Genetic characterization of *Babesia* and *Theileria* parasites in water buffaloes in Sri Lanka

Running Head: *Babesia* and *Theileria* in Sri Lankan water buffaloes

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ABSTRACT

Water buffaloes are thought to be the reservoir hosts for several hemoprotozoan parasites that infect cattle. In the present study, we surveyed Sri Lankan bred water buffaloes for infections with *Babesia bovis*, *B. bigemina*, *Theileria annulata*, and *T. orientalis* using parasite-specific PCR assays. When 320 blood-derived DNA samples from water buffaloes reared in three different districts (Polonnaruwa, Mannar, and Mullaitivu) of Sri Lanka were PCR screened, *B. bovis*, *B. bigemina*, and *T. orientalis* were detected. While *T. orientalis* was the predominant parasite (82.5%), low PCR-positive rates were observed for *B. bovis* (1.9%) and *B. bigemina* (1.6%). Amplicons of the gene sequences of the Rhoptry Associated Protein-1 (RAP-1) of *B. bovis*, the Apical Membrane Antigen-1 (AMA-1) of *B. bigemina*, and the Major Piroplasm Surface Protein (MPSP) of *T. orientalis* were compared with those characterized previously in Sri Lankan cattle. While the *B. bigemina* AMA-1 sequences from water buffaloes shared high identity values with those from cattle, *B. bovis* RAP-1 sequences from water buffaloes diverged genetically from those of cattle. For *T. orientalis*, none of the MPSP sequence types reported previously in Sri Lankan cattle (Types 1, 3, 5, and 7) were detected in the water buffaloes, and the MPSP sequences

46 analyzed in the present study belonged to types N1 or N2. In summary, in addition to
47 reporting the first PCR-based survey of *Babesia* and *Theileria* parasites in water
48 buffaloes in Sri Lanka, the present study found that the predominant variants of water
49 buffalo-derived *B. bovis* *RAP-1* and *T. orientalis* *MPSP* sequences were different from
50 those previously described from cattle in this country.

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52 **Keywords:** *Babesia*, PCR, Sri Lanka, *Theileria*, Water buffalo

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1. INTRODUCTION

Livestock play a critical role in fulfilling the nutritional requirements of human beings. Therefore, maintaining healthy livestock is essential for the supply of livestock products. However, livestock are often at risk of becoming infected with harmful infectious agents. Among them, piroplasmosis of cattle, which is caused by various species of *Babesia* and *Theileria* parasites, has a worldwide distribution (Uilenberg, 1995). Bovine piroplasmosis imposes huge economic losses to cattle farming operations.

B. bovis, *B. bigemina*, and *B. divergens* are described as the parasite species potentially involved in severe clinical babesiosis in cattle (Bock et al., 2004). Among these species, *B. bovis* is the most virulent and causes neurological and respiratory disorders that lead to death in the cattle affected (Everitt et al., 1986). However, *B. bigemina* and *B. divergens* infections can also be fatal when animals are not cared properly (Bock et al., 2004). While *B. bovis* and *B. bigemina* are found mostly in tropical and sub-tropical regions of the globe, *B. divergens* is present mainly in Europe (Zintl et al., 2003). Similar to the virulent *Babesia* spp., *Theileria parva* and *T. annulata* cause severe clinical disease in cattle (McKeever, 2009). The geographical distribution

of *T. parva* is limited to eastern, central, and southern Africa, whereas *T. annulata* is common in North Africa, southern Europe, and Asia (Lawrence et al., 1994). In contrast, a benign group of *Theileria* parasites including *T. orientalis*, *T. sergenti*, and *T. buffalo* has a wide host range and worldwide distribution (Sugimoto and Fujisaki, 2002). Although known to be benign, disease outbreaks in cattle caused by these species have been reported in several countries (Aparna et al., 2011; McFadden et al., 2011; Eamens et al., 2013).

Several studies have reported the presence of these *Babesia* and *Theileria* parasites in water buffaloes (Ferrerri et al., 2008; Altangerel et al., 2011; Khukhuu et al., 2011; He et al., 2012). Although clinical disease in water buffaloes from these parasites is not common, these animals might act as reservoir hosts (Karbe et al., 1979; McKeever, 2009; Oura et al., 2010; Altangerel et al., 2011); hence, detection of *Babesia* and *Theileria* parasites in these animals is equally important to that in cattle. It makes sense, therefore, that the control strategies used for livestock should be expanded to water buffaloes to minimize the infection rates among these animals.

