designing cocktail vaccines against *B. bovis* in an endemic country, as the *msa-1* is considered as a candidate antigen for vaccine development. A previous study that found marked differences between the *B. bovis msa-1* sequences from the vaccine strains and breakthrough isolates suggested that the immune escape could have been facilitated by the diversity of *msa-1* (Leroith et al., 2005). Therefore, the type-specific PCR assays could be used to determine the major *msa-1* genotypes prevalent among the cattle populations before selecting suitable *B. bovis* strains to be used as live attenuated vaccines in Asian countries.

Artificial recombinants can be generated during PCR when template DNA contains multiple genotypes (Liu et al., 2014). In the present study, the length of amplicons from each type-specific PCR assay was exactly that same as predicted. In addition, the nucleotide sequences did not differ between the two clones that were analyzed for a given PCR amplicon. These findings suggest that the artificial recombination was unlikely during the PCR process in the present study. The DNA samples analyzed in the present study had tested positive for *B. bovis* by highly sensitive nested PCR assays based on multi-copy genes of *B. bovis* (Altangerel et al., 2012; Figueroa et al., 1993; Sivakumar et al., 2012a; Yokoyama et al., 2015). Therefore, single-step type-specific PCR assays targeting single copy genes may not detect the *msa-1* genotypes if the *B. bovis* DNA concentration is low in the field DNA samples. This could explain why only 23 of 109 *B. bovis*-positive DNA samples tested positive in the type-specific PCR assays.

In addition to the type-specific PCR assays, other techniques such as PCR-restriction fragment length polymorphism (RFLP) and PCR-reverse line blot hybridization (RLB) have also been employed to analyze the genetic diversity of microbes (Genis et al., 2009; Godfroid et al., 2003). In particular, RFLP was effectively used to determine the *msa-2b* genotypes among Mexican *B. bovis* isolates (Genis et al., 2009). Therefore, the type-specific PCR assays should be coupled with RFLP and RLB in the future investigation to generate more meaningful data. Furthermore, there are several other Asian countries in which the genetic diversity of *MSA* genes has not been investigated, and the genotypes identified so far are based on a limited numbers of sequences. Therefore, the type-specific PCR assays must be updated whenever novel genotypes are detected. Nevertheless, the type-specific PCR assays developed in the present study are useful tools to determine the distribution of *B. bovis msa-1* genotypes in Asia.

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Figure legends

Fig. 1: Detection of *msa-1* genotypes. *Babesia bovis* *msa-1* gene sequences retrieved from the GenBank database were used to construct a maximum likelihood phylogenetic tree, based on a general time reversible substitution model. Note that the sequences, except those within genotype C, can be classified into Asian (AS1 – AS8), Australian (AU1 – AU6), and American (AM1 – AM3) genotypes. The plasmids containing the *msa-1* gene sequences that were indicated by boldface type letters in the phylogeny were used to analyze the specificities of type-specific PCR assays.

Fig. 2: Specificity testing of the type-specific PCR assays. The type-specific PCR assays were evaluated for their specificity for detecting *msa-1* genotype C (panel A), AS1 (panel B), AS2 (panel C), AS3 (panel D), AS4 (panel E), AS5 (panel F), AS6 (panel G), AS7 (panel H), and AS8 (panel I). Lanes 1 – 9, plasmids containing the inserts for *msa-1* genotypes C, AS1, AS2, AS3, AS4, AS5, AS6, AS7, and AS8, respectively, in all panels. Note that each PCR assay specifically detected the respective genotype.

Fig. 3: Phylogenetic analysis of *msa-1* gene fragments amplified by the type-specific PCR assays. The sequences determined in the present study together with those already reported in Asian countries were used to construct a maximum likelihood phylogenetic tree based on a general time reversible substitution model. The sequences determined from the amplicons of type-specific PCR assays are indicated by boldface type letters.

Note that the newly determined sequences clustered within the corresponding clades in the phylogeny.

Table 1. Primers used for the type-specific PCR assays

Genotype	Primers (5'–3')		Annealing temp. (° C)	Amplicon sizes (bp)
	Forward	Reverse		
C	CAATCGTCCTTCCCGAAGGATCA	GCATGTGCAAACGCATCAAAAGACT	52	517
AS1	CTTTTGCAACTTCTGTCCCGGGT	CATGACCGATAGATACTAAAGTTTTATTGG	59	504
AS2	CTGGGGAAAACAATGTGGATGCATT	TATCAACCCTTGATTGCTCACCGT	64	329
AS3	TGTTAATGATAGAGTAGGTAATGCCAC	CAGATGATTCACCAGCAGATGCC	57	519
AS4	CACTGATGGTTTCGTTACAGCG	CCTCTCTGTTGTTATAGACGTGCTTAAC	57	209
AS5	GAAGGGGAAACCCACATGAC	TGCATAACCGACACGTACTAAT	56	345
AS6	GATTAAGACTAATCCGAAGTTTACCGA	TGGTGCGCCACTACCGGGTGA	61	406
AS7	AAGGACTTGATGAAATGAAGAAAACA	GCCTAGTTTCAACACCGGAGC	53	402
AS8	GTTGCAGTATCCATTAAGGGCAATAGT	TTTTTTGGTAATACATAGCAGCAGCA	61	340

Table 2. *B. bovis msa-I* genotypes determined by the type-specific PCR assays in Sri Lanka, Mongolia, and Vietnam

Country	Sample No.	Genotypes (Accession numbers ^a)
Sri Lanka	P31	AS5 (LC066122)
	P58	AS5 (LC066123)
	P75	AS1 (LC066113)
	AM13	AS5 (LC066124)
	AM21	AS1 (LC066114)
	AK29	AS5 (LC066125)
	J6 ^b	C (LC066106), AS7 (LC066133), AS8 (LC066134)
Mongolia	BI-9	C (LC066107)
	BI-15	AS4 (LC066117)
	JA-14	C (LC066108)
	JA-27 ^b	C (LC066109), AS1 (LC066115), AS6 (LC066126)
	JA-41 ^b	C (LC066110), AS6 (LC066127)
	DA-8	C (LC066111)
	BO36	C (LC066112)
Vietnam	CA63	AS6 (LC066128)
	CA124	AS4 (LC066118)
	CA164	AS4 (LC066119)
	CA187 ^b	AS1 (LC066116), AS4 (LC066120)
	CA202	AS6 (LC066129)
	CA263	AS6 (LC066130)
	BU23	AS8 (LC066135)
	BU25 ^b	AS4 (LC066121), AS6 (LC066131)
	BU28	AS6 (LC066132)

^a Sequences of PCR amplicons were registered with GenBank.

^b These samples were co-infected with multiple *MSA-I* genotypes.

Fig. 1

Fig. 2

Fig. 3