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Research Article

NUCLEIC ACID-BASED DIAGNOSIS OF *BABESIA BIGEMINA* INFECTION IN BOVINES OF TELANGANA STATE, INDIA

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ABSTRACT: Bovine babesiosis due to *Babesia bigemina*, is an intra-erythrocytic, tick-borne protozoan disease affecting bovines. The recovered animals from acute infection remain carriers of these blood parasites posing a potential threat of infection to healthy, susceptible populations. A total of 158 whole blood samples each along with blood smears were collected from the bovines in different districts of Telangana for PCR assay and blood smear examination, respectively. PCR assay targeting the 18S rRNA gene of *Babesia* sp. and SpeI-AvaI restriction fragment of *B. bigemina* were conducted and compared with conventional blood smear examination for its efficacy in the diagnosis of babesiosis. PCR amplified the *B. bigemina* DNA with a sensitivity of 625 pg and could detect the pathogen in 16 out of 158 blood samples indicating a prevalence rate of 10.12%. The prevalence of babesiosis exhibited a significant increase (p=0.029) in correlation with the age of the animals. The infection rate was notably higher in animals aged 2-5 years (17.14%), followed by those older than 5 years (6%), compared to the younger animals below two years (2.63%) of age. Moreover, the occurrence of babesiosis was significantly higher (p=0.043) among crossbred animals (13.72%) in comparison to indigenous breeds (3.57%). Additionally, a distinct gender-based difference was observed, with females (13.27%) showing a significantly higher incidence (p=0.038) compared to males (2.22%). Giemsa-stained blood smear examination detected piroplasms in only 5 out of 158 blood smears screened indicating a prevalence rate of 3.16%. Hence, considering the superiority of PCR assay over conventional microscopic techniques, the test is recommended for the field diagnosis of bovine babesiosis.

Keywords: Bovine, *Babesia bigemina*, Diagnosis, PCR.

INTRODUCTION

Bovine babesiosis due to *Babesia bigemina* is a tick-borne intra-erythrocytic haemoprotozoan parasite causing substantial economic losses to the global livestock sector in temperate to tropical countries. The clinical signs of the acute disease include pyrexia, anorexia, depression, weakness, ataxia, hemoglobinuria, anemia, jaundice, and the presence of intra-erythrocytic parasites [1, 2]. The recovered animals become carriers of the infection for both ticks and naive cattle [3, 4].

Diagnostic techniques like microscopy, Quantitative Buffy Coat (QBC) analysis, and several serological methods such as Complement Fixation Test (CFT), Indirect Fluorescent Antibody Test (IFAT), Indirect Haemagglutination (IHA) test, and Enzyme-Linked Immunosorbent Assay (ELISA) are available for diagnosis of babesiosis but these have some limitations with low sensitivity and specificity [5]. Given the economic significance of carrier and sub-clinical infections and the limited sensitivity and specificity of conventional microscopy and serological techniques, the present study was undertaken to standardize a Polymerase chain reaction (PCR) aimed at the SpeI-AvaI restriction fragment of *B. bigemina* [6] and field validation of standardized PCR assay in the diagnosis of bovine babesiosis in Telangana state. The

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standardized PCR assay was employed to study the age-wise, sex-wise, and breed-wise prevalence of *B. bigemina* infection in cattle.

MATERIALS AND METHODS

Extraction of genomic DNA from Babesia-positive blood

Around 0.5 ml of blood was collected into EDTA-coated vacutainers (BD®) aseptically from the ear vein of a cross-bred Holstein Friesian cow exhibiting clinical signs of babesiosis such as pyrexia (105.7ºC), hemoglobinuria, anemia, tick infestation and found positive for *B. bigemina* on blood smear examination at Veterinary Dispensary, Nandigama, Mahabubnagar district of Telangana state. The genomic DNA was extracted from the positive blood sample [7]. Briefly, two hundred microlitres of whole blood were lysed with 330 µl of a cell lysis buffer (100 mM NaCl (SRL), 10 mM Tris HCl (Hi-media) (pH 8.0), 1 mM EDTA (SRL) (pH 8.0)), 20% Sodium dodecyl sulfate (SDS) (Hi-media) and 20 µl of proteinase K (Thermo Scientific) (20 mg/ml) by incubation at 56ºC for 4 hrs with periodic swirling followed by addition of another 10 µl of proteinase K and incubation overnight at 37ºC. Following overnight incubation, DNA was then purified using the standard Phenol-Chloroform extraction method followed by ethanol precipitation. Finally, the pellet was resuspended in 50 µl of TE buffer (pH 8.0) and stored at –20ºC.

