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PCR based diagnosis of *Babesia bovis* infection in bovines of Telangana State, India

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Abstract
A Polymerase Chain Reaction (PCR) assay targeting 18S rRNA gene of *Babesia sp.* was standardized to detect bovine babesiosis in Telangana state, India. The amplified products of *Babesia* genus specific PCR were further subjected to species-specific verification targeting Apocytochrome B gene of *Babesia bovis.* The genus specific PCR yielded a product of 440 bp whereas the species specific PCR assay yielded 711 bp length product on 2% agarose gel electrophoresis. A total of 158 bovine whole blood samples were collected simultaneously along with blood smears from different parts of Telangana state for PCR assay and blood smear examination, respectively. The analytical sensitivity of standardized PCR assay targeting Apocytochrome B gene detected as low as 390.62 pg. of *B. bovis* DNA. The conventional Giemsa staining technique could detect only 1 (0.63%) positive case out of 158 bovine blood samples collected. The present study recommends application of standardized PCR assay in the diagnosis of bovine babesiosis caused by *Babesia bovis* in the field.

Keywords: PCR, *Babesia bovis*, Bovines, Telangana state

1. Introduction
Babesiosis in bovine population, caused by *Babesia bigemina* and *Babesia bovis* is a potential concern in the Indian subcontinent, owing to huge economic losses (*i.e.*, estimated to be 57.2 million US$ annually, Minijauw and McLeod 2003) [15]. The recovered animals from acute infection act as carriers of these blood parasites, posing a risk of infection to healthy susceptible animals. *Boophilus sp.* acts as the main vector for both *Babesia* species, although transmission may occur by other tick species (Papadopoulos *et al.,* 1996) [18]. Walker and Edward (1927) [22] first reported babesiosis in India, and *B. bigemina* was considered to be predominant pathogenic species in the country (Setty and Rao, 1972; Kolte *et al.*, 2017) [20, 13]. Review of the literature turned up cases of Indian cattle and buffaloes having *B. bovis* related piroplasmosis. (Walker and Edward 1927; Idnani 1938; Gautam and Chhabra, 1983; Muraleedharan *et al.*, 1984; Shastri *et al.*, 1991; Kolte *et al.*, 2017; Ravindran and Gosh 2017) [22, 12, 10, 16, 21, 13, 19].

The clinical presentation of acute babesiosis include pyrexia, anaemia, weakness, icterus, jaundice, haemoglobinuria, presence of intra-erythrocytic parasites and nervous signs (Callow 1984, Muraleedharan *et al.,* 1991) [5, 17]. Following recovery, persistence of infection by *B. bovis* may remain for life time (Goff *et al.*, 2008) [11] resulting in carriers capable of infecting both ticks and naive cattle (Figueroa *et al.*, 1993; Calder *et al.*, 1996) [9, 4].

Keeping in view of the economic significance of carrier infections, lack of sensitivity and specificity of traditional microscopy and serological techniques, the present study was undertaken to standardize a Polymerase Chain Reaction (PCR) to detect *Babesia sp* by targeting the 18S rRNA gene and Apocytochrome b gene of *B. bovis* and the field validation of standardized PCR assay in the diagnosis of bovine babesiosis caused by *B. bovis* in Telangana state.

2. Materials and Methods
2.1 Extraction of genomic DNA from *Babesia* positive blood
Around 0.5 ml of blood was collected into EDTA coated Vacutainers (BD®) aseptically from ear vein of a graded murrah buffalo suspected for *Babesia* infection showing clinical signs of babesiosis such as pyrexia (104.8 °F), haemoglobinuria, complete recumbency, anaemia and tick infestation at Veterinary Polyclinic, Khammam district of Telangana state.

Prior to DNA extraction, preliminary screening was done using Giemsa stained thin blood smears made with a drop of blood from tip of the ear.

The genomic DNA was isolated from the positive blood sample by using DNeasy® Blood and Tissue kit (Qiagen, Germany), according to manufacturer's instructions with minor modifications. Briefly, 100 µl of whole blood was lysed with 200 µl of buffer AL by incubating the tubes at 56 ℃ for 10 minutes. Then, 200 µl of ethanol was added to the tube and the whole mixture was transferred to QIAamp mini spin column in 2 ml collection tube. Later, the QIAamp mini spin column was washed with buffer AW_1 and AW_2 followed by centrifugation of 12000 rpm for 2 minutes. Then the spin column was transferred to new microcentrifuge tube and DNA was eluted in 100 µl buffer AE by centrifugation at 8000 rpm for 1 minute after incubation of tubes at room temperature for 3 minutes. The DNA thus eluted was stored at -20 ℃ till further use.