Recently, we demonstrated the presence of *B. bovis*, *B. bigemina*, *T. annulata* and *T. orientalis* in cattle populations reared in Sri Lanka (Sivakumar et al., 2012b). The predominant parasite detected among the cattle was *T. orientalis*, followed by *B.*

bigemina, *B. bovis*, and *T. annulata*. When genetic diversity in the *T. orientalis* isolates from cattle was analyzed, four different genotypes (Types 1, 3, 5, and 7) were defined based on the (Homologous/allelic) Major Piroplasm Surface Antigen (MPSP) gene sequences (Sivakumar et al., 2013b). Type 7 found previously to be involved in several clinical theileriosis cases in southern India was the most common MPSP type in the Sri Lankan cattle populations. In addition, genetic diversity, as based on the Merozoite Surface Antigen (MSA) genes among the *B. bovis* isolates, was very high in Sri Lankan populations of the parasites (Sivakumar et al., 2013a). In Sri Lanka, water buffaloes are mainly bred in the dry zone and used to be in close contact with cattle, especially during grazing. However, the scientific literature is very sparse for hemoprotozoan parasites of Sri Lankan water buffaloes. Although *T. orientalis*-like parasites have been shown to be present in Giemsa-stained thin blood smears prepared from water buffaloes, the exact species was not determined (Weilgama et al., 1986).

In the present study, we collected blood samples from water buffaloes reared in three different districts of Sri Lanka, and conducted a series of PCR-based surveys of *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis*. Subsequently, the PCR amplicons were sequenced, and the resultant gene sequences were compared with those generated from cattle in previous investigations (Sivakumar et al., 2012b, 2013b).

2. Materials and methods

2.1. Blood samples

Blood samples were collected from 320 water buffaloes reared in three different districts (Polonnaruwa, Mannar, and Mullaitivu Fig. 1) of Sri Lanka in February 2013. All the animals surveyed in the present study were over 1 year of age and were apparently healthy during the sampling period. Approximately 2 ml of whole blood was drawn from each animal into a Vacutainer tube that contained EDTA (NIPRO, Osaka, Japan). The samples were labeled and then stored at -20°C until they were used for DNA extraction.

2.2. DNA extraction

DNA samples were extracted from each blood sample as described previously (Sivakumar et al., 2012a). Briefly, DNA was extracted from 200 μl of each blood sample using a commercial extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA samples were eluted in 200 μl of the elution buffer provided with the kit, and then stored at -20°C until use.

2.3. Polymerase chain reactions

Previously described parasite-specific PCR assays based on the Rhoptry Associated Protein-1 (RAP-1), Apical Membrane Antigen-1 (AMA-1), *Theileria annulata* Merozoite Surface Antigen-1 (Tams1), and MPSP genes were employed to screen all of the DNA samples for infections caused by *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis*, respectively. While *B. bovis* was examined by a nested PCR (Figuerola et al., 1993), all other parasites were screened by single-step PCRs (Kirvar et al., 2000; Ota et al., 2009; Sivakumar et al., 2012a). The PCR primers, PCR reaction mixtures, and cycling conditions have been described in a previous report (Sivakumar et al., 2012b). PCR products were subjected to gel electrophoresis, and bands were visualized under UV light after ethidium bromide staining. Detection of PCR amplicons close to the expected sizes (i.e., *B. bovis* 298 bp, *B. bigemina* 211 bp, *T. annulata* 788bp, and *T. orientalis* 776 bp) were considered positive for the respective parasites.

2.4. Cloning and sequencing

PCR amplicons with high band intensities were selected for DNA cloning and sequencing, following a previously described protocol (Sivakumar et al., 2013a). Briefly, the PCR products were cloned into a plasmid vector (PCR 2.1-TOPO, Invitrogen,

Carlsbad, CA, USA), and the nucleotide sequences of the inserted genes were determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. Sequencing and phylogenetic analyses

The nucleotide sequences generated in the present study were initially analyzed using GENETYX v. 7.0 software (GENETYX, Tokyo, Japan), and the basic local alignment tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/>). The identity values among the gene sequences were estimated using the EMBOSS NEEDLE software program, which is available online (<http://emboss.bioinformatics.nl/cgi-bin/emboss/needle>). The phylogenetic trees were constructed using MAFFT (Kato et al., 2002) and were based on the neighbor-joining method (Saitou and Nei, 1987) and the Jukes-Cantor substitution model (Jukes and Cantor, 1969). In addition to the sequences obtained in the present study, homologous gene sequences retrieved from GenBank were also incorporated into the phylograms.

