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Original Article

A longitudinal study of *Babesia* and *Theileria* infections in cattle in Sri Lanka

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ABSTRACT

Throughout the world, infections with the *Babesia* and *Theileria* parasites often result in economically significant clinical disease in cattle. We conducted a longitudinal survey of *Babesia* and *Theileria* infections in cattle from the Polonnaruwa (n = 75; dry zone) and Nuwara Eliya (n = 161; wet zone) districts of Sri Lanka. DNA from blood samples collected in June, September, and December 2014 and March 2015 was screened for *Babesia bovis*, *Babesia bigemina*, *Theileria annulata* and *Theileria orientalis* using specific polymerase chain reactions (PCRs). Additionally, serum samples collected from the animals were screened using enzyme-linked immunosorbent assays (ELISAs) to detect *B. bovis*- and *B. bigemina*-specific antibodies. All of the animals surveyed in Polonnaruwa and 150 (93.2%) of the animals surveyed in Nuwara Eliya were PCR-positive for *Babesia* and/or *Theileria* at least once during the study period. A greater percentage of the cattle in Polonnaruwa were positive for *T. annulata* and *T. orientalis* than *B. bovis* or *B. bigemina* at all time points. *T. orientalis* was the most common infection in Nuwara Eliya. Additionally, more cattle were seropositive for *B. bigemina* than *B. bovis* in both districts. Although significant variations were sometimes observed in the rates of animals that were positive for *B. bigemina*, *T. annulata*, and *T. orientalis* at the different sampling time points, the rates of new infections with these parasites (by PCR or ELISA) on second, third, and fourth time points among the parasite-negative samples at the first, second, and third time points, respectively, did not differ between the sampling in either district—suggesting that the parasite species infected cattle at a constant rate in these locations. However, in Polonnaruwa, the rates of new infection with *T. annulata* were higher than the rates of new infection with *T. orientalis*. The rates were also higher than those in Nuwara Eliya. In Nuwara Eliya, the rates of new infection with *T. orientalis* were higher than the rates of new infection with *T. annulata*. The rates were also higher than those in *T. orientalis* in Polonnaruwa. These differences might be due to variations in the density and activity of the specific tick vectors within and between the districts. Our findings suggest the need for year-round control measures against bovine *Babesia* and *Theileria* infection in Sri Lanka. Further studies to determine the densities of the vector tick species in the different geographical areas of the country are warranted.

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1. Introduction

Piroplasmids, such as *Babesia* and *Theileria* parasites, cause economically significant disease in livestock, especially cattle (Bishop et al., 2004; Bock et al., 2004). The prevalence of *Babesia* and *Theileria* in a locality is usually related to the distribution of their transmission vectors, the ixodid ticks. The lifecycles of *Babesia* and *Theileria* parasites in their host animals begin with the injection of sporozoites by infected ticks during their blood meal (Bishop et al., 2004; Hunfeld et al., 2008). *Babesia* sporozoites directly invade the host's red blood cells (RBCs), where they transform into trophozoites and then meronts, which undergo asexual reproduction to form merozoites (Homer et al., 2000). In contrast, *Theileria* sporozoites first infect the host's leukocytes, where they develop into schizonts. The subsequent rupture of the schizonts releases merozoites, the life stage that eventually infects host RBCs (Bishop et al., 2004). Hence, parasites can be detected in the blood samples of *Babesia*- and *Theileria*-infected animals (Mans et al., 2015; Mosqueda et al., 2012).