The presence of the extracted genomic DNA was confirmed through agarose (0.7%) gel electrophoresis. The concentration and purity of DNA extracted were assessed by a Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA). The 355.6 ng/µl of DNA thus obtained was made into aliquots of 50 ng/µl and utilized as template DNA in all PCR reactions. The DNA extracted from the blood of the newborn calf was utilized as a negative control in all PCR reactions.

Polymerase chain reaction

Genus specific PCR assay

Initially, the genomic DNA isolated from the blood samples was first analyzed using a genus-specific PCR assay aimed at the 18S rRNA gene of *Babesia* sp. The PCR reaction included 10 pmol each of forward and reverse primers, 12.5 µl of Emerald Amp® GT PCR master mix, 50 ng of template DNA, and nuclease-free water, bringing the total volume to 25 µl, and was carried out in an automated thermal cycler (Hi-media). [8, 9]. Details of primers and cycling conditions are given in Table 1.

Species-specific PCR assay

Later, samples that showed amplification in the genus-specific PCR assay were further subjected to a species-specific PCR aimed at the the SpeI-AvaI restriction fragment of *B. bigemina* with 10 pmol each of forward and reverse primers, 12.5 µl of Emerald Amp® GT PCR Master Mix, 50 ng of template DNA and the reaction volume was adjusted to 25 µl with nuclease-free water in an automated thermal cycler (Hi-media) [6, 8, 10]. Details of primers and cycling conditions are given in Table 1.

For sensitivity analysis, descending double-fold dilutions of template DNA concentration starting from 10 ng to 312.5 pg were tested. All the amplified PCR products were subjected to agarose gel electrophoresis (2%) and stained with ethidium bromide (0.5 µg/ml) alongside a 100 bp DNA ladder, run at 50 V for 2 hours. The results were visualized and documented in a gel documentation (syngene) system.

Collection and processing of blood samples from the field

A total of 158 blood samples (~0.5 ml from ear vein using 24 gauge needle) were collected in EDTA-coated vacutainers (BD®) from different districts of Telangana and transported to the laboratory at 4 ºC within 4-8 hrs of collection for further processing. DNA was isolated from the blood samples by using the DNeasy® Blood and Tissue kit (Qiagen, Germany), following the manufacturer's instructions, and stored at -20 ºC until further use. DNA extracted from 158 field-collected blood samples was checked for amplification of the genus-specific fragment of *Babesia* and a species-specific fragment of *B. bigemina* by PCR as described above.

Blood smear examination

Methanol-fixed thin peripheral blood smears collected from the tip of the ears of respective animals during the collection of blood samples were examined by Giemsa staining under a light microscope (Olympus CH30RF200). The detection of even a single piroplasm was classified as a positive case, and a minimum of 4000 red blood cells (RBCs) were examined before declaring negative for blood parasites [8].

Statistical analysis

The efficacy of laboratory standardized PCR assay was compared to the results of Giemsa stained blood smears for making recommendations to the field. The field results of the PCR assay were analyzed to study

the age-wise, sex-wise, and breed-wise prevalence of *B. bigemina* infection in cattle by chi-square test using SPSS software [11].

RESULTS AND DISCUSSION

The genus-specific standardized PCR assay for amplification of the gene encoding 18S rRNA of *Babesia* spp. revealed a single band of 440 bp length showing no amplicon in the negative control when run in 2% agarose gel electrophoresis stained with Ethidium bromide (0.5µg/ml). (Fig. 1)

Similarly, the species-specific standardized PCR assay for amplification of species-specific gene encoding SpeI-AvaI restriction fragment of *B. bigemina* in positive sample revealed a single band of 278 bp length, while the negative control showed no amplification when run in 2% agarose gel electrophoresis (Fig. 2). The analytical sensitivity studies with positive template DNA, could detect as low as 625 pg of parasite DNA which is lesser than that reported earlier [12, 13].

Table 1. Details of primers and cycling conditions.

Parasite	Primer sequence (5'-3')	Cycling conditions	Product size
Babesia sp.	F:GTTTCTGMCCCATCAGCTTGAC	94 °C/10 min	440 bp
18S rRNA gene	R:CAAGACAAAAGTCTGCTTGAAAC	(94 °C/30 sec, 62 °C/30 sec, 72 °C/45 sec) 40 cycles, 72 °C/10 min	[8]
Babesia bigemina	F:CATCTAATTTCTCTCCATACCCCTCC	95 °C/5 min, (95 °C/1 min,	278 bp
SpeI-AvaI restriction fragment	R:CCTCGGCTTCAACTCTGATGCCAAAG	65 °C/1 min, 72 °C/1 min) 35 cycles, 72 °C/15 min	[6, 9]

Table 2. Age wise occurrence of *Babesia bigemina* by PCR assay.