Agar gel electrophoresis (0.7%) was carried out for checking the presence of genomic DNA extracted from blood. Concentration and purity of the extracted DNA was checked by Nanodrop 1000 Spectrophotometer (Thermo scientific, USA). The DNA thus obtained was used as template in PCR reaction. Whereas, the DNA extracted from the blood of new born calf was used as negative control in all PCR reactions.

2.2 Polymerase chain reaction

Genus Specific PCR assay

The genomic DNA obtained from the blood samples was first subjected to genus specific PCR assay targeting 18S rRNA gene of *Babesia sp.* with 10 pmol each of forward (5'GTTTCTGMCCCATCAGCTTGAC3') and reverse (5'CAAGACAAAAGTCTGCTTGAAAC3') primers, 12.5 µl of EmeraldAmp® GT PCR master mix, 50 ng template DNA and the reaction volume was made up to 25 µl with nuclease free water in an automated thermal cycler (Hi-media) as per the protocol described by Hilpertshauser *et al.* (2007). The cycling conditions consisted of Initial incubation at 37 °C for 10 min, initial denaturation at 94 °C for 10 min followed by 40 cycles each of denaturation at 94 °C/30 sec, annealing at 62 °C/30 sec and extension at 72 °C/45 sec with a final extension at 72 °C for 10 min. The amplified products were subjected to gel electrophoresis (2%) along with 100 bp DNA ladder at 50v for 2hrs and visualized under gel documentation system.

Species-specific PCR assay

Subsequently, samples that demonstrated genus-specific PCR amplification were subjected to species-specific PCR assay targeting *Apocytochrome b* gene of *B. bovis* as per the method described by Fahirmal *et al.* (1992). The PCR conditions were standardized by using 10 pmol each of forward (5'GGGTTTATAGTCGGTTTTGT3') and reverse (5'ACCATTCTGGTACTATATGC3') primers, 12.5 µl of EmeraldAmp® GT PCR Master Mix, 50 ng template DNA and the reaction volume was made up to 25 µl with Nuclease Free Water. The cycling conditions consisted of initial hold at 94 °C for 5 min followed by 30 cycles each of denaturation at 94 °C/1 min, annealing at 55 °C/2 min and extension at 72 °C/3 min followed by final extension at 72 °C for 7 min. The amplified PCR products were run in Ethidium bromide (0.5µg/ml) stained, agarose (2%) gel electrophoresis along with 100 bp DNA ladder at 50 V for 2 hrs. The results were visualized and documented in gel documentation (syngene) system. For sensitivity analysis, descending double fold dilutions of template DNA concentration ranging from 12.5 ng to 195.31 pg were tested as described above.

2.3 Collection and processing of blood samples from field

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

2.4 Blood smear examination

During sample collection, methanol-fixed thin peripheral blood smears (from the tip of the ear) were also taken from the corresponding animals. The blood smears were stained with Giemsa at a 1:6 dilution in sterile distilled water for 45 minutes and observed under 100x objective lens of a light microscope (Olympus). A single piroplasm presence was considered indicative of a positive case and a minimum of 5000 red blood cells were screened, before proclaiming negative for blood parasites.

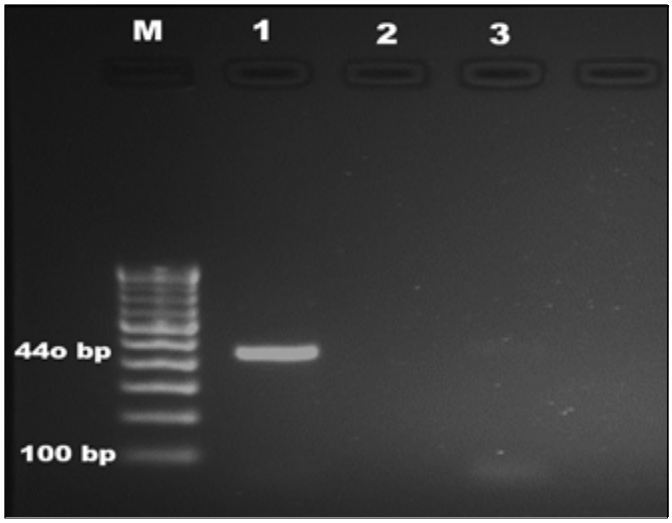
2.5 Statistical analysis

The results of PCR assay were compared with those of microscopic examination of Giemsa stained blood smear by chi square test using Graph pad prism 5 software.