2.6. Statistical analysis

The confidence intervals for the prevalence values of each parasite species

163 were calculated based on the method described by Wilson (1927) using the OpenEpi

164 online program (<http://www.openepi.com/v37/Proportion/Proportion.htm>).

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3. RESULTS

The present survey detected *B. bovis*, *B. bigemina*, and *T. orientalis* from water buffaloes reared in Sri Lanka, whereas none of the samples was positive for *T. annulata* (Table 1). While all three of the above parasites were found in Mannar and Mullaitivu districts, Polonnaruwa district had only *B. bovis* and *T. orientalis*. The most common parasite was *T. orientalis*, which was detected in 264 animals (82.5%). The highest *T. orientalis*-positive rate was in the Mannar district (94.5%) as compared with Polonnaruwa (78.8%) and Mullaitivu (58.9%) districts. In contrast to the high prevalence of *T. orientalis*, very low detection rates were observed with *B. bovis* (1.9%) and *B. bigemina* (1.6%). Because of the low prevalence rates for these parasites, co-infection with more than one *Babesia* species was also very low. Out of 265 DNA samples that were positive for at least one parasite species, only 10 were co-infected with two different species. All *B. bovis*- and *B. bigemina*-infected samples, except for one individual *B. bovis*-infected sample from the Mullaitivu district, were also infected with *T. orientalis*.

All the amplicons resulting from *B. bovis*- and *B. bigemina*-specific PCRs were cloned and sequenced. For *T. orientalis*, 49 PCR amplicons, which showed high band

intensities, from all three sampling locations were selected for sequencing. While 2 clones were sequenced per sample for *B. bigemina* and *B. bovis*, a single clone was sequenced for *T. orientalis* PCR amplicons. However, for each *B. bovis* and *B. bigemina* positive sample, sequences of both clones were identical to each other. The *B. bovis* *RAP-I* sequences (n=6) determined in the present study (AB845432–AB845437) were identical to each other, and to those from the United States (AF030059) and Argentina (AF030055 and AF030056). However, the *RAP-I* sequences from water buffaloes shared only 91.9–93.0% identity values with those from Sri Lankan cattle (AB690859–AB690861) that had been determined in a previous investigation (Sivakumar et al., 2012b). In the phylogenetic tree, *B. bovis* *RAP-I* sequences were dispersed across two major clades (Fig. 2). While the *RAP-I* sequences from Sri Lankan buffaloes were found in a clade with those from several other countries (clade 2), the previously described sequences (AB690859–AB690861), which had been obtained from *B. bovis*-positive DNA samples sourced from cattle, formed a separate clade (clade 1).

The *AMA-1* gene fragments amplified by the *B. bigemina*-specific PCR assay were 211 bp in length as expected. The Sri Lankan *AMA-1* sequences generated in the present study (AB845438–AB845442) were 99.1–100% identical to each other, and also

shared 99.5–100% sequence identities with the *B. bigemina* AMA-1 sequences obtained from Sri Lankan cattle in a previous study (AB690862), and with other database registered isolates (JN572801, HM543730, AB481200, AB742553, and JX860295).

Out of 49 *T. orientalis* MPSP gene sequences determined in the present study, 24 (AB845443 - AB845466), 16 (AB845467 - AB845482), and 9 (AB845483 - AB845491) sequences were from Polonnaruwa, Mannar, and Mullaitivu districts, respectively. Among these sequences, 42 (AB845444–AB845451, AB845453, AB845454, AB845456, AB845457, AB845460–AB845472, AB845474–AB845476, and AB845478–AB845491) were 779 bp in length, while six (AB845443, AB845452, AB845455, AB845458, AB845459, and AB845477) and one (AB845473) were 776 and 773 bp, respectively. The phylogenetic analysis revealed that the MPSP gene sequences from water buffaloes in Sri Lanka could be classified as belonging to two types, N1 and N2 (Fig. 3). All of the 42 sequences of 779 bp were MPSP type N1, while the remaining seven were type N2.

4. DISCUSSION

A recently conducted PCR-based study identified the presence of clinically significant *Babesia* and *Theileria* parasites among Sri Lankan cattle populations (Sivakumar et al., 2012b). Although clinical piroplasmosis has rarely been detected in water buffaloes bred in Sri Lanka, the potential for these animals to act as reservoir hosts for *Babesia* and *Theileria* parasites has not been investigated. In the present study, therefore, Sri Lankan water buffaloes were surveyed for *Babesia* and *Theileria* parasites, which have been detected in these animals previously (Sivakumar et al., 2012b).

