Prevalence and molecular detection of *Babesia bigemina* in water buffalo from southeastern region of Pakistan

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**Abstract**

*Babesia* spp. are tick-transmitted apicomplexan parasites that infect erythrocytes of a wide range of vertebrates including domestic animals, and can be associated with a considerable economic loss due to introduction of prophylactic measures, decreased production and rates of morbidity and mortality. An efficient detection assay needs to be set up for accurate diagnosis and prevention of disease. The present work is aimed at verifying the occurrence of *Babesia bigemina* infection in water buffaloes in the southeastern region of Pakistan by using both traditional blood smear and molecular techniques. A total of 100 blood samples were collected from tick-free and tick-infested animals (50 animals each group). The overall prevalence was recorded as 20% and 17% by using thin blood smear and PCR methods, respectively. Moreover, the analysis of infection in tick-infested and tick-free animals showed the infection rates of 36% and 4% by using blood smear method, and 30% and 4% with that of PCR method, respectively. Despite the higher prevalence rate recorded by blood smear method, which may be the result of non-specific identification of *Babesia* species, these results indicate that the PCR assay used in this study provides a useful tool for accurate diagnosis of the *Babesia bigemina* infection in bovines.

**Key words:** *Babesia bigemina*, diagnosis, PCR, prevalence, blood smear technique

**Introduction**

Babesiosis is a tick-transmitted disease caused by intraerythrocytic infection by several species of *Babesia* (Kjemtrup and Conrad, 2000; Schnittiger et al. 2012). The disease poses a major threat to livestock production in tropical and subtropical areas, and can have a significant impact on farming communities due to economic losses (Zintl et al., 2003; Bock et al., 2004; Canever et al. 2014). *Babesia bigemina* and *Babesia bovis* are the predominant and most clinically relevant species in bovines in endemic areas (Aulakh et al., 2005; Karunakaran et al., 2011; Venu et al., 2013), where the principal vector that matches with the distribution is *Boophilus* (*Rhipicephalus*) species. However, in rare cases *Ixodes* are also reported to transmit infections (Friedhoff, 1988; Estrada-Pena et al., 2004; OIE, 2008).

Bovine babesiosis is highly pathogenic, especially in cattle and buffaloes, and causes chronic to severe infections. The severity of the *Babesia* infection and clinical signs are variable depending on the infected species, host immune status and age. The disease is usually characterized by anemia, fever, jaundice and hemoglobinuria. The increase in global transportation of animals, which facilitates the introduction of vectors into new areas has enhanced transmission of babesiosis in bovines (Rani et al., 2010; Karunakaran et al., 2011; Patel et al., 2011; Venu et al., 2013). Along with *Babesia bigemina* and *Babesia bovis*, other *Babesia* species infecting bovines, *Babesia major* and *Babesia occultans*, are also present worldwide and can result in the infection similar but less pathogenic compared to the former two species (Ros-García et al., 2011; Zulfiqar et al., 2012).

Clinical diagnosis of babesiosis in bovines is usually based on the vector exposure, risk of transmission and prevalence of infection in an endemic area, and upon the use of some conventional laboratory diagnostic methods, specifically Giemsa-stained blood smear examination, which enables the detection of piroplasms in the red blood cells. However, the detection of the piroplasms by this method is not reliable in early or carrier stages. Moreover, the low sensitivity and the difficulty of distinguishing morphologically similar strains and related species or even other piroplasms further limits the use of this traditional diagnostic method (Krause et al., 1996; Passos et al., 1998).

Recently, PCR-based molecular detection methods have become the preferred methods for diagnosis of blood parasites due to their higher sensitivity and accuracy compared to conventional methods (Nagore et al., 2004, Altay et al., 2005). The molecular-based techniques are preferred over conventional methods because of their ability to identify genotypically distinct but morphologically indistinguishable piroplasm species in the infected blood.
Asian breeds of water buffalo (*Bubalus bubalis*) are considered to be one of the best sources of milk in the Indo-Pak region, with 34.6 million heads of buffalo producing 31,252 thousand tons of milk for human consumption only in Pakistan (Bilal et al., 2006; Farooq, 2014). Being in the endemic areas, these breeds are highly susceptible to tick-transmitted diseases, which results in the severe economic losses (Jabbar et al., 2015; Saad et al., 2015). However, due to the lack of accurate diagnostic methods, only limited data is available regarding the epidemiology of *Babesia* in Pakistan (Jabbar et al., 2015). Therefore, rapid and accurate diagnosis of the babesiosis with immediate introduction of chemotherapy will increase the chances for a favourable prognosis and prevention of an outbreak in non-endemic areas. In the present study, we have detected the *Babesia bigemina* infection in water buffalo using PCR-based DNA amplification method, and compared it to the conventional blood smear examination of blood samples collected from buffalo in Hyderabad city with the aim of early and accurate diagnosis of *B. bigemina* infections.