3. Results and Discussion

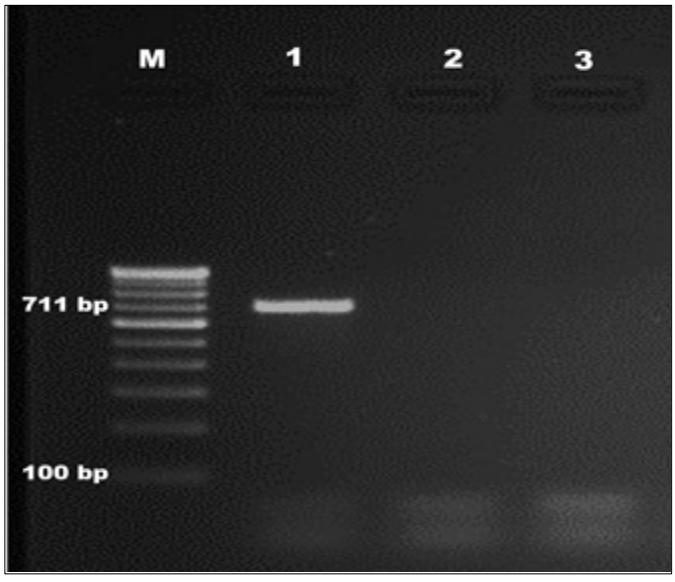
Due to low number of circulating parasites and the shortcomings in sensitivity and specificity associated with conventional detection methods like microscopy and serological techniques, the identification of carrier and subclinical cases of babesiosis has become a challenging task. Molecular based approach of detection would help in overcoming the difficulties in detection owing to their high accuracy, sensitivity and reliability. The standardized PCR assay to detect *Babesia sp.* targeting 18S rRNA gene, yielded a single band of 440 bp length without any amplification in negative control when run in 2% agarose gel electrophoresis (Figure 1). Later, PCR assay encoding Apocytochrome B gene of *B. bovis* in *Babesia sp.* positive sample yielded a single band of 711 bp length without any amplification in negative control when run in 2% agarose gel electrophoresis (Figure 2). The primers used in this study did not amplify the leucocyte DNA of bovine calf indicating the specificity of primers towards the DNA of *Babesia* sp. as well as *B. bovis*. Out of 158 blood samples screened from different districts of Telangana, only one sample (0.63%) showed specific signals of amplification by PCR assay (Figure 4). Almost similar observations of *B. bovis* infection were reported by Almeria *et al.* (2001) as 0.75% by PCR assay in Minorca. Higher incidence of *B. bovis* by PCR than our present studies were reported earlier by Chaudhry *et al.* (2010) [6], Zulfiqar *et al.* (2012) [23] and Kolte *et al.* (2017) [13] as 11%, 18% and 3% in Pakistan, Punjab and Maharashtra, respectively. The variation in incidence reported by different workers could be due to changes in vector geographical distribution and different agro-climatic conditions conducive to vector propagation.

Besides that, sensitivity evaluations using positive template DNA revealed amplification with an analytical sensitivity level of 390.62 pg. of parasite DNA (Figure 3). Further, this is the first report on *B. bovis* infection in bovines of Telangana state, India. When the laboratory standardized PCR assay was compared with the conventional staining techniques *viz.*, Giemsa stained thin blood smear revealed presence of *B. bovis* piroplasms in only 1 (0.63%) (Figure 4) out of 158 blood smears examined. Though there is no significant difference (P=1) between PCR and conventional staining techniques in our study, the sensitivity of blood smear examination is said to be comparatively low and it requires certain amount of skill to detect parasites in the blood smear, even in acute infection and in severe parasitemia. Besides that PCR could amplify *B. bovis* DNA up to a sensitivity of 390.62pg. Hence, considering the sensitivity and specificity of gene targeted PCR assay, the test is recommended for the diagnosis of acute, chronic and carrier state of babesiosis due to *B. bovis* infection in the field.