With the exception of *T. annulata*, all the other parasites surveyed (i.e., *B. bovis*, *B. bigemina*, and *T. orientalis*) were detected in the water buffaloes. Similar to the cattle populations (Sivakumar et al., 2012b), *T. orientalis* was identified as the predominant parasite among the water buffaloes. However, the prevalence of *T. orientalis* (82.5%) in the water buffaloes was higher than that reported for cattle (53.5%). In contrast, the parasite-positive rates for *B. bovis* and *B. bigemina* (1.9% and 1.6%, respectively) were much lower in water buffaloes than in cattle (13.9% and 30.1%, respectively). In addition, all of the buffaloes surveyed were *T. annulata*-negative, despite this parasite being detected in Sri Lankan cattle populations (9.8%) (Sivakumar et al., 2012b). Although the reasons for these discrepancies are not clear, low parasitemia in water

buffaloes or differences between the tick species that infest cattle and water buffaloes could account for these observations.

Sequencing and phylogenetic analyzes confirmed that the PCR assays detected only the target species. Additionally, the nucleotide sequences determined in the present study were compared with the respective sequences previously determined from the parasite-positive DNA samples of cattle in Sri Lanka (Sivakumar et al., 2012b, 2013a). In the *RAP-1* gene-based phylogram, the sequences from the Sri Lankan water buffaloes were clearly separated from the *RAP-1* sequences from *B. bovis* in Sri Lankan cattle (Sivakumar et al., 2012b). Furthermore, the water buffalo sequences shared low identity values with those from cattle, suggesting that the *B. bovis* population in water buffaloes might be different from that in cattle in Sri Lanka. However, *RAP-1* sequences obtained in the present study clustered together with several cattle-derived sequences from other countries. Therefore, there may be no host specificity for these variants, while the present findings might be unique to Sri Lanka. Although the *B. bovis RAP-1* sequences from cattle formed a single clade in the phylogram, the gene sequences of the merozoite surface antigens (MSA) from these samples were recently shown to be highly diverse and dispersed across several clades in their respective phylograms (Sivakumar et al., 2013a). Therefore, an attempt was made to amplify the MSA genes (*MSA-1*,

MSA-2b, and MSA-2c) from the *B. bovis*-positive water buffalo DNA samples. However, visible PCR amplicons for the targeted MSA genes were not detected in any of the DNA samples, possibly because of the low concentration of *B. bovis* DNA in the field samples. Therefore, large numbers of *B. bovis*-positive DNA samples from Sri Lankan water buffaloes are required to generate sufficient MSA gene sequences for comparative genetic diversity analysis of *B. bovis*.

In the phylogenetic analysis, the *B. bigemina* AMA-1 sequences from water buffaloes obtained in the present study formed a monophyletic clade together with those from Sri Lankan cattle (data not shown). A recently conducted study demonstrated that the relationship between the diversity of *Plasmodium falciparum* AMA-1 gene sequences and their antigenicity profiles was inconsistent and moderate (Drew et al., 2012). Therefore, monophyletic clade formed by AMA-1 gene sequences from Sri Lankan water buffaloes and cattle does not necessarily mean that the sequence variants from both host animals might be originated from same sub-populations of *B. bigemina*.

When the *T. orientalis* MPSP gene sequences were analyzed, only N1 and N2 types were identified in the Sri Lankan water buffaloes. The distribution of types N1 and N2 in Sri Lankan water buffaloes is different from those reported in other Asian countries. For example, in Thailand, out of 24 *T. orientalis* MPSP sequences from water

buffaloes, only a single sequence was determined to be type N2, while type N1 was not detected at all (Altangerel et al., 2011). Previously, four kinds of MPSP sequence types (1, 3, 5, and 7) were detected in Sri Lankan cattle (Sivakumar et al., 2013b). In contrast to the present findings, types 1, 3, 5, and 7 have been reported in water buffaloes in several other countries (Altangerel et al., 2011; Khukhuu et al., 2011), suggesting that, similar to the *B. bovis* *RAP-1* variants, there may be no specific barrier for these MPSP types to infect water buffaloes.

