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MOLECULAR PREVALENCE OF *Babesia bigemina* INFECTION IN CATTLE IN WESTERN SEMI-ARID AND FOOTHILL REGIONS OF PUNJAB (INDIA)

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ABSTRACT

A nested PCR based prevalence study was done from June, 2011 to May, 2013 to determine the carrier status of *Babesia bigemina* infection in apparently healthy crossbred cattle from Western semi-arid and Foothill regions of Punjab (India). Of the 278 blood samples collected, an amplicon of 170 bp size was detected in 16.19% animals by nested PCR aa compared to the routine blood smear examination which showed parasitic piroplasms in only 1.80% samples. Regarding various risk factors, statistically non-significant variation was observed with respect to zone-wise, sex-wise and age-wise prevalence whereas season-wise disease prevalence showed significant variation ($p<0.05$).

Key words: *Babesia bigemina*, cattle, nested PCR, prevalence, risk factors

INTRODUCTION

Bovine babesiosis is a tick borne disease caused by apicomplexan protozoan parasites belonging to genus *Babesia* (Bock *et al.*, 2004). *Babesia bigemina* and *B. bovis* are economically important parasites in Asia, Africa, Central and South America, Southern Europe and Australia (Spickler and Roth, 2008). Among the various species, *B. bigemina* most commonly infects cattle in tropical and sub-tropical regions of the world including Indian subcontinent and is transmitted transovarially by *Rhipicephalus* (*Boophilus*) *microplus* ticks (Chauvin *et al*., 2009). In India, the annual cost of controlling ticks and tick-borne diseases is estimated to be 498.7 million US $ (Minjauw and McLeod, 2003).

The clinical signs of babesiosis include high fever, anaemia, haemoglobinuria, ataxia and sometimes death (Bock *et al*., 2004). Subclinical infections may continue for long periods (Brown *et al*., 2006) with infected animals acting as carriers with low parasitaemia virtually undetectable by microscopy. Hence, specific and sensitive diagnostic methods need to be used to monitor prevalence of these infections if efficient control strategies are to be implemented. Bovine babesiosis is traditionally diagnosed microscopically by identification of piroplasms in Giemsa stained blood smear which still remains the "gold standard" but lacks sensitivity (Bose *et al*., 1995). Alternative diagnostic methods like indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) are capable of detecting antibodies in carrier animals but suffer serious drawbacks of cross-reactions and lack of discrimination between previous exposure and current infections (Wagner *et al*., 1992). Owing to these limitations, several researchers have

reported PCR-based diagnosis of the disease due to high sensitivity and specificity of these assays (Oliveira *et al.*, 2005; Ravindran *et al.*, 2008; Sharma *et al.*, 2013).

There is scarcity of information on babesiosis in India especially in apparently healthy cattle as only a few reports of this disease have been made across the country in general and Punjab state in particular. Also, in earlier studies workers have either relied on conventional techniques or study was based on clinical samples. Previously the authors have employed PCR assay to determine the prevalence of babesiosis from one agro-climatic zone of Punjab i.e. Central Plain zone (Bhat *et al.*, 2014). Therefore, to ascertain the status of disease from other parts of Punjab state, the present study was aimed to assess the prevalence of *B. bigemina* infection in apparently healthy cattle from Foothill and Western arid regions of Punjab which comprises four agro-climatic zones i.e. Sub-mountain zone, Undulating Plain zone, Western zone and Western Plain zone, by employing both conventional and molecular assays.

MATERIALS AND METHODS

Blood samples (n = 278) were collected aseptically in vacutainer containing 3.2% sodium citrate as anti-coagulant from apparently healthy cattle from various districts of Western semi-arid [Western zone (n = 125), Western Plain zone (n = 53)] and Foothill [Sub-mountain Undulating zone (n = 49), Undulating Plain zone (n = 51)] regions of Punjab (India) from June, 2011 to May, 2013. The samples were collected in each season [pre-monsoon (n = 84), monsoon (n = 115) and post-monsoon (n = 79)] of a calendar year from above agro-climatic zones, and categorized as: 0-6 months age (n = 76) and >6 months age (n = 202); male (n = 53) and female (n = 225). Peripheral thin blood smears were prepared from the collected samples, stained by standard Geimsa staining protocol and examined under oil immersion for the demonstration of *Babesia* piroplasms, if any. The blood samples were further stored at -20°C for DNA extraction.

