



Short communication

Molecular survey of *Babesia* infections in cattle from different areas of Myanmar



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ABSTRACT

Cattle babesiosis is one of the most important tick-borne diseases worldwide. The present study reports a molecular survey of *Babesia* infections in cattle in Myanmar. Nested PCR assays based on the *Babesia bigemina* apical membrane antigen-1 gene (*AMA-1*) and *B. bovis* rhoptry associated protein-1 gene (*RAP-1*) revealed that the overall percentage of *B. bigemina* and *B. bovis* infection were 9.8% (70/713) and 17.1% (122/713), respectively. A mixed infection was detected in 4.6% (33/713) of animals. Animals <1 year (OR = 13.66, CI = 5.15–36.26) and 1–5 years of age (OR = 3.91, CI = 1.50–10.17) were identified as potential risk factors for *B. bigemina* infection. For *B. bovis* infection, age <1 year (OR = 3.06, CI = 1.63–5.75) and 1–5 years (OR = 2.08, CI = 1.21–3.57), Friesian–Zebu crossbreeds (OR = 2.04, CI = 1.26–3.30) and grazing (OR = 1.59, CI = 1.06–2.38) were identified as potential risk factors. This is the first report on a nationwide survey of bovine *Babesia* infections in Myanmar, providing useful information for the management and control of the disease.

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1. Introduction

Babesiosis caused by infection with protozoan parasites of the genus *Babesia* is one of the most important tick-borne diseases in cattle. Among *Babesia* parasites, *B. bigemina* and *B. bovis* are the main species in cattle, with high prevalence in tropical and subtropical regions of the world (Bock et al., 2004; Uilenberg, 1995). Clinical signs caused by these species include anemia, fever, hemoglobinuria, and in many cases death (Brown et al., 2006). Nervous signs and respiratory distress syndrome are sometimes observed in *B. bovis* infection (Everitt et al., 1986; Wright and Goodger, 1988). However, cattle may remain persistently infected with no clinical symptoms, and thus play an important role in parasite transmission. Therefore, effective control strategies must

include the detection of asymptomatic carrier animals (Bock et al., 2004).

The microscopic detection of *Babesia* parasites in blood smears has always been considered the gold standard for the diagnosis of acute babesiosis, but this detection is difficult in carrier animals with low parasitemia levels (Almeria et al., 2001). Molecular diagnosis by PCR-based techniques has been developed for the detection of *Babesia* DNA and used for epidemiological surveys in livestock populations (Figuerola et al., 1992, 1993). Recently, an improved PCR method targeting the apical membrane antigen-1 gene (*AMA-1*) was demonstrated for specific detection of *B. bigemina* in many countries, including Egypt, Mongolia, Sri Lanka, Vietnam and the Philippines (Elsify et al., 2015; Sivakumar et al., 2012a,b, 2013; Ybañez et al., 2013). A PCR method based on the rhoptry associated protein-1 gene (*RAP-1*) has been useful for the detection of *B. bovis* in Egypt, South Africa, Brazil, Portugal, Sri Lanka and the Philippines (Elsify et al., 2015; Mtshali and Mtshali, 2013; Ramos et al., 2012; Silva et al., 2009; Sivakumar et al., 2012b; Ybañez et al., 2013).

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In Myanmar, the molecular survey of bovine *Theileria orientalis* infection in different areas has been reported recently (Bawm et al., 2014), suggesting that other tick-borne pathogens were also distributed in Myanmar. In the present study, infection of cattle with *B. bigemina* and *B. bovis* was investigated by nested PCR based on the *AMA-1* and *RAP-1* genes, respectively, since no previous data referring to these parasites are available so far.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all reagents used in this study were of molecular biology grade and purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan) or Sigma–Aldrich Co. (St. Louis, MO, USA).

2.2. Study area

A molecular survey was conducted at 43 cattle farms in 9 cities located in different areas of Myanmar, Bagan/Nyaung U, Mandalay, Myitkyina, Nay Pyi Taw, Nyaungshwe, Patheingyi, Pyin Oo Lwin, Taunggyi and Yangon (Bawm et al., 2014). A questionnaire was used to obtain the characteristics and management of cattle. The items on the questionnaire included age, sex, breed, health status and grazing habit. In most commercial farms, Holstein (Friesian)–Zebu cross breed is raised with a semi-intensive system and fed roughage, such as rice straw and concentrate of rice bran, ground nut cake and sesame cake. Zebu breed is usually raised with traditional methods under a free range system. The feed relies on dry and green roughage from natural grazing land. In dry areas, among the conventional feed resources, butter bean residue, natural grass and sesame residue were used as roughage sources in the diet when rice straw was unavailable (Aung et al., 2015). In general, dairy cattle were injected with Ivermectin at every 4–5 months interval and draught cattle received manual washing every day, but no acaricide or tick control measures were applied to most local cattle. Precise tick control measure at each farm is unknown in this study.

