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Molecular Detection of *Babesia bigemina* and *Babesia bovis* in Crossbred Carrier Cattle Through PCR

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Abstract.- Bovine babesiosis and other tick borne diseases are considered responsible for more than 50% losses in the crossbred cattle. Carrier cattle infected with babesiosis are challenge to current diagnostic methods and are 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. Here, we report molecular detection of *Babesia bovis* and *Babesia bigemina* in cross bred carrier cattle. For this study, 100 blood samples were randomly collected and analyzed using light microscopy and small subunit ribosomal RNA gene based PCR. Screening by LM showed that 18% of samples were positive. PCR analysis of samples diagnosed 29% positive, out of which 11% were positive for *B. bovis* and 18% for *B. bigemina*. Hence, 11% of the animals apparently healthy through routine LM diagnosis were carriers posing threat for the healthy herd population.

Keywords: *Babesia bigemina*, *Babesia bovis*, SS rRNA gene PCR, bovine babesiosis, carrier cattle.

The major economic impact of babesiosis is on the cattle industry, infections also occur in other domestic animals, including horse, sheep, goats, pigs and dogs. Bovine babesiosis is considered one of the most important tick-borne diseases of cattle worldwide, and of the 1.2 x 10^9 cattle in the world in the world, over 500 million of these cattle are potentially at risk of having bovine babesiosis. Animals that survive infection generally become low level carriers of the parasite and serve as a reservoir for transmission (Fahrimal *et al*., 1992).

Babesiosis is a tick transmitted disease of cattle caused by protozoan parasites of species *Babesia bovis* (*B. bovis*), *Babesia bigemina* (*B. bigemina*), and others. *Boophilus* species of ticks are major vector for the transmission of *B. bovis* and *B. bigemina* (O.I.E., 2005).

The vector of *Babesia, i.e*., *Boophilus microplus* is wide spread in tropics and subtropics. Generally, *B. bovis* is more pathogenic than *B. bigemina* and *B. diverges*. Infections are characterized by high fever, anorexia, general circulatory failure and sometimes, nervous signs as a result of sequestration of infected erythrocytes in the cerebral capillaries. In acute cases, maximum parasitemia (% of erythrocytes infected) in circulating blood is less than 1% in *B. bovis* in contrast to 10.30% in *B. bigemina* (Friedhoff, 1994).

Carrier cattle infected with *Babesia* are difficult to detect because of the low numbers of parasites that occur in peripheral blood. However, diagnosis of low-level infections with the parasite is important for and epidemiological studies (Fahrimal *et al*., 1992). Indirect Fluorescent Antibody Test (IFAT) is the most widely used test for the detection of antibodies to *B. bovis* and *B. bigemina* but serological cross reactions make species diagnosis difficult (O.I.E., 2005). Parasites can be found in the blood easily during acute infections. *Babesia* organisms could be detected under oil immersion (minimum x 8 eyepiece and x 60 objective lens) in erythrocytes. In blood and tissue smears. *Babesia bovis* could be hard to find in blood samples; brain biopsies may be helpful in detecting this species. In chronic infections, parasites are uncommon in blood samples and diagnosis is usually made by serology. Antibodies to *Babesia* can also be detected by ELISA. Carriers might be identified by transfusing blood into a test animal. *In vitro* culture can also be helpful. DNA probes and PCR have occasionally been used (O.I.E., 2005).

PCR has proven to be very sensitive particular in detecting *B. bovis* and *B. bigemina* in carrier cattle (Calder, 1996; Salem *et al.*, 1999). To

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overcome the economic losses early proper diagnosis of babesiosis is important in carrier cattle. The present project was aimed at standardizing the molecular diagnostic PCR technique for the early and accurate diagnosis of babesiosis in cattle and to differentiate *Babesia bovis* from *B. bigemina,* using species specific primers.

Materials and methods

The blood samples were collected from the crossbred cattle kept at Livestock Experimental Station, Qadirabad, from June to August, 2005. Microscopic examination of the blood smears was conducted at hematology Laboratory and DNA from the samples was isolated in the Postgraduate Molecular Biology Laboratory of Pathology Department, University of Veterinary & Animal Sciences, Lahore; Polymerase Chain Reaction was conducted at School of Biological Sciences, University of the Punjab, Lahore.

