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# Molecular diagnosis of sub-clinical Babesia infection in cattle by PCR

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**Abstract**
Babesiosis is an important tick-borne haemo-protozoan disease of various domestic and wild animals throughout the world and characterized by anorexia, dullness, depression, pale mucus membranes, coffee colored urine. Microscopic examination is not suitable for detecting the carrier or chronic phases of piroplasmosis. However, it remains the most rapid confirmatory method for detecting this infection in acute phase of the disease. While PCR turned out to be a sensitive and accurate method for diagnosis of Babesiosis in animals in the early phase of infection and in carrier animals by DNA amplification. In the present investigation, a total of 105 Holstein Friesian cows (irrespective of age) were examined for diagnosis of sub-clinical Babesiosis by PCR. Out of these 105 blood samples, 23 positive cases (21.90%) have been observed for the existence of Babesiosis by PCR technique. It indicates a higher sensitivity of PCR over traditional blood smear examination, especially for detecting latent infections. This sub-clinical state is challenge to current diagnostic methods and is difficult to detect because of the low number of parasites in circulation.

**Keywords:** Babesiosis, hemoprotozoan, PCR, latent infections

## 1. Introduction
Bovine Babesiosis is economically the most important tick-borne disease of cattle caused by protozoan parasites of the genus Babesia [14]. Babesia parasites cause both acute and persistent sub-clinical disease in cattle. Accurate and correct diagnosis of Babesial infections plays an important role in monitoring, management and control of infection. Ticks of Boophilus species are responsible and major vectors for the transmission of *B. bovis* and *B. bigemina* [8]. Haemo-protozoan parasites cause economically important vector-borne diseases of tropical and sub-tropical parts of the world including India [12]. After trypanosomes the second most parasite found in the blood of mammals is Babesia [15]. The most commonly used method for the diagnosis of Babesiosis is blood smear in acute cases but in carrier stages where the infection is too low, where more sensitive tools are needed; because microscopy does not detect the infection in early stage or carrier stage and PCR is most reliable and sensitive method, which diagnose the low grade infections [10]. After recovery from Babesiosis, the animals generally become carriers and considered as a reservoir for natural transmission of the disease [3]. Serological examination for detection of the carrier state in animals lacks specificity and sensitivity, especially for the diagnosis of infection status [5]. Numerous molecular techniques have been developed, but the PCR has become as a rapid sensitive assay which are capable of genomic detection [2]. The PCR assay is believed to be a powerful tool for the diagnosis of Babesiosis [1]. The present study was conducted to focus the prevalence and molecular detection of sub-clinical Babesiosis in cattle population presenting at the dairy farms.

## 2. Materials and Methods
In the present investigation, a total of 105 Holstein Friesian cows (irrespective of age) were examined for diagnosis of sub-clinical Babesiosis. Blood sample was collected from jugular vein with all aseptic precautions in sterilized test tubes having disodium salt of ethylene diamine tetra acetic acid (EDTA) as an anticoagulant added at the rate of 1 mg/ml of blood as recommended by Jain (1986). Blood samples collected in EDTA vacutainers were stored at -20°C until DNA extraction. Genomic DNA was isolated from whole blood using HiPura™ Blood Genomic DNA Miniprep Purification Kit (HIMEDIA) as follows: Add 200 µl blood sample to the microcentrifuge tube.

After that we added 20 µl of the reconstituted Proteinase K solution (20 mg/ml) into 2.0 ml collection tube containing 200 µl of the whole blood. Then vortex for 10-15 seconds to ensure thorough mixing. Then add 200 µl of the Lysis Solution to the sample, vortex thoroughly for a few seconds to obtain a homogenous mixture. Incubate at $550^0$C for 10 minutes. Then add 200 µl of ethanol to the lysate obtained from the above step for preparation of lysate for binding to the spin column. Mix thoroughly by gentle pipetting. After that transfer the lysate into the spin column provided. Centrifuge at 6,500 x g (10,000 rpm) for 1 minute. Discard the flow-through liquid and place the column in a new 2.0 ml collection tube. Then we added 500 µl of diluted Prewash Solution to the column and centrifuge at 6,500 x g (10,000 rpm) for 1 minute. Discard the flow-through liquid and re-use the same collection tube with the column. Then we added 500 µl of diluted Wash Solution to the column and centrifuge at 12,000-16,000 x g (13,000-16,000 rpm) for 3 minutes to dry the column. Discard the flow-through liquid and spin the empty column for another minute at the same speed if residual ethanol is observed. Discard the collection tube containing the flow through liquid and place the column in a new 2.0 ml collection tube. Then pipette 100 µl of the Elution Buffer directly onto the column without spilling to the sides. Incubate for 1 minute at room temperature (15-25°C). Centrifuge at 6,500 x g (10,000 rpm) for 1 minute to elute the DNA. Transfer the eluate to a fresh capped 2 ml collection tube for longer DNA storage.