Age group	Animals		
	No. of animals examined	No. of animals positive	Percentage (%) of positive animals
Young (<2 years)	38	1[a]	2.63
2-5 years	70	12[b]	17.14
Old (>5 years)	50	3[c]	6
Total	158	16	10.12

*The values superscripted with dissimilar alphabets in a column are significantly (p=0.029) different.

Table 3. Sex wise occurrence of *Babesia bigemina* by PCR assay.

Sex	Animals		
	No. of animals examined	No. of positive animals	Percentage (%) of positive animals
Male	45	1[a]	2.22
Female	113	15[b]	13.27
Total	158	16	10.12

*The values superscripted with dissimilar alphabets in a column are significantly (p=0.038) different.

Table 4. Breed wise occurrence of *Babesia bigemina* by PCR assay.

Breed	Animals		
	No. of animals examined	No. of positive animals	Percentage (%) of positive animals
Nondescript animals	56	2[a]	3.57
Crossbred animals	102	14[b]	13.72
Total	158	16	10.12

*The values superscripted with dissimilar alphabets in a column are significantly (p=0.043) different.

The PCR assay performed on 158 blood samples collected from different districts of Telangana showed specific signals of amplification in 16 samples (10.12%) for *Babesia* sp. and *B. bigemina* (Fig. 3). Higher incidence of *B. bigemina* by PCR than our present study was reported earlier as 18%,15.46%, 76.1%, and 25.96%, in Pakistan, Syria, South Africa and Punjab, respectively [14, 15, 16]. Whereas, the lower incidence was reported in Sudan, Thailand, and Punjab *i.e.*, 4%, 3.6%, and 7.35%, respectively [17, 18, 19]. The variations observed in the incidence reported by various workers could be due to the differences in geographical

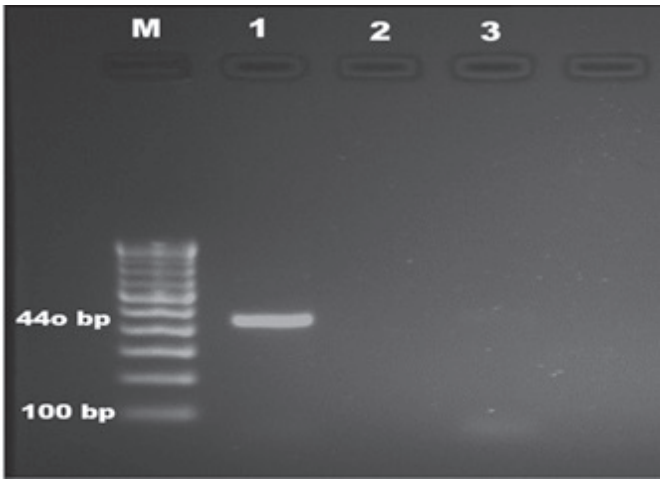

Fig. 1. Standardization of PCR assay for the diagnosis of *Babesia* sp. [M:100 bp DNA ladder; 1: *Babesia* sp. DNA, 2: Cattle (leucocyte) DNA, 3: Negative control].

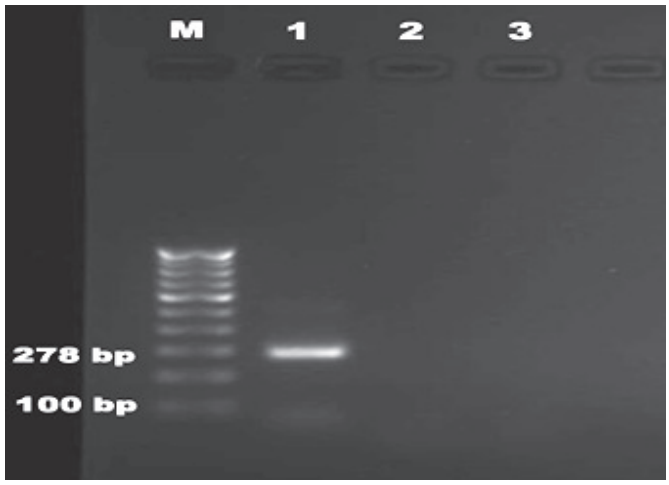

Fig. 2. Standardization of PCR assay for the diagnosis of *B. bigemina*. [M: 100 bp DNA ladder, 1: *Babesia bigemina* DNA, 2: Cattle (leucocyte) DNA, 3: Negative Control].