Microscopic blood smear examination

The dried blood smears were fixed in absolute methyl alcohol for one minute. Staining was performed using Giemsa as described by Benjamin (1986). Thick and thin blood smears were stained with one fourth dilution of commercially available giemsa stain for four minutes and observed with the help of oil immersion lens (100X) to detect the presence of *B. bigemina* and *B. bovis*. The measurements of the parasites were performed by micrometry using stage and ocular micrometers at 100X having a conversion factor of 1.0µm per unit space scale as described by Foreyt (2001). Pictures were taken from tri-ocular microscope (Micros Austria) with digital camera attachments.

DNA isolation

DNA was isolated from the whole blood samples by commercial kit (GENTRA PUREGENE). Nine hundred microliters of RBC lysis solution was added to 300µl of whole blood sample to disrupt the RBCs and release of parasite in the medium. Samples were incubated for 1-3 minutes at room temperature, centrifuged at 14,000 rpm for one minute and supernatant was discarded leaving behind 10-20µl of residual liquid containing the parasite of interest *i.e. Babesia* sp. Afterwards 300µl cell lysis solution was added, vortexed for 10 seconds and 100µl protein precipitation solution was added and vortexed for 20 seconds. Centrifugation step was repeated before adding 300µl 100% Isopropanol and tubes were gently inverted 50 times for thorough mixing. Samples were centrifuged and supernatant was discarded and 300µl 70% ethanol was added. After brief incubation the sample was centrifuged at 14,000 rpm for 1 minute for obtaining proper concentration of parasite DNA. Ethanol was discarded and tubes were dried before adding 50µl of DNA hydration solution to the DNA pellet. Isolated DNA solution was stored at 4^0C for immediate processing, while at -40^{o}C for till further analysis.

PCR amplification

For specific detection of *B. bigemina* and *B. bovis* primers shown in Table I were used for ribotyping. PCR was performed under the conditions as suggested by Guido *et al*. (2002).

PCR reaction was performed to obtain the 1,124bp and 541bp amplified products over 35 cycles by 94ºC for 5 min., 94ºC for 30 sec., 50ºC for 30 sec., 72ºC for 45 sec. and completed with a final extension step of 7 min. at 72ºC. Finally the amplified DNA fragments were analyzed after electrophoresis on 1% agarose gel.

Results and discussion

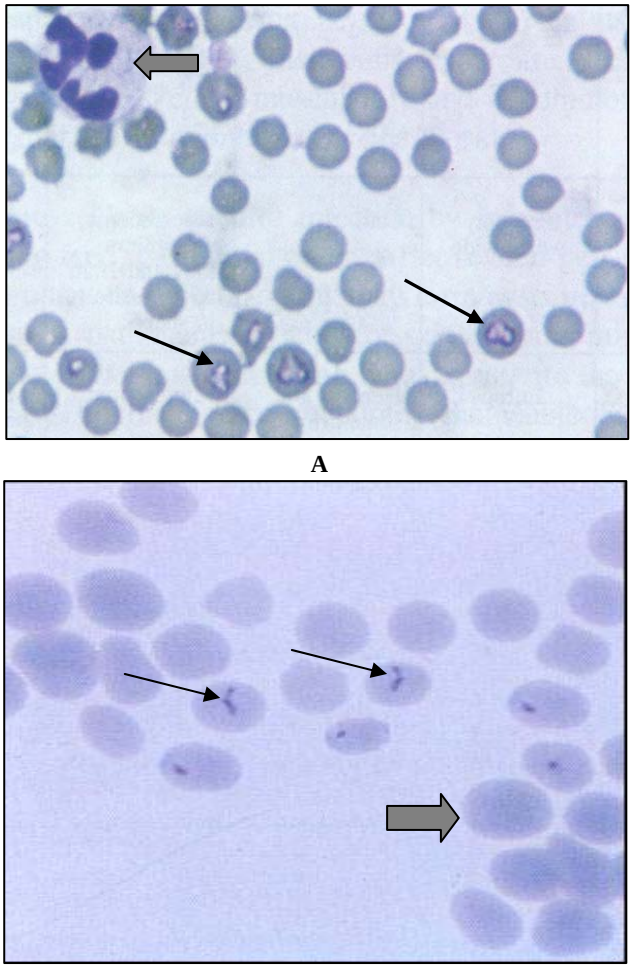
Of the 100 blood samples, 18 were positive for *Babesia* by microscopic examination. *Babesia bigemina* is larger in size and having a paired structure at an acute angle to each other. *Babesia bovis* is smaller in size and having paired form at an obtuse angel to each other. However, it is hard to differentiate the two species by microscopic examination (Fig. 1).

