Phylogenetic Analysis of Local Isolate of Canine Babesiosis

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Abstract

Canine Babesiosis is a very important parasitic disease of dogs caused by an intra-cellular protozoan called as Babesia. Different species of Babesia are involved in this infection. Microscopy has been used as a diagnostic tool since long time ago for the diagnosis of blood parasites. Microscopy cannot really help us to differentiate among different species of Babesia. For this purpose, techniques of molecular parasitology can really help us. One of such techniques is PCR i.e. Polymerase Chain Reaction. The aim of current study was to develop PCR for the diagnosis of canine babesiosis so that different species of Babesia causing canine babesiosis can be differentiated. The first step in this direction was to detect genus Babesia. For this purpose, genus specific primers were designed and were used in an attempt to detect genome of Babesia in blood samples found positive through microscopic examination i.e. the blood samples containing intra-erythrocytic bodies. With these genus specific primers, we were able to amplify a PCR product of less than 0.2kb in size. The sequencing of these amplicons followed by their phylogenetic analyses gives us the information about the species of Babesia present in Pakistan.

Keywords: Canine babesiosis, Molecular characterization, Phylogeny

Introduction:

Canine babesiosis; an important tick-borne infectious disease of dogs, has been described an emerging veterinary problem worldwide (Irwin 2009). Victor Babes in 1888 described the first intra-erythrocytic piroplasms in cattle in Romania (Hunfled et al. 2008).This disease is caused by an intra-erythrocytic protozoan parasite of genus babesia. After trypanosomes, babesia is thought to be the second most common blood parasites that have a major impact on health of domestic animals in areas without severe winters (Telford et al. 1993). Abdulssalam (1959) reported that the Indo-Pakistan Sub-continent is rich in tick's manifestation due to variation in topography and climatic condition.

Transmission of babesia parasite to the canine host is by the bite of an Ixodid tick that acts as a biological vector. More than 100 species of babesia exist, off the species affecting dogs, three –

Babesia canis (B.canis), B.gibsoni and *B.vogeli*- are greatly assigned in nature and of great concern in tropical and subtropical regions of the world (Amuta et al. 2010).

B.canis is regarded as more threatening in dogs as compared to other species (Ahmed et al. 2011). *B.canis* was described in dogs in Italy by Piana and Galli-valerio (Durbaum et al. 2000). Historically, *babesia* infection in dogs was identified on the basis of morphologic appearance of the parasite within the erythrocytes. Canine babesial organisms are morphologically classified into large and small forms, both exhibiting a worldwide distribution. *Babesia canis* and another novel, as yet unnamed babesia spp. detected in the USA (large babesia) and *B.gibsoni* and *B.annae* (small babesia) have been documented to infect dogs (Birkenheuer et al. 2004). All large forms of *Babesia*were designated *B.canis* (4-5µm*)*, whereas all small forms of *babesia* were considered to be *B.gibsoni* (1-2.5µm) (Salona et al. 2011).

Tick species are the vectors of babesia (Bock et al. 2006). *Rhipicephalus sanguineous* (Brown Dog Tick) is the principal vector that picks up the trophozoites of babesia. These trophozoites multiply by binary fission, producing piriform bodies and induce antibody–mediated cytotoxic destruction of RBC's leading to remittent fever, progressive anemia, jaundice and haemoglobinuria (Red water) and in some cases death (Kubelova et al., 2011). Cerebral babesiosis is uncommon but carries a poor prognosis (Mathe A et al. 2006). Other ticks like *Hyalomma, Demacentor* can also spread this infection. This parasite cannot live outside the dogs or tick vector (Ahmed et al. 2011). Mortality due to Babesiosis is extremely variable and may be upto 50% or higher .The incident rate of canine Babesiosis is more during the month from June to September (anonymous 2004). Prevalence of Babesiosis has been reported by different researchers from 13% to 20.4% in canines (Cabannes et al. 2002; Ahmad et al. 2007), variations depending upon factors like age, breed, season and activity of ticks.

Usually, the diagnosis is made upon size and morphological appearance of the intra-erythrocytic forms in peripheral blood smears, stained with Giemsa stain in acute cases. Serology for identification of carrier state lacks specificity and sensitivity (Mahmoud et al. 2008; Iseki et al. 2010). The most powerful tool for the diagnosis of Babesiosis is DNA amplification test which are more reliable method than others (Aktas et al. 2005). Despite the importance of tick infestation and babesiosis in domestic animals, very less study has been done on the molecular level in Pakistan.

This study was aimed to determine the comparison of Molecular and Light microscopy diagnosis, Optimization of molecular diagnosis for detection at genus level followed by phylogenetic analysis.

