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Molecular prevalence of *Babesia bigemina* in *Rhipicephalus microplus* ticks infesting cross-bred cattle of Punjab, India

S.A. Bhat[a,*], N.K. Singh[a], H. Singh[a], S.S. Rath[a]

[a] *Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, 141 004, Punjab, India*

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ABSTRACT

Babesiosis is an economically important tick-borne apicomplexan protozoan disease of cattle in tropical and subtropical regions. In the present study, *Rhipicephalus microplus* engorged female ticks were collected from 135 apparently healthy cattle from different agro-climatic zones of Punjab, India, to investigate the carrier status of *Babesia bigemina* infection in vector tick by using microscopy and PCR based assays. PCR when applied on DNA extracted from the egg masses harvested from ticks showed 1.48% (2/135) samples as positive, whereas 4.44% (6/135) samples were positive when product of primary PCR was used as template in nPCR. Further, among the DNA samples isolated from the unfed larval stages that emerged from egg masses laid by ticks, only 1.48% (2/135) samples were detected as positive for *B. bigemina* in PCR, while 7.41% (10/135) samples were detected positive in nPCR assay. Statistically, non-significant ($p > 0.05$) difference in prevalence rates was observed across different agro-climatic zones and between different age groups of cattle from which engorged ticks were collected. It can, thus, be concluded that prevalence of *B. bigemina* in the vector tick, *R. microplus* in Punjab state of India indicates an endemic status of the organism and a further study is needed for the management and control of the bovine babesiosis.

1. Introduction

Bovine babesiosis is an important tick-borne intra-erythrocytic apicomplexan protozoan disease worldwide caused by the parasites of genus *Babesia*, infecting a wide range of domesticated and wild cattle (McCrosker, 1981). The major species of genus *Babesia* infecting cattle in tropics and subtropics are *B. bigemina* and *B. bovis* with *B. bigemina* having wider distribution (OIE, 2010), and is transmitted by cattle fever tick, *Rhipicephalus microplus* (Murrell et al., 2001). The transmission of *Babesia* organism to a susceptible bovine host depends on the successful infection of the tick midgut epithelium through the sexual stages of the parasite (Howell et al., 2007).With the reports of replacement of multi-host ticks by the one host tick, there is an increasing concern over the diseases transmitted by the common one host tick, *R. microplus*, particularly babesiosis and anaplasmosis in Punjab state, India (Singh and Rath, 2013).

The most commonly encountered clinical signs induced by these parasites include high grade fever, anemia, hemoglobinuria, ataxia, and sometimes death (Bock et al., 2004). A characteristic feature of *Babesia* infection is that animals which recover from acute infection become carriers, creating a potential source of infection to healthy susceptible population. Babesiosis is traditionally diagnosed by identification of the parasites in Giemsa stained peripheral blood smear, but it is often very difficult to detect these

* Corresponding author.
E-mail address: hodvp@gadvasu.in (S.A. Bhat).

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latent infections microscopically due to low levels of parasitaemia. Alternatively, indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) are widely used for detection of antibodies against *Babesia* infections in cattle. Further, for detection of *Babesia* organism in vector ticks, its parasitic forms like ookinetes and sporozoites have been observed under microscope using different staining methods. Giemsa stained hemolymph of adult ticks along with egg and larval squashes have also been used for diagnosis in ticks (Bock et al., 2004; Oliveira-Sequeira et al., 2005). However, the main drawbacks of microscopic detection of *Babesia* spp. in blood smears, hemolymph of adult ticks, and in tick egg and larval squashes are the low sensitivity and the difficulty of differentiating between the species involved (Bock et al., 2004). Similarly, sero-diagnostics suffers from the problem of cross-reactivity and sometimes low antigen output.

Therefore, application of molecular biology techniques as PCR-based assays to study the epidemiology of babesiosis is very helpful but still incipient. These assays are characterized by high sensitivity and specificity as has been verified by several authors for the detection of infection both in the vertebrate hosts and ticks (Oliveira-Sequeira et al., 2005; Smeenk et al., 2000; Gayo et al., 2003; Oliveira et al., 2008; Tavassoli et al., 2013). However, in India, there are only few published reports regarding the use of molecular methods for the detection of *B. bigemina* in cattle (Singh et al., 2007; Silva et al., 2009; Chaudhry et al., 2010; Singh et al., 2013) and vector tick (Ravindran et al., 2006). Therefore, the present study was undertaken with the objective of determining the molecular prevalence of *B. bigemina* infection in vector tick, *R. microplus* in Punjab, India.