Babesia bigemina has dimensions of 3.24±0.22 x 1.29±0.17 as against 1.29±0.21 x 0.71±0.17 of *B. bovis.* Ribotyping analysis (Fig. 2) showed that 29 samples were positive for babesiosis, in which 18 were positive for *B. bigemina* and 11 were positive for *B. bovis*.

Through this study it was confirmed that PCR is more sensitive and specific in detecting low level of infections if carrier animals as compared to light microscopy and the results of our study are in

Table I.- Selected primers sequences from SS rRNA gene A region as reported by Guido *et al*. (2002).

		Target position on genome	Predicted amplicon size(PAS)
Babesia bigemina			
GAU5 (Forward)	5'- TGGCGGCGTTTATTAGTTCG- 3'	409-428	1,124 bp
GAU6 (Reverse)	5'- CCACGCTTGAAGCACAGGA- 3'	1532-1515	
Babesia bovis			
GAU9 (Forward)	5'- CTGTCGTACCGTTGGTTGAC-3'	675-694	541 bp
GAU10 (Reverse)	5'- CGCACGGACGGAGACCGA-3'	1215-1198	



A

B

Fig. 1. Giemsa stained microplate showing characteristics of *babesia bigemina* (A), and *B. bovis* infection. Thin arrow shows *Babesia* sp. Inside the bovine erythrocytes, whereas thick arrow shows a bovine neutrophil in A a uninfected bovine erythrocyte in B.

relevance with Smeek *et al*. (2000). Similar observations were reported by Fahrimal *et al*. (1992) who studied the carrier cattle infected with babesia by using PCR amplification of a portion of the apocytochrome b gene from the parasite. The results in this study are also in relevance with the studies conducted by Figueroa *et al*. (1992a,b, 1993a,b) regarding carrier animals.

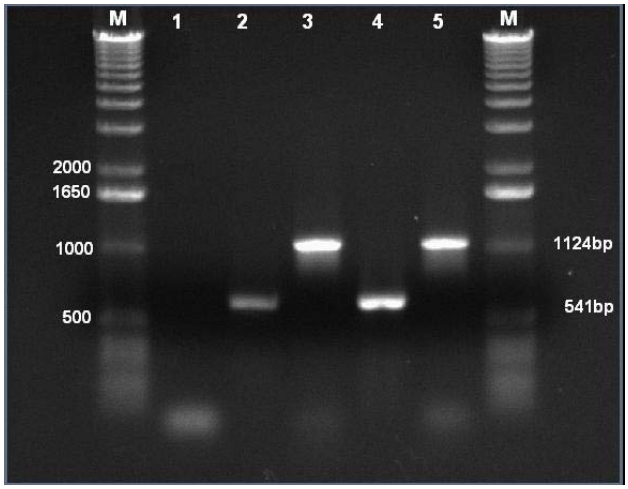


Fig. 2. Representative gel showing the specificity of SS rRNA PCR for the efficient detection of bovine babesiosis.
Lane 1, negative bovine genomic DNA control; Lane 2, positive control for *B. bovis*; Lane 3, positive control for *B. bigemina*; Lane 4, positive sample for *B. bovis* at 541bp and Lane 5, positive sample for *B. bigemina* at 1124bp; Lane M, 1000bp DNA ladder marker.

The present study was conducted in the months of June-August, when the seasonal prevalence of vector ticks is very high, which transmit the infection from carrier animals to other healthy animals in the herd, the similar observations were made by Hermans *et al*. (1994) in Cost Rica

where they assessed the infection transmitted from the ticks by the use of PCR analysis.

It is concluded that the methodology used in the current study seems to be helpful for the diagnosis of babesiosis in animals in the early phase of infection and in carrier animals by DNA amplification, and it may be used as a tool for epidemiological investigation and subsequent disease eradication.

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