### Material and methods

#### Sample collection

One hundred whole blood samples were randomly collected from the Kundhi breed of the water buffalo (50 animals each from tick-infected and non-infected animals) from different animal farms present in the cattle colony of Hyderabad, Pakistan. Three ml of blood from the jugular vein of each animal were collected in the vacutainer tubes supplied with EDTA (ethylenediaminetetraacetic acid) as an anticoagulant to avoid coagulation of the blood and rupture of erythrocytes. The samples were then brought to Molecular Parasitology Laboratory, Department of Veterinary Parasitology, Sindh Agriculture University, Tandojam for further analysis.

#### Microscopic examination

Thin blood smears were prepared from the fresh blood obtained from all animals in order to determine the presence of parasite. Briefly, a tiny drop of blood was spread on a slide and fixed with absolute methanol for 1-2 min, and subsequently stained with 10% Giemsa stain by immersing the slide for 30 min. The stained slides were then screened for the presence of infection under immersion oil at 100× magnification.

#### Genomic DNA extraction

The genomic DNA was extracted from the blood samples using GF-1 Nucleic Acid Extraction Kit (Cat. No. GF-BD-100; Vivantis, Malaysia) according to the manufacturer’s instructions. Briefly, 200 µl of buffer BB was added to 200 µl of the whole blood and mixed thoroughly by vertexing to obtain a uniform suspension. After homogenization, 20 µl of Proteinase-K was added and mixed immediately in 2 ml collection tube. The suspension was then incubated at 65°C for 10 min. Afterwards, 200 µl of absolute ethanol was added and mixed immediately by vertexing. The suspension was then transferred to a Genomic DNA extraction column inserted in a collection tube and centrifuged at 5000×g for 1 min. The tube containing flow-through was discarded and the column was washed with 500 µl of the wash buffer 1 and centrifuged at 5000×g for 1 min. The flowthrough was discarded and column was washed with 500 µl of the wash buffer 2 and centrifuged at 5000×g for 1 min. The column was then placed onto a new 2 ml collection tube and again washed with 500 µl of the wash buffer 2 and centrifuged at 5000 ×g for 3 min. Finally, genomic DNA was eluted using 50 µl of elution buffer by incubating for 2 min at room temperature followed by centrifugation at 5000 ×g for 1 min. The purified genomic DNA was immediately used or stored at −20°C until further processing.

#### PCR amplification

*Babesia bigemina* infection in water buffalo was detected by using PCR amplification performed using genomic DNA extracted from the collected blood samples targeting 18S ribosomal RNA (rRNA) gene. The PCR was performed using previously published primers specific for *Babesia bigemina*, Bi-F: AATAAACATACAGGGCTTTCGTCT and Bi-R: ACGCCAGGCTTGAATACAC (Kim et al., 2007). The PCR amplifications were performed in the thermal cycler (Applied Biosystem 2720, USA) with 25 µl PCR mixture consisting of 12.5 µl of PCR Master Mix (GoTaq1 Green Master Mix, Promega, USA), 2.5 µl of each forward and reverse primers (25 pmol each), 50 ng of genomic DNA and the remaining volume that was adjusted with nuclease free water. PCR was performed using the following cyclic conditions: initial denaturation for 5 min at 94°C followed by 40 cycles with denaturation for 45 sec at 94°C, annealing for 45 sec at 54°C and extension for 45 sec at 72°C, and a final extension for 7 minutes at 72°C. After PCR amplification, 5 µl of each PCR product was separated by electrophoresis with 1.5% agarose gel stained with ethidium bromide and visualized by using UV transillumination under Gel Documentation System, Cleaver Scientific, Ltd, UK.