Genomic DNA isolation

Genomic DNA was isolated from the blood sample using QIAamp® DNA blood mini-kit (QIAGEN, GmbH, Germany) following the manufacturer's instructions with minor modifications. In brief, approximately 200 µL blood sample was mixed with 25 µL proteinase-K and 200 µL lysis buffer in a 2.0 mL micro-centrifuge tube. The homogenous suspension was thoroughly vortexed and incubated at 56°C for 15 min. and centrifuged for 30 sec. at 5000 rpm. Subsequently 200 µL ethanol was added to the lysate and again vortexed. The mixture was applied to QIAamp spin column and centrifuged at 8000 rpm for 1.5 min. Thereafter, 2 washings were given with wash buffers and DNA was eluted in 150 µL elution buffer and stored at -20°C till further use. Genomic DNA of *B. bigemina* was isolated from infected blood showing high parasitaemia in Giemsa stained blood smear examination and utilized as positive control. Genomic DNA was also isolated from the whole blood of infection-free, 3-day-old bovine calf and used as a negative control.

PCR assays

The primary and nested PCR assays were carried out using the sequences of oligonucleotide primers specific for *B. bigemina* as described by Figueroa *et al.* (1992). The sequences of primary PCR primers used were BiIA: 5′-CATCTAATTTCTCTCCATACC CCTCC-3′ and BiIB: 5′-CCTC GGCTTCAACTCTGATGCCAAAG-3′ and those nested PCR primers were; BiIAN 5′-CGCAAGC CCAGCACGCCCCGGTGC-3′ and BiIBN 5′-CCGACCTGGATAGGCTGTGTGATG -3′. Two rounds of PCR in a final volume of 25 µL were carried out in a PCR thermal cycler (Eppendorf, Germany). In primary PCR assay, the master mix consisted of 2.5 µL 10X PCR buffer (MBI Fermentas), 0.5 µL 10 mM dNTP mix (MBI Fermentas), 2.0 µL 25 mM $MgCl_2$ (MBI Fermentas), 1.0 U recombinant Taq DNA polymerase (MBI Fermentas), 1 µL each (15 pmol) of BiIA and BiIB

primers and 5 µL template DNA isolated from field samples. The volume was made up to 25 µL with nuclease-free water. The cycling conditions were: initial denaturation at 94°C for 5 min., 37 cycles of denaturation at 94°C for 1 min., annealing at 59°C for 1 min. and extension at 72°C for 1 min. and the final extension was performed at 72°C for 10 min. In order to carry out the nested PCR, the PCR conditions were same as described above, but instead of template DNA, 1 µL primary PCR product was used as template and amplified with 15 pmol, each of BiIAN and BiIBN primers and annealing temperature was kept at 70°C. The PCR products (primary as well as nested) were checked for amplification by electrophoresis on a 1.5% agarose gel and visualized using gel documentation system (Syngene, UK). To check the specificity of assay, genomic DNA of *Theileria annulata, Anaplasma marginale* and *Trypanosoma evansi,* isolated from microscopically positive cases by standard protocols, 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.

Statistical analysis of data was done by SPSS 13.0 software by applying χ^2 test regarding association of various risk factors such as zone, sex, age and season with prevalence of the disease.

RESULTS AND DISCUSSION

Examination of Giemsa-stained peripheral thin blood smears revealed 1.80% (5/278) animals positive for the piroplasms of *B. bigemina* (Fig. 1). However, of the total samples subjected to primary PCR, 4.68% (13/278) were found positive for *B. bigemina* infection as revealed by the amplification of a 278-bp fragment (Fig. 2). The primary PCR products, when employed as template in nested PCR produced the amplicons of desired size (170-bp), in 16.19% (45/278) samples (Fig. 2). This validates that nested PCR, when coupled with primary PCR, results in increased sensitivity of the assay. The products of primary PCR assay (32 samples) that were not visualized in the ethidium bromide-stained agarose gel electrophoresis, when subjected to nested PCR, could be detected. The primers used in the present assays did not amplify any product when the genomic DNA of *T. annulata*, *A. marginale* and *T. evansi* were used as template.