Among the bovine *Babesia* parasites, *Babesia bovis*, *Babesia bigemina*, and *Babesia divergens* are known to be virulent species, while species with low pathogenicity—such as *Babesia ovata* and *Babesia major*—are also infective to cattle (Bock et al., 2004). Acute infections with *B. bovis* and *B. bigemina*, which are the major causative agents of clinical babesiosis in the tropical and subtropical regions of world, are characterized by anemia and anemia-related syndromes, which are associated

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with extensive intravascular hemolysis (Ristic, 1981). Additionally, *B. bovis* infections can sometimes be associated with neurological and respiratory syndromes caused by the cytoadherence of parasite-infected RBCs to endothelial cells in the capillary beds (Everitt et al., 1986; Wright and Goodger, 1988). On the other hand, *Theileria* parasites, which comprise the bovine *Theileria parva* and *Theileria annulata* transforming species types, are more pathogenic than *Theileria orientalis,* which is a non-transforming species (Dobbelaere and Heussler, 1999; McKeever, 2009; Sivakumar et al., 2014a). Geographically, the parasite responsible for East Coast Fever (*T. parva*) has a limited distribution in Africa, while the causative agent of tropical theileriosis (*T. annulata*), is endemic in parts of Asia and Africa (Weir et al., 2010). In contrast, *T. orientalis* has a worldwide distribution and occasionally has a clinically significant impact (Eamens et al., 2013; McFadden et al., 2011; Sivakumar et al., 2014a).

Control strategies against *Babesia* and *Theileria* parasites largely depend on immunization with live vaccines and tick-control measures (Bishop et al., 2004; Bock and de Vos, 2001; Bock et al., 2004; Oura, 2007). In addition to tick surveys, longitudinal studies on *Babesia* and *Theileria* infections may provide information on the high-risk season(s) for infection—in terms of when the tick vectors are most active—thereby enabling the advanced application of systematic tick-control measures (Shimizu et al., 2000). However, while numerous cross-sectional surveys of *Babesia* and *Theileria* parasites have been carried out in a number of countries where these parasites are endemic (Altay et al., 2008; Elsify et al., 2015; García-Sanmartín et al., 2006; Ybañez et al., 2013), longitudinal studies to monitor parasite infections have rarely been conducted in cattle populations. *Babesia* and *Theileria* parasites are endemic in the cattle and water buffalo populations of Sri Lanka (Jorgensen et al., 1992; Sivakumar et al., 2012b, 2014b; Weilgama et al., 1986, 1989). Recent polymerase chain reaction (PCR)-based investigations in Sri Lanka have confirmed the nationwide occurrence of *B. bovis*, *B. bigemina*, and *T. orientalis* in both cattle and water buffaloes, and *T. annulata* in cattle (Sivakumar et al., 2012b, 2014b). However, these studies were cross-sectional in nature. Thus, they did not provide any information on the temporal dynamics of *Babesia* and *Theileria* epidemiology in Sri Lanka. In the present study, we conducted a longitudinal survey of *Babesia* and *Theileria* infections in cattle that were reared in two distinct geographical locations in Sri Lanka: the Polonnaruwa (dry zone) and Nuwara Eliya (wet zone) districts.

2. Materials and methods

2.1. Study areas and animals

The study animals were cattle that had been reared in the Polonnaruwa (hereafter referred to as "P") and Nuwara Eliya (hereafter referred to as "NE") districts of Sri Lanka, which are located in dry and wet zones, respectively (Sivakumar et al., 2012b). The vegetation in the dry zone is characterized by scrub forest, interspersed with tough bushes. In contrast, the common types of vegetation in the wet zone include evergreen forest, savannah, and wet patana grassland. The mean annual rainfall in wet zone is over 2500 mm, whereas the mean annual rainfall of the dry zone is <1750 mm. In the P district, the mean daily maximum and minimum temperatures (in the hottest and coldest months) were 33 °C and 21 °C, respectively; those in the NE district were 28 °C and 18 °C, respectively. In the P district, local cattle (*Bos indicus*) and their crosses are maintained using extensive or semi-intensive management practices. In the NE district, European breeds (*Bos taurus*) are maintained by an intensive management system (Abeygunawardena et al., 1997). In the present study, blood samples were collected from 75 animals in six cattle farms in the P district (21, 5, 5, 6, 11, and 27 animals) and 161 animals in three cattle farms in NE district (58, 87, and 16 animals, respectively) in June 2014; resampling was performed in September 2014, December 2014, and March 2015. Two milliliters of blood were collected from each animal in vacutainer tubes with or without ethylene-diaminetetraacetic acid (EDTA; NIPRO, Osaka, Japan). All of the animal protocols were approved by the Ethical Review Committee of the Veterinary Research Institute, Sri Lanka.