### Results

With the advancement in diagnostic techniques and use of molecular approaches for accurate diagnosis, amplification of total DNA extracted from the blood of domestic animals provides a feasible way to arrive at definitive diagnosis. In the present study, out of 100 blood samples, 20% were found positive for *Babesia bigemina* by thin blood smear microscopic examination (Fig. 1). The genomic DNA extracted from the whole blood samples was used for the PCR amplification with the primers specific to 18S ribosomal RNA (rRNA) gene. It resulted in the fragments of 170 bp, which was similar to previous reports (Kim et al., 2007), thus providing the evidence for using this molecular approach to accurately diagnose *Babesia bigemina* infection in bovines (Fig. 2). The analysis of 100 blood samples from water buffalo that were subjected to PCR showed that a total of 17% of sampled animals were positive for *Babesia bigemina* infection in water buffalo kept in Hyderabad cattle colony (Fig. 3).
Figure 1. Thin blood smear microscopic examination of intra-erythrocytic *Babesia bigemina* infection (100X magnification).

Figure 2. Accuracy and specificity of the PCR method. Agarose gel electrophoresis of amplification of 18S ribosomal RNA gene from *Babesia bigemina* using specific primers. Ladder DNA marker; lane 1 and 2, uninfected buffalo blood (negative control); lane 3 and 4, *B. bigemina* positive blood samples showing bands of about 170 bp.

Figure 3. Prevalence of *Babesia bigemina* infection in water buffalo as observed by thin blood smear microscopic examination, and using genomic DNA amplification method by PCR.
Further effect of persistent tick infection on the prevalence of *Babesia bigemina* infection in water buffalo was determined. This can be used as an evidence whether an animal is tick-infested or not at the time of the sample collection, although the previously infested animals can demonstrate *Babesia bigemina* infection. The blood samples collected from tick-infested and tick-free buffaloes demonstrated the infection in 36% of tick-infested and 4% tick-free buffaloes during the examination by the blood smear method. However, when the same blood samples were processed via PCR, 30% buffaloes were shown to be infected with *Babesia bigemina*, whereas the infection in tick-free buffaloes was observed in only 4% of the samples collected (Fig. 4).

**Figure 4.** Prevalence of *Babesia bigemina* infection in blood samples collected from tick-infested and tick-free water buffalo in Hyderabad cattle colony, and analyzed by thin blood smear microscopic examination and PCR amplification method.

**Discussion and conclusions**

Accurate diagnosis of *Babesia* infections leads to a better understanding of their epidemiology, which will further provide useful information to establish effective control programs and management of the diseases. In the present study, thin blood smear microscopic examination and PCR-based molecular detection tools were used for the first time to detect *Babesia bigemina* in the blood collected from water buffalo in a previously uncharacterized southeastern regions of Pakistan.

A higher infection frequency of *Babesia* infections in water buffaloes indicate a situation of stable endemicity, which can only be controlled through early and accurate diagnosis. Recently developed several molecular and serological methods along with conventional traditional methods have enabled quick and accurate diagnosis of an infection, which can be beneficial to control and treat *Babesia* infection in an endemic area. The PCR-based molecular detection method is now commonly used for the detection of several parasites but it needs specific primers and optimization of PCR assay. The accuracy and specificity of the primer pairs used in this study to detect *Babesia bigemina* were determined by successful amplification of a 170 bp product, as observed by gel electrophoresis from the positive samples collected from buffaloes. In contrast, no PCR amplification was observed on the samples that were negative for the infection, as observed during blood smear examination. The successful amplification of particular *Babesia bigemina* products shows that the primers used in this assay are specific for *Babesia bigemina* DNA. PCR assay used in this study proved to be highly sensitive, and can be simultaneously used for detection of *Babesia bigemina* and differentiation of *Babesia* species. In the present study, it was observed that a total of 20% of animals were infected with *Babesia bigemina* when the samples were examined by thin blood smear microscopic examination, while only 17% were found positive when analyzed using PCR-based DNA amplification method. Indeed, the PCR assay can detect small amount of parasites in the sample, however, the present study demonstrated that more positive samples were detected by thin blood smear microscopic examination than PCR method. Even more, some samples found positive on blood smear proved to be negative at PCR detection, which may be the result of inaccuracy of blood smear diagnostic method to differentiate several species of *Babesia*, sometimes even mistakenly confused with other piroplasms. However, subspecies identification is impossible by blood smear examination. Furthermore, the highest prevalence was found in tick-infested animals (36% and 30%) using both blood smear and PCR methods, respectively. On the other hand, the lowest prevalence was found in tick-free animals where no tick was found to be attached to the skin of the animal. Our findings indicate that bovine babesiosis can persist only in the endemic areas with significant number of vectors present that can serve as a source for disease transmission.