The gene sequences analyzed in the present study encode antigens, which are known to be under constant immune pressure. The differences in the immune profiles between water buffaloes and cattle, therefore, might influence the variants of the parasites that infect these animals. The epidemiological studies related to *T. parva* demonstrated that the variants in cattle were sometimes different from those observed in cape buffaloes. One of the reasons for these discrepancies was suggested to be the selection of variants in tick vectors (McKeever, 2009). Our recent study suggested the possibility that a parasite MPSP type-specific transmission pattern exists in different tick species (Yokoyama et al., 2012). Additionally, similarities and differences among the tick species that infect cattle and water buffaloes have been observed (Dovaudi et al., 2008; Kariuki et al., 2012). Hence, it is possible that the tick species that transmit *T.*

289 *orientalis* to cattle might not feed on water buffaloes bred in Sri Lanka. Similar to *T.*
290 *orientalis*, selection of variants in tick vector might be one of the possible explanations
291 for the differences between *B. bovis* variants in water buffaloes and cattle in Sri Lanka.
292 Clearly, additional studies to identify the tick vectors that transmit different variants of *T.*
293 *orientalis* and *B. bovis* to cattle and water buffaloes are needed to confirm this
294 possibility.

295 Although the present study suggested that *B. bovis* and *T. orientalis* variants in
296 water buffaloes might be different from those in cattle, this observation can be
297 influenced by several confounding factors. For instance, sequencing of 1 or 2 clones per
298 PCR amplicon could have detected only the predominant variants and left the minor
299 variants undetected. The possibility that these undetected variants in water buffaloes
300 might be similar to those observed in the cattle may not be entirely ruled out. In addition,
301 the present investigation is a cross-sectional study, and therefore, it detected the variants
302 which could have been predominant at the time of sampling. However, Lau et al. (2010)
303 demonstrated that a cattle herd can be infected with several variants of *B. bovis* at a
304 given time and that the changes in the genotypes can be observed with time not only
305 within a herd but also in individual animals. Therefore, year-round sampling from both

306 cattle and water buffaloes and sequencing analysis of protozoan genes are now a
307 priority in Sri Lanka.

308 In conclusion, the first PCR-based study conducted on water buffaloes in Sri
309 Lanka detected *B. bovis*, *B. bigemina*, and *T. orientalis* parasites. The findings from the
310 sequencing analyses warrant further studies to determine the role of water buffaloes in
311 the epidemiology of *Babesia* and *Theileria* parasites in Sri Lankan cattle.

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Conflicts of interest

The authors have no conflicts of interest concerning the work reported in this paper.

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FIGURE LIGENDS

Fig. 1. Sampling locations in Sri Lanka. Blood samples were collected from water buffaloes in three different districts.

Fig. 2. *B. bovis* RAP-1 gene-based phylogenetic tree. The phylogenetic tree was constructed using the *B. bovis* RAP-1 sequences determined in the present study (bold type) and those from several other countries. Bootstrap values are provided at the beginning of each branch. Note that the sequences generated from the *B. bovis*-positive DNA samples from Sri Lankan water buffaloes clustered together with those from other countries and formed a monophyletic clade (clade 2), while the previously determined RAP-1 sequences from *B. bovis* of cattle formed a separate clade (clade 1).

Fig. 3. *T. orientalis* MPSP gene-based phylogenetic tree. In addition to the MPSP gene sequences determined in the present study (boldface), representative sequences of each genotype were used to construct the phylogram. Bootstrap values are provided at the beginning of each branch. Note that the sequences generated from the *T.*

485 *orientalis*-positive DNA samples from Sri Lankan water buffaloes clustered with other
486 MPSP N1 and N2 types.

Table 1. PCR screening results

Districts	No. of Samples	<i>B. bovis</i>		<i>B. bigemina</i>		<i>T. orientalis</i>	
		No. Positive	% (CI ^a)	No. Positive	% (CI)	No. Positive	% (CI)
Polonnaruwa	118	1	0.8 (0.1 - 4.6)	0	0	93	78.8 (70.6 - 85.2)
Mannar	146	3	2.1 (0.7 - 5.9)	4	2.7 (1.1 - 6.8)	138	94.5 (89.6 - 97.2)
Mullaitivu	56	2	3.6 (1.0 - 12.1)	1	1.8 (0.3 - 9.4)	33	58.9 (45.9 - 70.8)
(Total)	320	6	1.9 (0.9 - 4.0)	5	1.6 (0.7 - 3.6)	264	82.5 (78.0 - 86.3)

^a 95% Confidence interval

Figure

Fig. 1

Fig. 2

Fig. 3