Materials and methods:

Collection of Blood Samples

Blood sample were collected in anti-coagulant (EDTA) tubes. The blood sample was brought to the Department of Parasitology from Pet Centre. Each blood sample was subjected to microscopic examination. The positive samples were immediately subjected to DNA extraction by using commercial kit (TIANamp Blood DNA Kit, TIANGEN).

Primer design:

A pair of oligonucleotides was designed for targeting the 18S ribosomal RNA gene amplification by means of PCR.

Babesia-Forward	AACTGTGAGCGACTCAATGG
Babesia-Reverse	CTGACCATGAGTAGACATTCA

DNA Amplification:

Amplification was performed on T100 thermal cycler (BIO-RAD, Singapore). For the primary round of amplification, an initial denaturation step at 95^0C for 10 min, followed by 35 cycles of denaturation at 95^0C for 30s, annealing at 56^0C for 30s and extension at 72^0C for 30s.Final extension was done at 72 ^{0}C for 5 min followed by a hold step at 4^0C. Amplified DNA was subjected to gel electrophoresis for visual examination.

Results:

DNA alignment was done by using MegaAlign Software of Lasergene DNAstar. A consensus sequence was found after alignment of DNA sequences of 18S ribosomal RNA of the babesia spp. causing Canine Babesiosis by using EditSeq software of Lasergene DNAstar.

The PCR was standardized by changing the concentration of DNA template, dNTPs and primers. A 193 bp band was obtained when pcr product was electrophoroesed at 2 % agarose gel. This band indicated the positivity of genus babesia spp. causing Canine Babesiosis. These species included were Babesia canis, Babesia canis canis, Babesia canis vogeli, Babesia gibsoni.

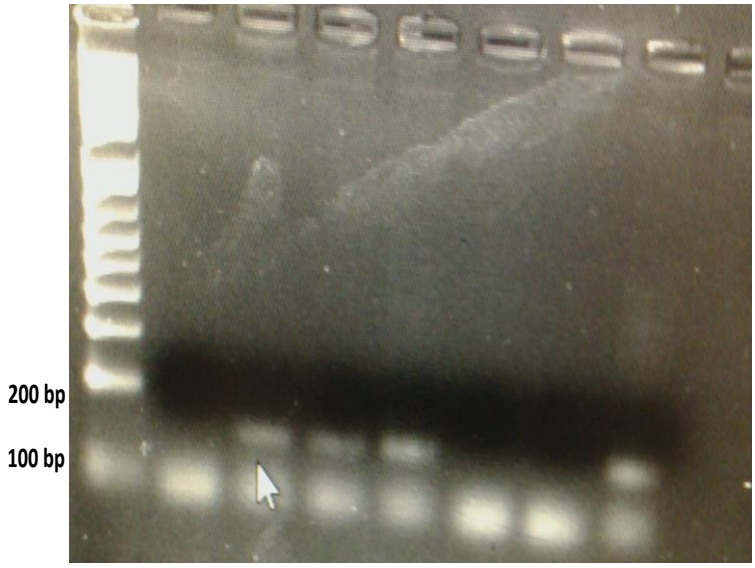

Figure 1: DNA amplified band of slightly less than 0.2kb obtained by PCR

Phylogenetic analysis:

The samples found positive on PCR were selected for sequencing. The DNA was extracted from the gel after running the positive samples in the electrophoresis and was sent to the Biosciences,…… for sequencing. The sequences obtained were compared using DNA star lasergene software with a bootstrap of 1,000 replications and evolutionary distances adjusted. The phylogenetic tree obtained is shown in figure 2.