Blood smear examination revealed a low prevalence rate (1.80%) of bovine babesiosis which corroborates with the findings of Singh *et al*. (2012) who reported 1.56% prevalence of babesiosis in other parts of Punjab. Aulakh *et al.* (2005) through blood smear examination found an overall infection rate of 8.33% in clinical samples of crossbred cattle from different parts of Punjab. A very low prevalence of *B. bigemina* (0.48%) from Punjab using blood smear examination has been reported (Sharma *et al.*, 2013).

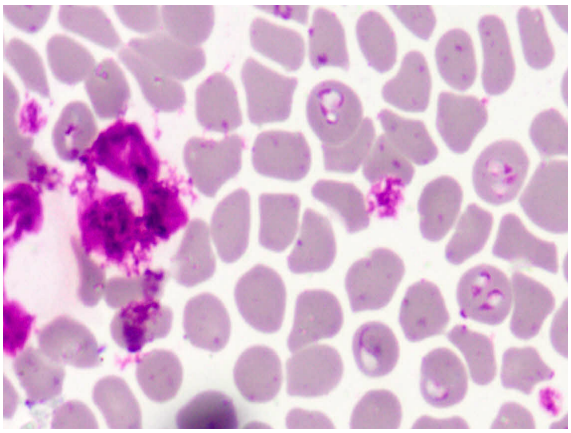

Fig. 1: *Babesia bigemina* piroplasms in Geimsa stained thin blood smear

In terms of molecular prevalence of bovine babesiosis, 4.68% samples were positive by primary PCR while 16.19% samples were positive by nested PCR. Sharma *et al.* (2013) reported 2.43% prevalence of *B. bigemina* in Punjab using duplex PCR. Ananda *et al*. (2009) recorded 12.1% prevalence in Bangalore region and Nair *et al*. (2011) reported 0.6% prevalence in north Kerala by using PCR assay. In a similar study, 34% infection of *B. bigemina* has been reported in clinically healthy individuals from Portugal (Silva *et al*., 2009). However, a comparatively higher prevalence of bovine babesiosis has been reported by microscopy (18%) and PCR analysis (29%) from Pakistan by

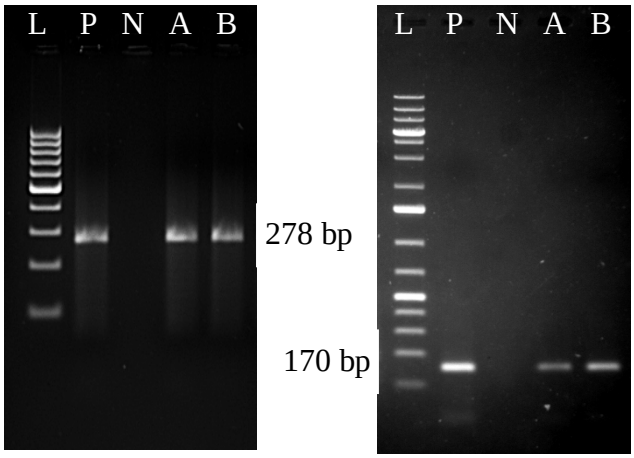

Fig. 2: Amplification of 278 bp fragments of *Babesia bigemina* by primary PCR (left) and 170 bp fragment of *B. bigemina* by nested PCR (right). Lane L: 100 bp DNA ladder, Lane P: Positive control, Lane N: Negative control, Lanes A and B: Field collected blood samples

Chaudhry *et al*. (2010) and from Zimbabwe (35%) by Smeenk *et al*. (2000). The availability of vector tick, *R. (B.) microplus* is a major determining factor for the presence and degree of infection in any geographical area.

