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PCR BASED DETECTION OF SUBCLINICAL BOVINE BABESIOSIS IN PUNJAB

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ABSTRACT

A PCR based assay was standardized for the specific and sensitive detection of *Babesia bigemina* infection in bovine blood samples. Among the 104 samples collected from apparently healthy carrier cattle, an amplicon of 278 bp size specific for *B. bigemina* was detected in 25.96% animals against the routine blood smear examination, which revealed parasitic piroplasms in only 3.84% of samples. The use of PCR resulted in significantly higher efficacy of detection of the parasite compared to microscopic examination of blood smears. These results clearly demonstrate that bovine babesiosis is common and endemic in the state and PCR is the optimal approach for the detection of subclinical cases.

Key words: *Babesia bigemina*, Cattle, PCR, Punjab.

INTRODUCTION

The state of Punjab contributing a significant percentage (10.0) of national cattle population extends from the latitudes 29.30° N to 32.32° N and longitudes 73.55° E to 76.50° E with excessively hot and dry climate in summer and cool winter with frost and an average annual rainfall of 565.9 mm. Existence of such environmental conditions, intensive animal husbandry practices and strong genetic selection pressure on animals for higher milk production favours tick infestation, thus increasing chances of tick borne haemoprotozoan diseases. Although in the past a large number of Ixodid tick species (21) were reported from the state (Ghosh *et al*, 2007), but in a recent study dominance (85.28%) of *Rhipicephalus (Boophilus) microplus* in cattle of Punjab was recorded (Haque *et al*, 2011).

Among one of the most frequent and economically important tick-borne diseases of cattle, babesiosis caused by *Babesia bigemina* transmitted by *R. (B.) microplus* is widely prevalent in tropical and subtropical countries of the world including India. It has been estimated that more than 900 million cattle worldwide are at risk to this disease (Montenegro-James and James, 1998) and in terms of numbers and distribution of species in animals, stands second only to the trypanosomes in the world (Levine, 1988; Telford *et al*, 1993). The estimated annual loss due to bovine babesiosis in India is around US$ 57.2 million (McLeod and Kristjanson, 1999).

The presented case history, signs and symptoms are considered to be indicative of bovine babesiosis, but a definitive diagnosis can only be achieved by laboratory methods. Diagnosis by microscopy still remains the "gold standard" but lacks sensitivity (1 parasite per 10^5-10^6 erythrocytes), while thick blood films stained with acridine orange (sensitivity approximately 10^{-7}) and the quantitative buffy coat (QBC) analysis system (10^{-7} to 10^{-8}) are applicable to diagnose the infection in the laboratory (Bose *et al*, 1995). The introduction of molecular or DNA based methods have added precision in diagnostic parasitology in form of enhanced sensitivity and specificity for detection of low-grade parasite infections (Ramos *et al*, 1992). In this regard, polymerase chain reaction (PCR) has been used successfully for diagnosis of bovine babesiosis by various workers worldwide (Patarapadungkit *et al*, 2004; Singh *et al*, 2007; Silva *et al*, 2009; Chaudhry *et al*, 2010).

Although for many years, several sporadic reports of clinical cases of bovine haemoglobinuria associated with tick infestation have been observed,

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studies to ascertain the status of babesiosis in healthy carrier animals exhibiting low parasitaemia has not been carried yet especially from this part of the country. The present study thus provides the first epidemiological data regarding subclinical bovine babesiosis by PCR analysis of blood samples obtained from cattle exposed to ticks in field conditions. The diagnostic performance of PCR assay in terms of sensitivity has also been compared with the conventional detection of piroplasms in blood smears by light microscopy.

MATERIALS AND METHODS

Collection of blood samples: A total of 104 blood samples were collected aseptically in vacutainers containing 3.2% sodium citrate as anticoagulant from random population of apparently healthy cattle exposed to tick infestation in field conditions of Ludhiana (69), Moga (25) and Mansa (10) districts of Punjab state. For, all the collected samples, peripheral blood smears were made and examined under oil immersion power of microscope after staining with Giemsa and the results obtained were compared to that of PCR assay.

Genomic DNA extraction: For conducting the PCR, whole genomic DNA was isolated from citrated whole blood using QIAamp® DNA blood mini kit (QIAGEN, GmbH, Germany) following the manufacturer's recommendations with minor modifications. In brief, 200 µL of the blood sample was mixed with 20 µL of Proteinase K and 200 µL of lysis buffer and incubated at 56°C for 10 min. Then, 200 µL of ethanol was added to the sample and the mixture was applied to QIAamp Mini spin column and centrifuged at 8000 rpm for 1 min. Thereafter, two washings were given with 500 µL each of wash buffer 1 and 2. At the end 150 µL of elution buffer was added to the column and DNA was collected in 1.5 mL eppendorf tube after centrifugation and stored at -20°C till use.

DNA amplification: The PCR was carried out using the sequences of oligonucleotide primers specific for *B. bigemina* as described by Figueroa *et al.* (1992). The sequences of the primers were Bbig278Forward: 5' CAT CTA ATT TCT CTC CAT ACC CCT CC-3' and Bbig278Reverse: 5'-CCT CGG CTT CAA CTC TGA TGC CAA AG-3'

The PCR was set up in 25 µl reaction consisting of 2.5 µl of 10X PCR buffer (MBI Fermentas), 0.5 µl of 10 mM dNTP mix (MBI Fermentas), 1.5 µl of 25 mM $MgCl_2$ (MBI Fermentas), 1.0 U of recombinant *Taq* DNA polymerase (MBI Fermentas), 1 µl each (20 p mol) of the primers and 5 µl of template DNA isolated from field samples. The volume was made up to 25 µl with nuclease free water. The cycling conditions were as: Initial denaturation at 94°C for 5 min, 34 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min and the final extension was performed at 72°C for 10 min. Purified *B. bigemina* DNA isolated from clinically infected cow served as reference positive control, while leukocyte DNA isolated from a three day-old bovine calf (Sambrook *et al.*, 1989) served as reference negative control.