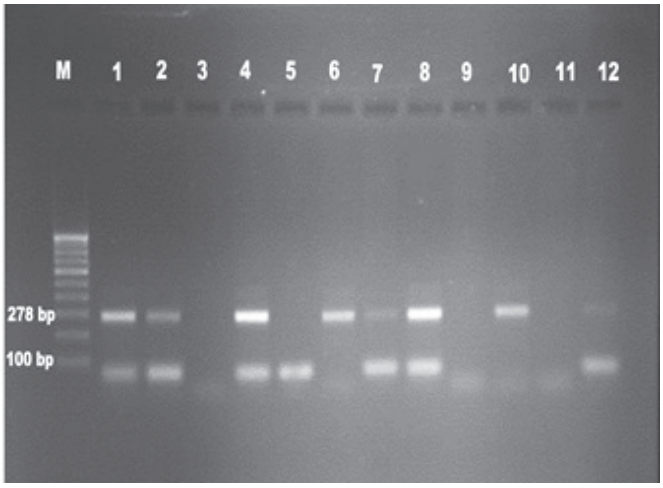

Fig. 3. Diagnosis of *B. bigemina* infection in field samples by PCR assay. [M- 100 bp DNA ladder, 1: *Babesia bigemina* positive DNA; 2, 4, 6, 7, 8, 10, 12: *Babesia bigemina* positive field samples; 3, 5, 9, 11: *Babesia bigemina* negative field samples].

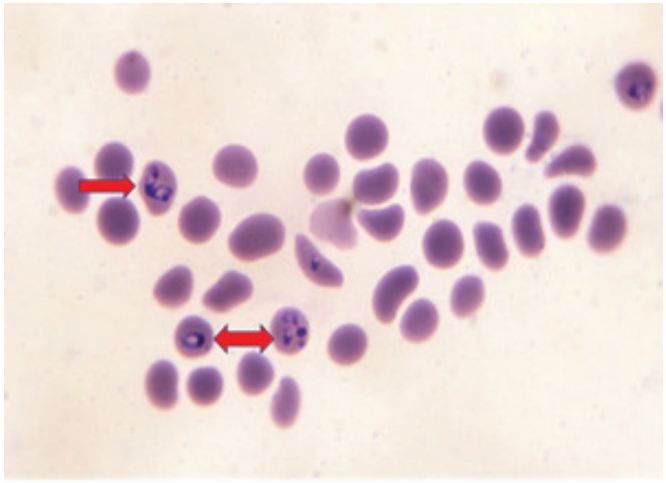

Fig. 4. *Babesia bigemina* (1000x, Giemsa staining).

distribution patterns of vectors and agroclimatic conditions favoring the propagation of vectors [20].

Further, the data was categorized and analyzed for age-wise (Table 2), sex-wise (Table 3), and breed-wise (Table 4) occurrence of babesiosis. Studies on the age-wise occurrence of babesiosis found a significantly (p=0.029) elevated rate of infection in animals of 2-5 yrs age (12/70), followed by >5 yrs age (3/50) than young animals below two yrs (1/38) of age indicating 17.14%, 6% and 2.63% of infection respectively. Earlier studies also observed a higher prevalence of babesiosis in adults compared to young animals [21, 22]. An inverse age susceptibility occurring in Babesia infections, where young animals exhibit natural resistance while the older animals are completely susceptible [23] could be the reason for the present observation.

Using the data collected, we observed that cross-bred animals revealed a higher prevalence (13.72%) of babesiosis compared to indigenous breeds (3.57%) with a significant difference (p=0.043). Similar findings were reported as the highest prevalence among crossbred and least in Nondescript breeds of animals [24, 25].

A significantly (p=0.038) higher incidence of babesiosis was observed in female animals (13.27%) than in male (2.22%) animals. Female animals were recorded to have a higher risk of infection as compared to males according to previous reports [25, 26, 27]. The difference in sex could be attributed to high

hormonal stress levels among females, especially during pregnancy and lactation, which makes them more susceptible to infection compared to males [28]. Elevated levels of prolactin and progesterone in females increase their susceptibility to infection [29].

CONCLUSION

When the laboratory standardized PCR assay was compared with the conventional staining technique, examination of Giemsa-stained thin blood smears revealed the presence of *B. bigemina* piroplasms in only 5 (3.16%) (Fig. 4) out of 158 smears examined, indicating the superiority of PCR assay over blood smear examination regarding its sensitivity and specificity. Further, the PCR assay (16/158) showed significantly higher sensitivity ($p=0.013$ *i.e.*, $p<0.05$) in the detection of babesiosis than conventional blood smear examination (5/158) and is thus recommended for field diagnosis of bovine babesiosis.

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