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Analysis of the genetic diversity of *Babesia bovis msa-1* in Thailand using type-specific polymerase chain reaction

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ABSTRACT

Babesia bovis, a tick-borne hemoprotozoan parasite, causes fatal bovine babesiosis in cattle. Merozoite surface antigens, encoded by *msa* genes, of *B. bovis* play an important role in the initial attachment of the merozoite to host erythrocytes. In the present study, *B. bovis*-positive blood DNA samples (n = 162) sourced from cattle in Thailand were analyzed using type-specific polymerase chain reaction (PCR) assays targeting nine Asian *msa-1* genotypes (genotypes C and AS1–AS8). All nine *msa-1* genotypes were detected in Thai cattle, and at least one *msa-1* genotype was detected in 154 of the 162 samples. The most common genotype was AS7 (n = 139), followed by AS5 (n = 61), AS1 (n = 37), AS8 (n = 32), C (n = 11), AS4 (n = 8), AS3 (n = 5), AS6 (n = 2), and AS2 (n = 1). Co-infection with parasites of different genotypes was also commonly detected, and with 90 (58.4%) of the 154 PCR-positive DNA samples showing infection with more than one genotype. Overall, the detected genetic diversity of *msa-1* from *B. bovis* was much higher than that previously determined in Thailand. These findings warrant further investigation to determine the nature of antigenic variation between the different *B. bovis* genotypes detected in the cattle population of Thailand.

Keywords: *Babesia bovis*, Genetic diversity, *msa-1*, Thailand, Type-specific polymerase chain reaction

INTRODUCTION

Babesia bovis is a hemoprotozoan parasite that causes severe clinical babesiosis in cattle, leading to huge economic losses in tropical and subtropical regions of the world (Bock *et al*., 2004; Ristic, 1981). In addition to the fever, hemoglobinuria, anemia, and jaundice that are commonly associated with *Babesia* infections, *B. bovis* may result in respiratory and neurological syndromes caused by the sequestration of infected red blood cells (RBCs) in capillaries of host animals (Everitt *et al*., 1986).

The lifecycle of *B. bovis* in cattle begins with the injection of sporozoites during the blood meal of infected tick vectors (Hunfeld *et al*., 2008). The sporozoites then invade the RBCs, where they transform into

merozoites (Bock *et al*., 2004). The mature merozoites are released from the RBCs, invading new RBCs, where they undergo asexual multiplication (Bock *et al*., 2004). The RBC invasion of merozoites is mediated by several surface-exposed parasite antigens (Yokoyama *et al*., 2006). Among these, merozoite surface antigens (MSAs) play a crucial role in the initial attachment of the merozoite to the host RBC membrane (Yokoyama *et al*., 2006). MSAs, which are highly immunogenic, induce neutralization-sensitive antibodies in the infected cattle (Hines *et al*., 1992). Therefore, MSAs have been considered as candidates for use in subunit vaccines against *B. bovis* infection (Brown and Palmer, 1999; Hines *et al*., 1992). However, the high level of genetic diversity amongst the genes that encode the MSAs hinders the use of these antigens in vaccines (Berens *et al*., 2005; Leroith *et al*., 2005; Sivakumar *et al*., 2013a). Although development of a cocktail vaccine containing antigens encompassing the genetic variations could overcome this constraint, a thorough knowledge of the genetic variation between *B. bovis msa* genes is essential before such an idea can be executed.