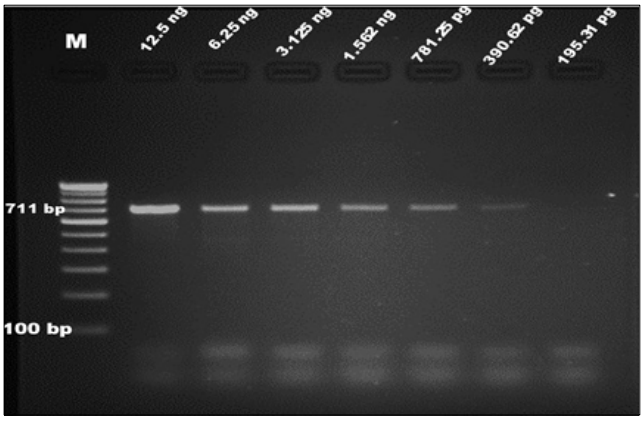
M: 100 bp DNA ladder; 1: *Babesia* spp. DNA; 2: Cattle (leucocyte) DNA; 3: Negative Control

Fig 1: PCR assay for the diagnosis of Genus specific *Babesia* spp. in Cattle



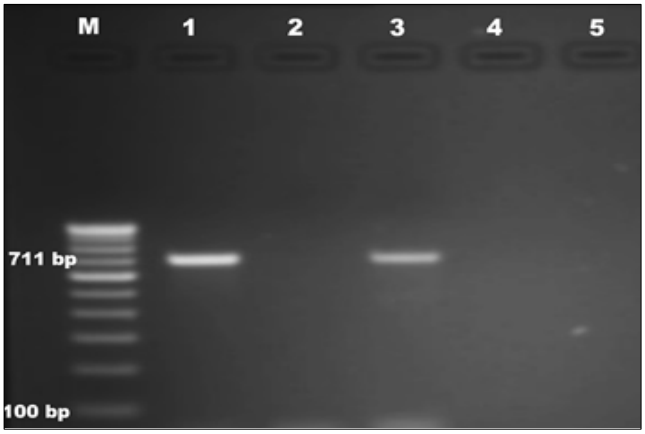
M: 100 bp DNA ladder; 1: *Babesia bovis* DNA; 2: Buffalo (leucocyte) DNA; 3: Negative Control

Fig 2: PCR assay for the diagnosis of *Babesia bovis* in Cattle



M: Gene ruler of 100 bp DNA ladder, Lanes: 1 to 7 descending 2 fold dilutions of *Babesia bovis* genomic DNA

Fig 3: Analytical Sensitivity of PCR assay for *Babesia bovis*



M: 100 bp DNA ladder; 1: *Babesia bovis* positive control; 3: *Babesia bovis* positive field sample 2, 4, 5: *Babesia bovis* negative field samples

Fig 4: Diagnosis of *B. bovis* infection in field samples by PCR assay

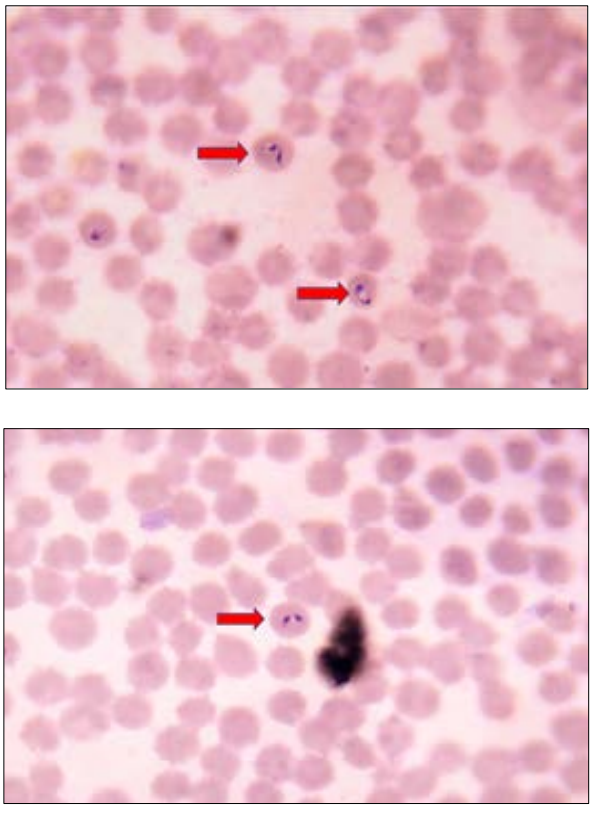


Fig 5: Giemsa staining of *Babesia bovis* (1000x)

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5. Conflict of interest
The Authors affirm that there are no conflicts of interest to disclose.

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