The PCR products were checked for amplification by electrophoresis on a 1.7% agarose gel and visualized using gel documentation system (Syngene, U.K.). In order to check the specificity of the assay, genomic DNAs of *Theileria annulata*, *Anaplasma marginale* and *Trypanosoma evansi* were also employed in the PCR to see the amplification, if any. The results of PCR assay were compared with that of Giemsa stained blood smear examination and statistical analysis of the data obtained was carried out by Chi square test using SPSS 16.0 software.

RESULTS AND DISCUSSION

The PCR assay for detection of *B. bigemina* in three districts of Punjab state was standardized. An amplification of 278 bp fragment was observed in positive control (parasite DNA), while no amplification was observed in negative control as well as DNAs isolated from *T. annulata*, *A. marginale* and *T. evansi* determining the specificity of the test (Fig. 1). The sensitivity of the assay was found to be 500 pg of genomic DNA by serial dilution which is in agreement with the results obtained by Ravindran (2002). The overall positivity of bovine babesiosis was recorded to be 25.96% (27/104) as revealed by the amplification of 278 bp amplicon. Among the positive samples Ludhiana (20/69), Moga (4/25) and Mansa (3/10) consisted of 28.98%, 16.0% and 30% respectively. However, blood smear examination showed only 04 (3.84%) animals positive for the piroplasms of *B. bigemina* with low parasitaemia (never exceeding 1%). The sensitivity

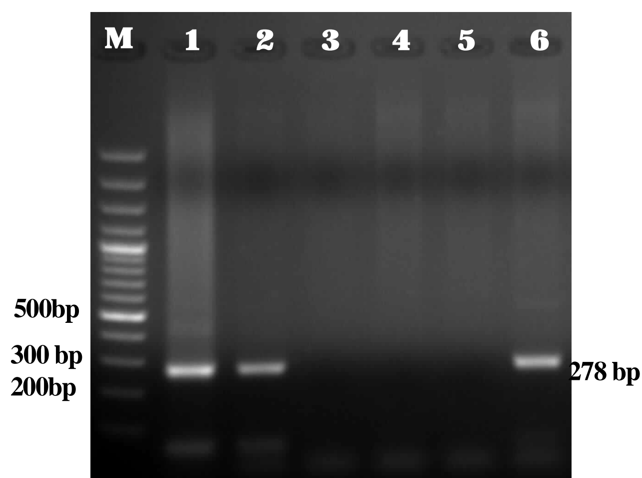

FIG. 1: Amplification of 278 bp fragment of *Babesia bigemina*.

Lane M: 100 bp DNA Ladderplus
Lane 1 to 4: Field Collected blood samples
Lane 5: Negative Control
Lane 6: Positive Control

of PCR assay was recorded to be significantly higher ($p< 0.01$) than that of blood smear examination (χ^2 value 20.053).

The piroplasms were found at low levels indicating the carrier status of the studied animals. Such carrier animals are poor in productivity leading to huge economic losses to the livestock owners and also act as important contributors to transmission of disease to susceptible animals by ticks, especially in endemic areas and thus, detection of infection in carrier animals becomes an important epidemiological parameter (d'Oliveira *et al*, 1995). This would be of utmost help in designing better and effective control strategies against the disease. Microscopic techniques are best suited to diagnose acute babesiosis but these are not ideally suited for detection of the parasite in carrier animals or recovered animals, exhibiting low parasite numbers in the peripheral blood. Previous reports by different workers have clearly indicated the higher sensitivity of PCR-based techniques compared to other diagnostic techniques, such as IFAT or blood smear observations in the diagnosis of *Babesia* species (Sparagano, 1999). In the present study, the PCR technique for *B. bigemina* resulted in a significantly higher sensitivity of detection of carrier animals than microscopic examination (27 animals versus 04, respectively, $p< 0.01$).

Sensitive diagnosis of bovine babesiosis utilizing PCR based assay has been attempted by many workers with high degree of sensitivity and specificity worldwide (Salem, 1998; Smeenk *et al*, 2000; Santana *et al*, 2001; Linhares *et al*, 2002). A PCR based assay for sensitive detection of *B. bigemina* was originally described by Figueroa *et al* (1992), targeting 278 bp fragment specific of parasite DNA. They reported that the PCR product could be detected in DNA samples purified from 200 µl of blood with a parasitaemia as low as 1 in 10^8 cells containing an estimated 30 *B. bigemina* infected erythrocytes. Recently by use of PCR based assays 34% infection of *B. bigemina* has been reported in clinically healthy individuals from different districts from Central and Southern regions of Portugal (Silva *et al*, 2009). However, a comparatively higher prevalence of bovine babesiosis has been reported by microscopic (18%) and PCR analysis (29%) from Pakistan (Chaudhry *et al*, 2010). In India, only few reports are available (Ravindran, 2002; Singh *et al*, 2007) employing PCR based assay to know the status of the disease in other areas of the country.

The present study provided epidemiological data relating to subclinical bovine babesiosis in Ludhiana (28.98%), Moga (16.0%) and Mansa (30%) districts of Punjab state, India, indicating the prevalence of an endemic situation in this region.

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