The higher prevalence rate detected by blood smear method as compared to PCR method may be attributed to the presence of *Babesia* species other than *B. bigemina*. However, according to the morphological characteristics we are unable to accurately differentiate *Babesia* species, indicating the need for molecular approaches to detect...
and accurately differentiate the prevalent species in a population.

Our results are comparable to the earlier epidemiological studies conducted to determine the prevalence of Babesia species in the Indo-Pak region. For instance, the overall prevalence of Babesia bovis and Babesia bigemina in cattle and buffaloes observed in northwestern parts of Pakistan indicated a total of 24% prevalence rate when samples were examined by using thin blood smear method, and 37% by PCR. 11% samples were found positive for Babesia bovis, and 21% were infected with Babesia bigemina. At the same time, in 5% of the cases, both species caused the infection at the same time (Saad et al., 2015). Similarly, Bal et al., (2016) found overall 56% and 30% Babesia bigemina infection rate in cattle using PCR and blood smear method, respectively, in the blood samples collected from western parts of India. The differences in the infection rate in different animal groups and geographic locations may be attributed to the distribution of vectors. Also evident from the present study is that several factors including environmental factors, farm management and the origin of the animals are considered risk factors given that all animals brought to the cattle colony originate from different regions, which may serve as an infection source during sampling time.

Continuous and stable presence of Babesia infection in an area is characterized by the presence and successful reproduction of the ticks over time. However, slight fluctuation in the prevalence might be attributed to the environmental factors and vector-control practices. Therefore, in our study, the presence of Babesia infection is also related with the presence of the transmitting vectors. It has been observed that the ticks are widely distributed in different ecological and geographical regions of Pakistan including northern parts characterized with very low temperature during many months, and southeastern parts characterized with very high temperature during summer. Moreover, previous data showed the presence of Rhipicephalus species in most parts of Pakistan, which may be associated with the Babesia infection (Karim et al., 2017).

This is the first epidemiological study investigating the prevalence of Babesia bigemina infection in southeastern parts of Pakistan using both, conventional blood smear method and PCR-based DNA amplification method. Therefore, these results can serve as a base for further research, and prove itself beneficial for the management and control programs of the Babesia infection.

References

Prevalence and molecular detection of Babesia bigemina in water buffalo from southeastern region of Pakistan —30/30


Prevalenca i molekularna detekcija Babesia bigemina kod vodenog bivola na jugoistoku Pakistana

Sažetak

Babesia spp. je apikompleksni parazit kojeg prenose krpelji, a koji inficira eritrocite velikog broja kičmenjaka uključujući domaće životinje. Izaziva značajne ekonomske gubitke izazvane uvođenjem profilaktičkih mjera, smanjenjem proizvodnje i stopama morbidity i mortalitet. Za preciznu dijagnostiku i prevenciju bolesti potrebno je koristiti odgovarajući test. Cilj ovog istraživanja jeste otkriti slučajeve infekcije sa Babesia bigemina kod vodenih bivola na jugoistoku Pakistana koristeći tradicionalnu tehniku krvnog razmaza i molekularne tehnike. Prikupljeno je ukupno 100 krvnih uzoraka, 50 od životinja infestiranih krpeljima, a 50 od neinfestiranih. Ukupna prevalenca dobivena metodom tankog krvnog razmaza iznosi 20%, a PCR metodom 17%. Nadalje, analiza infestiranih i neinfestiranih životinja je koristeći metodu krvnog razmaza pokazala stopu infekcije od 36% kod infestiranih i 4% kod neinfestiranih životinja, dok je PCR metoda pokazala stopu od 30% kod infestiranih i 4% kod neinfestiranih životinja. Uprkos višoj prevalencii zabilježenoj metodom krvnog razmaza, a što se može objasniti nespecifičnom identifikacijom Babesia spp, rezultati pokazuju da je PCR test korišten u ovoj studiji korisna metoda za precizno dijagnosticiranje infekcije goveda sa B. bigemina.

Ključne riječi: Babesia bigemina, dijagnoza, PCR, prevalenca, tehnika krvnog razmaza