### 2.1 PCR Amplification

PCR was carried out by using following sets of primers as reported by Mahmmod and Yasser (2012). PCR assay was carried out by using the Babesia genus specific primers for targeting the small sub-unit ribosomal RNA (SSU r-RNA) gene supplied by (Xcelris Ltd.) as given below:

644 (F): 5’ TGG AAC TTT AGG GTT TAT ACG 3’
644 (R): 5’ GGT AAT TAC TCC ATA AGT TA 3’

PCR reactions for each sample were performed in a volume of 50 µl using puregene kit (PCR Master Mix (2x) supplied by Genetix Brand containing PCR Master Mix (2x) 4 x 1.25 ml vial, nuclease free water 4 x 1.25 ml vial and instruction leaflet.). The reaction mixture used per reaction is as follows:

**Table 1:** The reaction mixture used per reaction is shown in the table.

| S. No. | PCR Components | Quantity |
|---|---|---|
| 1. | 2x PCR assay buffer $MgCl_2$ (4mM), dNTP (0.4mM), Taq DNA polymerase(0.05UµL) | 25 µl |
| 2. | Primer-F (100pmol/ µl) | 1 µl |
| 3. | Primer-R (100pmol/ µl) | 1 µl |
| 4. | Template DNA | 4 µl |
| 5. | Nuclease free water | 19 µl |
| 6. | Total | 50 µl |

The thermo cycle profile was as described: Ist step is initialization by denaturation at 94°C for 5 minutes. Then 2nd step is the denaturation which is the regular cycling event and consists of heating the reaction chamber to 94°C for 30 seconds for 35 cycles. In the next step, annealing is done by lowering the reaction temperature at 51.1°C for 1 minute for 30 cycles. The next step is extension/elongation at 72°C which is the optimum temperature for 1 minute for 30 cycles. Finally, extension is done at 72°C for 10 minutes. The final step or the final hold cools the reaction chamber to at 4°C until the samples were taken out from thermal cycler. The amplified PCR products (644 bp) were then visualized by using 1.5% agarose gel electrophoresis (Syngene, UK).

## 3. Results and Discussion

A total of 105 Holstein Friesian cows were screened for the presence of sub-clinical Babesiosis. Out of these 105 blood samples, 23 positive cases (21.90%) have been observed for the existence of Babesiosis by PCR technique. It indicates a higher sensitivity of PCR over traditional blood smear examination, especially for detecting latent infections. In the present study, the results obtained from PCR revealed that 21.90 % were positive for Babesia. Similar results were obtained by Cringoli *et al*. (2002) [4], Niu *et al*. (2015) [7]. Fluctuation in the prevalence rates might be due to the variation of environmental conditions that affect both parasites and vectors. Variation in prevalence rates could possibly be attributed to an abundance of the vectors as a result of high temperature and humidity.

This sub-clinical state is challenge to current diagnostic methods and is difficult to detect because of the low number of parasites in circulation. However, diagnosis of carrier animals in herd is important for preventing outbreaks by transmission through vector ticks to healthy animals and for obtaining epidemiological data of disease. The diagnosis of ruminant piroplasmosis is generally based upon the microscopic examination of Giemsa stained blood smears and by clinical symptoms in acute cases.