2.2. DNA extraction and serum separation

The blood samples that were collected with EDTA were subjected to DNA extraction using a commercial kit (Qiagen, Hilden, Germany). Briefly, DNA was extracted from 200 μl of whole blood, according to the manufacturer's instructions. The extracted DNA samples were stored until further use at −20 °C. The serum samples were prepared from the blood samples that had been collected in tubes without any anticoagulants, as previously described (Munkhjargal et al., 2013).

2.3. The PCR detection of Babesia and Theileria parasites

Previously described *B. bovis*-, *B. bigemina*-, *T. annulata*-, and *T. orientalis*-specific PCRs were used to screen all of the DNA samples. A nested PCR, based on the rhoptry-associated protein-1 (*rap-1*) gene, was used to screen for *B. bovis* (Figueroa et al., 1993). Single-step PCRs based on apical membrane antigen-1, *T. annulata* merozoite surface antigen-1, and major piroplasm surface protein genes were used to specifically detect *B. bigemina*, *T. annulata*, and *T. orientalis*, respectively (Kirvar et al., 2000; Ota et al., 2009; Sivakumar et al., 2012a). The PCR primers, reaction mixtures, and cycling conditions were described in a previous report (Sivakumar et al., 2012b). After separation by agarose gel electrophoresis, the PCR products were stained with ethidium bromide, and then visualized under UV illumination. The detection of bands of similar size to those observed from the positive controls was considered evidence of parasite positivity.

2.4. The enzyme-linked immunosorbent assays (ELISAs)

All of the serum samples were analyzed by ELISAs using *B. bovis*- and *B. bigemina*-recombinant RAP-1 (rRAP-1) antigens. Briefly, 300-bp gene fragments, encoding the 100 amino acid residues located within the previously characterized *B. bovis*- and *B. bigemina*-specific N-terminal regions of the RAP-1 antigens (Boonchit et al., 2004, 2006), were amplified from PCR 2.1 plasmids containing inserts from *B. bovis* (GenBank accession number: LC157851) and *B. bigemina* (GenBank accession number: LC157860) *rap-1* genes that had been isolated in Sri Lanka (unpublished data). The *Bam*HI and *Xho*I restriction sites (underlined) were added to the forward (*B. bovis*, 5′-gcggatccAACTATCTGAAAGCCAATG-3′; *B. bigemina*, 5′-gcggatccCCTCACTACCTTTCTAAGGC-3′) and reverse (*B. bovis*, 5′-gccctcgagtcaAGCAATATTCTCGCCTAGG-3′; *B. bigemina*, 5′-gccctcgagtcaATCTTCATTTTTGGGGTCATC-3′) primers, respectively (the uppercase letters indicate the regions corresponding to the template sequences). A reverse-complement stop codon TGA (TCA) was also added to the 5′ end of the reverse primers. PCR amplification and cloning, and protein expression and purification were essentially performed according to previously described methods (Tattiyapong et al., 2016). Briefly, the PCR-amplified target gene fragments were digested with their respective restriction enzymes, ligated to a similarly digested pGEX4T-1 (*B. bovis*) or pGEX6p2 (*B. bigemina*) plasmid vector (GE Healthcare, Uppsala, Sweden), expressed as glutathione S-transferase (GST)-fusion proteins, purified using a Glutathione Sepharose 4B column (GE Healthcare), and finally cleaved using Thrombin or PreScission Protease (GE Healthcare) to isolate the rRAP-1 antigens. The purified *B. bovis*–rRAP-1 and *B. bigemina*–rRAP-1 antigens were then used in ELISAs to screen the serum samples that were collected in the present study, as described previously (Terkawi et al., 2011). A serum sample was considered to be positive if the OD value was greater than the cutoff value, which was the sum of the mean OD value of the five negative controls (non-infected serum samples) that were used in each ELISA plate and 5× the standard deviation.

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