The genetic diversity of *msa* genes, including *msa-1*, *msa-2b*, and *msa-2c*, has been analyzed in several *B. bovis*-endemic countries (Altangerel *et al*., 2012; Molad *et al*., 2014; Sivakumar *et al*., 2013a; Tattiyapong *et al*., 2014; Yokoyama *et al*., 2015). The most common method used to determine the diversity of *msa* genes is the analysis of sequences generated from polymerase chain reaction (PCR)-amplified gene fragments. However, this methodology, which requires expensive equipment and reagents, is ineffective in detecting co-infection with multiple genotypes. On the other hand, type-specific PCR assays, which eliminate the need for sequencing, have been useful in analyzing genetic variation of microbial genes, and are now commonly used in *Theileria* research (Sivakumar *et al*., 2013b; Yokoyama *et al*., 2011, 2012). Recently, type-specific PCR assays were developed for analysis of *msa-1* from *B. bovis*, and were successfully used to evaluate *B. bovis*-positive DNA samples from Sri Lanka, Mongolia, and Vietnam (Liyanagunawardena *et al*., 2016). A previous investigation found that *B. bovis msa-1* was highly conserved in Thailand; however, the findings were not conclusive as only a few sequences were analyzed (Nagano *et al*., 2013). Therefore, in the present study, we analyzed the genetic diversity of *msa-1* from large numbers of *B. bovis*-positive blood DNA samples sourced from cattle in Thailand using type-specific PCR assays.

MATERIALS AND METHODS

DNA samples

DNA samples used in the present study were extracted from blood collected from 162 dairy cattle that had been reared in 12 provinces of Thailand in 2010 (Simking *et al*., 2013). All DNA samples were positive for *B. bovis* based on results from a nested PCR assay targeting the spherical body 2 gene *sbp-2* (Simking *et al*., 2013).

Type-specific PCR assays for Asian *msa-1* genotypes

All 162 *B. bovis*-positive DNA samples were analyzed using type-specific PCR assays that target Asian *msa-1* genotypes (genotypes C and AS1–AS8), as described by Liyanagunawardena *et al*. (2016) with

minor modifications. The PCR assays were performed in a total volume of 10 µl, containing 2 µl of template DNA, 1 µl of 10× PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 1 µl of dNTPs (2 mM each; Applied Biosystems), 0.5 µl of each primer (10 µM; Liyanagunawardena *et al*., 2016), 0.5 U of *Taq* DNA polymerase (Applied Biosystems), and 4.9 µl of distilled water. Thermal cycler parameters consisted of an initial enzyme activation step at 95°C for 5 min, followed by 45 cycles of 95°C for 30 s, an appropriate annealing temperature (Liyanagunawardena *et al*., 2016) for 1 min, and 72°C for 1 min. After the final extension at 72°C for 7 min, PCR products were separated by 1.5% agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light. Detection of a band of the expected size was considered a positive result for each type-specific PCR assay (Liyanagunawardena *et al*., 2016).

RESULTS AND DISCUSSION

The results of type-specific PCR assays demonstrated the presence of all nine Asian *B. bovis msa-1* genotypes targeted in the present study (Table 1). This indicates that the genetic diversity of *msa-1* is much higher than that determined in a previous study of a small number of samples from Thailand (Nagano *et al*., 2013). Among the 162 *B. bovis*-positive DNA samples, 154 were positive at least for one genotype. Genotype AS7, which was detected in 139 samples, was the most common, followed by AS5 (n = 61), AS1 (n = 37), AS8 (n = 32), C (n = 11), AS4 (n = 8), AS3 (n = 5), AS6 (n = 2), and AS2 (n = 1) (Table 1). The four major genotypes, AS7, AS5, AS1, and AS8, were detected in cattle from all four geographic regions (north, northeast, central, and south) of Thailand. In contrast, the remaining minor genotypes (C, AS4, AS3, AS6, and AS2) were not detected in some regions, possibly because of the overall rarity of these genotypes (Table 1). We also observed co-infection with multiple genotypes: of the 154 type-specific PCR-positive samples, 90 (58.4%) showed co-infection with more than one genotype (Table 2). While a single sample showed the presence of five genotypes, eight, 32, and 49 samples were co-infected with four, three, and two genotypes, respectively. The high rate of co-infection might lead to the emergence of new genotypes through genetic recombination in the tick vectors (Lau *et al*., 2010).