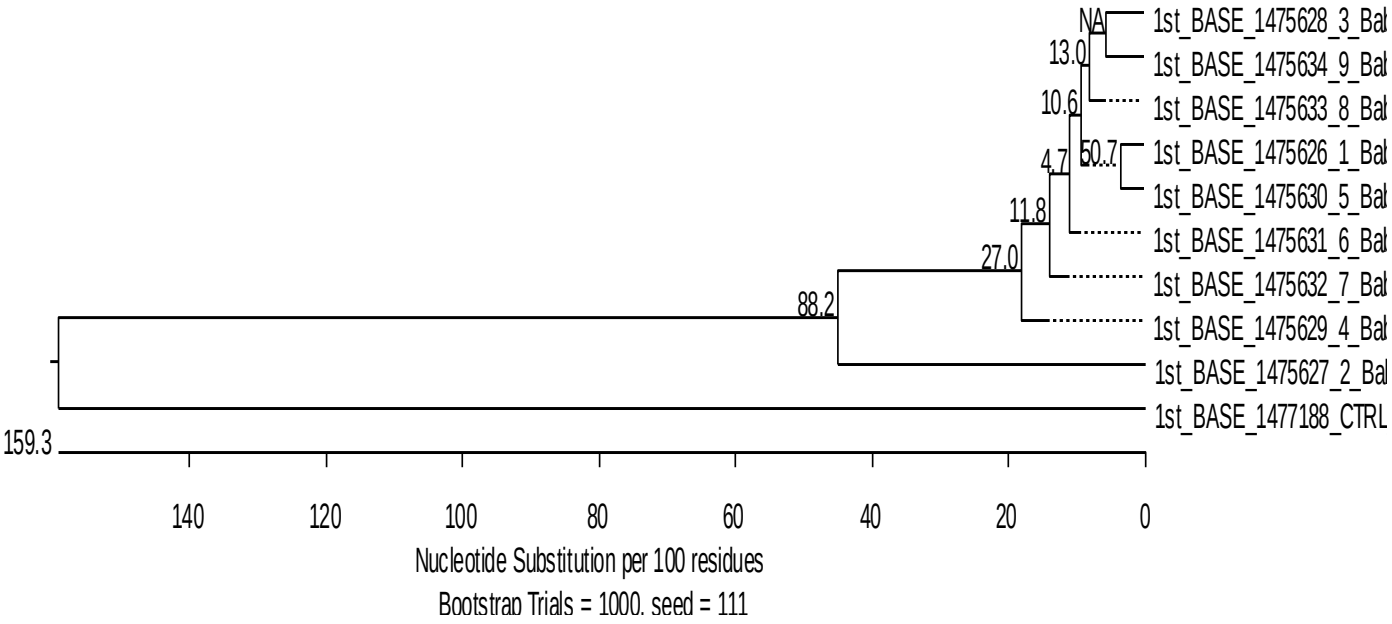

Figure 2: Phylogenetic tree

Discussion:

The diagnosis based on thin smear microscopy of blood has several disadvantages not only in differentiating intra-erythrocytic bodies but also the difficulties in differentiating different babesia spp. causing canine babesiosis. The PCR is more sensitive and specific, hence being the only confirmatory diagnostic test currently possible in Pakistan for the diagnosis of canine babesiosis. For the molecular differentiation of these species, it requires multi-plex PCR which has several limitations regarding problems with primer designing or their dimer formation. The aim of this study was to develop PCR test able to diagnose canine babesiosis in a single step

followed by sequencing of the amplified products to perform phylogenetic analyses in order to find out any sequence variations in the form of insertions, deletions or mutations related to the local geographical conditions. For this purpose, special primers were designed for the detection of babesia genus. At first, 18S rRNA sequences were aligned using online MegaAlign software and then a consensus sequence was developed by using online available software i.e. EditSeq software of Lasergene DNAstar followed by designing of special primers ableto detect babesia genus that included *Babesia canis canis, Babesis canis vogeli and Babesia gibsoni.* It is the first study of this type in Pakistan, destined to diagnose one of the most pathogenic blood protozoans i.e. Babesia in canines using advanced molecular technique i.e. PCR.

PCR technique with aim of designing primers for the genus babesia causing canine babesiosis has never been employed in Pakistan. Molecular confirmation will help us to differentiate between drug-resistant strains of babesia as well as other organsisms causing intra-erythrocytic bodies.

The PCR-mediated confirmation of Babesia genome in the DNA samples extracted from dog blood samples is not only useful from diagnostic point of view but is also very useful on the way of a very diverse type of studies e.g. the study of genes coding different antigenic proteins of Babesia, genetic studies about antigenic variations among different strains of babesia, studies about geographic markers in genome of babesia like in other parasites as Pneumocystis (Akbar et al. 2012). Identification of these different genes coding antigenic proteins would help us to move forward in the direction of recombinant protein productions of different Babesia antigens. This will pave the way for the development of perhaps more effective vaccines comprising recombinant proteins of Babesia in near future Insha Allah.

Conclusion:

The development of molecular diagnostic technique for the diagnosis of a highly pathogenic blood protozoan i.e. babesia help us a lot to improve our diagnostic abilities thus contributing a lot towards improvement in animal health. As babesia is a parasite that can infect a large number of animals including cattle, buffalo, sheep, goat, horses, cats and a wide variety of wild animals, the development of this molecular diagnostic technique i.e. PCR in our laboratory help us to conduct further studies using this technique in other animal hosts and its tick vectors. This also helps us to know more about the circulation of the parasite in our local environment.

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