2.3. Blood sample collection and DNA extraction

Collection of blood samples from cattle was approved by the Ministry of Livestock, Fisheries and Rural Development of Myanmar. In this cross-sectional study, systematic random sampling was performed. The animals were healthy during sampling. A total of 713 cattle blood samples were collected onto an 11 mm-disk of a FTA® Elute Micro Card (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), and DNA was extracted from the cards in accordance with the manufacturer's instructions with minor modifications (Terao et al., 2015). In this study, these DNA samples were previously used for the PCR detection of *T. orientalis* (Bawm et al., 2014).

2.4. PCR

Nested PCR targeting the *AMA-1* gene of *B. bigemina* and the *RAP-1* gene of *B. bovis* were carried out (Sivakumar et al., 2012a; Figueroa et al., 1993). For the *AMA-1* gene, the primary PCR mixture in a volume of 10 µl contained 0.8 µM of each primer (forward of BI-AMA-FO: GTATCAGCCGCCGACCTCCGTAAGT and reverse of BI-AMA-RO: GGCGTCAGACTCCAACGGGAACCG), 1× Ampdirect® Plus buffer (Shimadzu Corp. Kyoto, Japan), 2 µl of eluted DNA and 0.025 U/µl of BIOTAQ™ HS DNA polymerase (Bioline Ltd., London, UK). After initial denaturation at 95 °C for 10 min, the reaction was carried out with 35 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 1 min and extension at 72 °C for 1 min,

followed by a final extension at 72 °C for 10 min using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems Japan, Tokyo, Japan). For nested PCR, 0.5 µl of primary PCR product and primer set (forward of BI-AMA-FI: TACTGTGACGAGGACGGATC and reverse of BI-AMA-RI: CCTCAAAGCAGATTCGAGT) were used for the initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. For the *RAP-1* gene, the primary PCR mixture in a volume of 10 µl contained 0.5 µM of each primer (forward of BoF: CACGAGCAAGGAAGTACCAGT-GTTGA and reverse of BoR: CCAAGGACCTTCAACGTACGAGGTCA), 1× Ampdirect® Plus buffer, 2 µl of eluted DNA and 0.025 U/µl of BIOTAQ™ HS DNA polymerase. After initial denaturation at 95 °C for 10 min, the reaction was carried out with 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min using a GeneAmp PCR System 9700 thermal cycler. For nested PCR, 0.5 µl of primary PCR product and primer set (forward of BoFN: TCAACAACGTACTCTATATGGCTACC and reverse of BoRN: CTACC-GACCAGAACCTTCTCACCAT) were used for the initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The PCR products were electrophoresed in 2% Tris–acetic acid–EDTA (TAE) agarose gels, stained with Red Safe Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Sungnum, Korea), visualized under LED light and analyzed using a bio-image analyzer (ImageQuant LAS 4000, GE Healthcare Japan, Tokyo, Japan). For sequencing the nested PCR products, KAPA Blood PCR KIT (Cape Town, South Africa) was used. For the *AMA-1* gene, the primary PCR mixture in a volume of 25 µl contained 2.5 µM of each primer, 5 µl of eluted DNA and 1× KAPA Blood PCR MixB. After initial denaturation at 95 °C for 5 min, the reaction was carried out with 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. For nested PCR, 0.1 µl of primary PCR product was used for 40 cycles with annealing at 60 °C for 30 s. For the *RAP-1* gene, the primary PCR mixture in a volume of 25 µl contained 2.5 µM of each primer, 5 µl of eluted DNA and 1× KAPA Blood PCR MixB. After initial denaturation at 95 °C for 5 min, the reaction was carried out with 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. For nested PCR, 0.1 µl of primary PCR product was used for 40 cycles with annealing at 55 °C for 30 s. The amplified products were purified using a FastGene™ Gel/PCR Extraction kit (NIPPON Genetics Co. Ltd., Tokyo, Japan) and submitted for direct sequencing using an Applied Biosystems 3130 Genetic Analyzer with a BigDye v3.1 Terminator cycle sequencing kit (Applied Biosystems, Inc., Carlsbad, CA, USA) and CleanSEQ (Beckman Coulter Inc., Tokyo, Japan).

2.5. Statistical analysis

Animals were grouped into three age categories, <1 year, 1–5 years and >5 years old. Data obtained from the interviews were tabulated using the statistical package Epi Info™ 7 (<http://www.cdc.gov/epiinfo/7/>) and analyzed by Chi-square test for significant differences ($P < 0.05$) in infection rate in animals among various parameters along with Yates' correction or Fisher's exact test. The odds ratios (ORs) of the univariate analysis were calculated using measures of association along with 95% confidence intervals (CIs).

3. Results

Approximately 200 bp and 300 bp species-specific nested PCR products were obtained from the *AMA-1*- and *RAP-1*-based PCR

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