The genetic variation of *msa* genes has been linked to different immune profiles in infected animals (Berens *et al*., 2005; Leroith *et al*., 2005). We speculated that the immunity induced by one *msa-1* genotype might prevent subsequent infection with a closely related genotype. However, the present findings did not support this assumption. For example, 26 (81.3%) of 32 AS8-positive DNA samples were co-infected with AS7 (Table 2), which was shown to be a sister clade of AS8 in a previous phylogenetic analysis (Liyanagunawardena *et al*., 2016). This observation suggests that the immunity induced by *B. bovis* strains that belong to even closely related genotypes might not be cross-protective. Additionally, the findings infer that the different genotypes could have been simultaneously transmitted by co-infected ticks, as the tick vectors may ingest parasites with different genotypes when they feed on co-infected cattle. Therefore, additional studies are needed to determine the immunological cross-reactivity among *B. bovis* MSA-1 antigens belonging to different genotypes, and to investigate the tick vectors for possible co-infection with

multiple genotypes. Such studies should also identify any cross-reacting antigens that belong to different *msa-1* genotypes to assist with vaccine development.

In summary, the present study investigated the genotypic diversity of *B. bovis msa-1* using type-specific PCR assays. The results showed that *msa-1* is highly diverse among *B. bovis* isolates in Thailand, and that co-infection with multiple genotypes is common amongst parasite-infected animals. The findings of the present study have potential implications for the development of control measures for *B. bovis* in Thailand.

Table 1. *Babesia bovis msa-1* genotypes determined by type-specific PCR assays among *B. bovis*-positive DNA samples from cattle populations in different geographical regions of Thailand.

Regions	No. of samples	No. of positive samples								
		C	AS1	AS2	AS3	AS4	AS5	AS6	AS7	AS8
North	56	1 (1.8%)	9 (16.1%)	1 (1.8%)	1 (1.8%)	4 (7.1%)	18 (32.1%)	1 (1.8%)	50 (89.3%)	8 (14.3%)
Northeast	9	0	3 (33.3%)	0	0	0	6 (66.7%)	0	9 (100%)	2 (22.2%)
Central	96	10 (10.4%)	24 (25%)	0	4 (4.2%)	4 (4.2%)	36 (37.5%)	1 (1.0%)	79 (82.3%)	21 (21.9%)
South	1	0	1 (100%)	0	0	0	1 (100%)	0	1 (100%)	1 (100%)
Total	**162**	**11 (6.8%)**	**37 (22.8%)**	**1 (0.6%)**	**5 (3.1%)**	**8 (4.9%)**	**61 (37.7%)**	**2 (1.2%)**	**139 (85.8%)**	**32 (19.8%)**

Table 2. Co-infection of *B. bovis msa-1* genotypes as determined by type-specific PCR assays

Combination	No. of samples
5 genotypes	
C + AS3 + AS5 + AS7 + AS8	1
4 genotypes	
AS1 + AS5 + AS7 + AS8	5
AS1 + AS2 + AS4 + AS5	1
C + AS1 + AS7 + AS8	1
C + AS5 + AS7 + AS8	1
3 genotypes	
AS1 + AS5 + AS7	12
AS5 + AS7 + AS8	8
C + AS5 + AS7	3
AS1 + AS7 + AS8	3
C + AS1 + AS7	1
AS1 + AS4 + AS7	1
AS1 + AS5 + AS8	1
AS1 + AS6 + AS7	1
AS3 + AS5 + AS7	1
AS4 + AS5 + AS7	1
2 genotypes	
AS5 + AS7	18
AS7 + AS8	10
AS1 + AS7	7
C + AS7	3
AS4 + AS7	3
AS1 + AS5	2
AS3 + AS7	2
C + AS5	1
AS1 + AS4	1
AS3 + AS4	1
AS5 + AS8	1
1 genotype	
AS7	58
AS5	5
AS6	1

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