2. Materials and methods

2.1. Geographical area

The study was conducted from June 2011 to May 2013 in the northwestern Punjab state of India, covering a total area of 50,362 km^2 located between 29″30′N to 32″32′N latitude and 73″55′E to 76″50′ E longitudes. The state has been divided into 5 major agro-climatic zones i.e. Central Plain Zone, Undulating Zone, Western Zone, Western plain Zone and Sub-mountain Zone. Punjab has an inland subtropical location and its climate is continental, being semi-arid to sub-humid. Summers are very hot and winters very cold with annual temperatures in range from 1 °C to 46 °C (min to max) with average annual rainfall of 565.9 mm. These environmental conditions provide favorable conditions for the survival and propagation of ticks. The major tick species infesting cross-bred cattle of the region are *R. microplus* and *Hyalomma anatolicum* (Singh and Rath, 2013; Haque et al., 2011).

2.2. Sample collection

Engorged adult female *R. microplus* ticks were collected from 135 cattle of different agro-climatic zones of Punjab reared under intensive production system with periodic use of chemical acaricides (cypermethrin, deltamethrin, amitraz and ivermectin) as the only tick control measure adopted in the region. A total of 10 fully engorged females ticks were collected from each animal in separate vials, closed with muslin cloth to allow air and moisture exchange and brought to the laboratory. Ticks were washed, kept individually in labeled plastic tubes covered with muslin cloth and kept in desiccators placed in BOD incubator maintained at 28 ± 1 °C and 85 ± 5% relative humidity for oviposition.

2.3. Maintenance and processing of tick

Hemolymph was collected from four ticks collected from each individual animal at 8–10 days post incubation by removing both distal legs and blotting the exuding droplet of hemolymph on separate clean microscopic glass slide. Eight hemolymph smears were made from the ticks collected from each animal, fixed in methanol for 2–3 min, stained with Giemsa and observed under oil immersion microscope for presence of any parasitic stage. The remaining engorged female ticks were incubated till completion of oviposition. The eggs laid by all the ticks collected from each animal were pooled. Approximately, 25–30 eggs from the pooled egg mass were utilized for the preparation of egg squash in duplicate, fixed in methanol for 2–3 min, stained with Giemsa and observed under oil immersion microscope for presence of any parasitic stage. A small proportion of pooled egg mass was transferred to a microcentrifuge tube and kept at − 20 °C for DNA isolation. The remaining major portion of egg mass was transferred to labeled plastic tubes covered with muslin cloth and kept in desiccators placed in BOD incubator maintained at 28 ± 1 °C and 85 ± 5% relative humidity for hatching of larvae. Around 300–400, 10–14 day old unfed larvae harvested from the pooled eggs were stored in freezer at − 20 °C and utilized for DNA extraction.

2.4. Genomic DNA isolation from tick larvae and eggs

Whole-genomic DNA was isolated from egg samples and unfed larvae of *R. microplus* using QIAamp® DNA mini kit (QIAGEN, GmbH, Germany) following the manufacturer's recommendations with minor modifications. In brief, approximately 100 eggs/larvae (kept at − 20 °C) were triturated in 170 µl phosphate buffer saline (pH = 7.2) and homogenate was transferred into the 1.5 ml micro centrifuge tube to which 40 µl of proteinase-K and 150 µl of ATL buffer was added and vortexed thoroughly. The homogenous suspension was incubated overnight at 56 °C, vortexed at intervals and then centrifuged for 30 s at 5000 rpm. Then, 200 µl of AL buffer was added to the lysate, vortexed and incubated at 70 °C for 20 min. Further, 200 µl of ethanol was added to the lysate, vortexed thoroughly and centrifuged at 5000 rpm for 30 s. The mixture was transfered to QIAamp spin column and centrifuged at 8000 rpm for 1.5 min. Thereafter, 2 washings were given with wash buffers (AW1 and AW2) at 8000 rpm for 2 min and 14,000 rpm