After acute infections, recovered animals frequently sustain subclinical infections, which are microscopically undetectable (Perez-Llaneza *et al*. 2010, Schneider *et al*. 2011) [9, 13]. The study has indicated that the use of PCR in the surveillance of Babesiosis will enable the detection of asymptomatic carrier animals that could not be detected using conventional methods. Subclinical Babesiosis lead to the affected livestock, including cattle and small ruminants, becoming chronic carriers and in turn sources of infection for tick vectors, and cause natural transmission of the disease. Therefore, latent infections are the target in the epidemiology of the diseases. They can be considered as a source of infection for the potential vector causing natural transmission of the disease. In this study, the conserved primers for Babesia derived from SSU rRNA gene were used in PCR amplification for the detection of Babesia DNA, which amplified a fragment of 644 bp in the examined bovine samples (Salem *et al*. 1999) [11]. The results of this study showed that the samples of cattle served as reservoirs of Babesiosis. Consequently, it could be stated that the animal reservoirs increase the risk of the potential spread of disease to other animals and especially humans, and this deserves special attention.

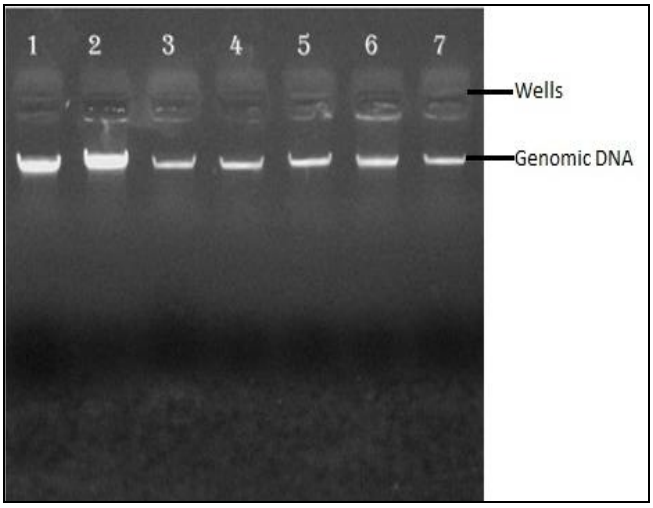


**Fig 1:** After that we added 20 µl of the reconstituted Proteinase K

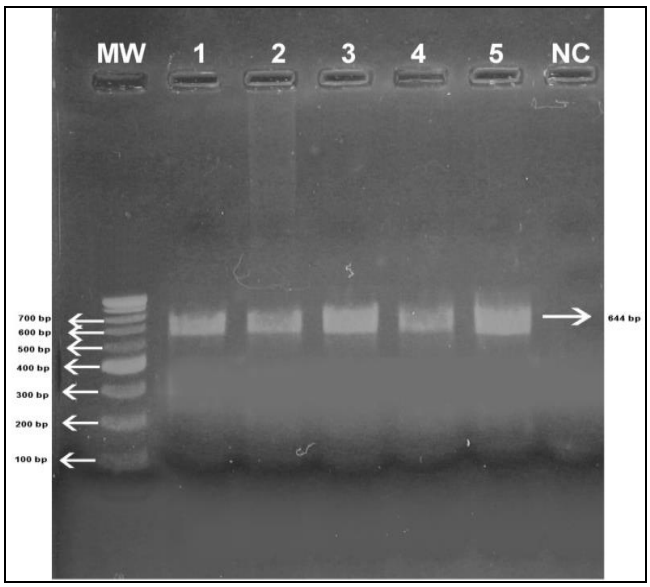


**Fig 2:** Confirmatory identification of the Babesia genus through PCR using SSU rRNA gene primer. Legends: MW Molecular weight marker (1 Kb DNA Ladder) Wells 1-5 amplicons (644 bp) NC Negative Control

## 4. Conclusion

The carrier and subclinical infections of Babesiosis are difficult to detect because of the low number of parasites in circulation and lack of sensitivity and specificity of conventional microscopic and serological techniques. Hence, early diagnosis and subsequent treatment of these infections in carrier animals is important to overcome the economic losses as well as to prevent transmission. Molecular detection based on nucleic acid identification and their amplification are the most sensitive and reliable techniques to accurately detect carrier and sub clinical infections. Therefore, a study was undertaken to check the sensitivity of PCR to ascertain the status of sub-clinical Babesiosis in cows.

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