Zone-wise a relatively high prevalence was recorded in Western zone (21.6%) followed by Western Plain zone (16.98%), Sub-mountain zone (10.20%) and Undulating Plain zone (7.84%) but the difference was insignificant (Table 1). Studies on epidemiology and distribution of tick species in Punjab showed *R. (B.) microplus* to be predominant tick responsible for the transmission of *B. bigemina* in cattle and buffalo (Haque *et al*., 2011; Singh and Rath, 2013). Therefore, owing to higher sensitivity of nested PCR (Oliveira *et al*., 2005) and abundance of specific vector, higher prevalence of *B. bigemina* infection in the cattle of Punjab is not surprising.

Age-wise, 17.3% (35/202) adults and 13.15% (10/76) calves were positive for infection but differences in prevalence values were non-significant (Table 1). The finding is in line with Ananda *et al*. (2009) who reported high prevalence in animals aged > 3 years followed by lower prevalence in animal < 1 year age. Kamani *et al*. (2010) also observed higher prevalence in adult than young cattle. Annetta *et al*. (2005) reported an inverse age rresistance of the disease where adult cattle showed more susceptibility than calves.

In terms of seasonal variation, the prevalence of *B. bigemina* infection was highest in monsoon [21.73% (25/115)] followed by post-monsoon [17.72% (14/79)] and pre-monsoon [7.14% (6/84)] season and the variation was significant (Table 1). Higher prevalence during monsoon and post-

Table 1: Prevalence of *B. bigemina* infection in apparently healthy crossbred cattle from Punjab

Risk factors		No. of samples	Blood smear examination	Primary PCR	Nested PCR
Agro-climatic zone	a) Western zone	125	3 (2.40)	6 (4.80)	27 (21.60)
	b) Western Plain zone	53	1 (1.89)	4 (7.55)	9 (16.98)
	c) Sub-mountain Undulating zone	49	1 (2.04)	2 (4.08)	5 (10.20)
	d) Undulating plain zone	51	0 (0.00)	1 (1.96)	4 (07.84)
	χ^2 (p>0.05)		1.21	1.87	6.77
Age	a) 0-6 months	76	1 (1.92)	3 (5.77)	10 (13.15)
	b) > 6 months	202	4 (1.98)	10 (4.95)	35 (17.33)
	χ^2 (p>0.05)		0.14	0.13	0.71
Season	a) Pre-monsoon	84	1 (1.19)	1 (1.19)	6 (7.14)
	b) Monsoon	115	3 (2.61)	9 (7.82)	25 (21.73)
	c) Post- monsoon	79	1 (1.26)	3 (3.80)	14 (17.72)
	χ^2 (p>0.05)		0.73	4.99	7.82*
Sex	a) Male	53	0 (0.00)	2 (3.77)	6 (11.32)
	b) Female	225	5 (2.22)	11 (4.89)	39 (17.33)
	χ^2 (p<0.05)		1.12	0.12	1.14
	Total	278	5 (1.80)	13 (4.68)	45 (16.19)

Figures in parenthesis are percent prevalence

monsoon months could be attributed to abundance of vector ticks during this period due to high temperature and humidity. This is in congruous with the observations of Ananda *et al*. (2009) who recorded highest prevalence in monsoon months. Radostits *et al*. (2000) have observed seasonal variation in the prevalence of clinical babesiosis in which greatest incidence usually occurs soon after a peak in tick population i.e. monsoon season.

Sex-wise, female cattle showed relatively higher prevalence [17.33% (39/225)] than males [11.32% (6/53)] and the difference was statistically non-significant ($p>0.05$) (Table 1). The higher percentage positivity recorded in female cattle in this investigation is in conformity with the reports of Kamani *et al*. (2010), Alim *et al*. (2012) and Terkawi *et al*. (2012). As most of the dairies use to cull male animals at their early age due to economic reasons, therefore, the number of blood samples collected from male cattle, were comparatively lesser than their counterparts.

In conclusion, the significant variation in the prevalence rates of *B. bigemina* infection across seasons, in the apparently healthy crossbred cattle in Semi-arid and Foothill regions of Punjab state highlights the association between season and carrier status.

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