Detection of *Babesia ovis* Using Polymerase Chain Reaction

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Summary

A procedure was developed for detection of *Babesia ovis* (*B.ovis*) infection in blood samples containing infected erythrocytes by polymerase chain reaction (PCR). In order to detect the DNA parasite in blood sample two sets of oligonucleotides were designed according to the nucleotide sequences of the 18S rRNA and ATP-binding protein genes of *B.ovis*. These genes were amplified at 286 and 453bp fragments, respectively. Samples from eight isolates of experimentally infected lambs to *B.ovis* were analyzed. PCR analysis showed that all of the samples were infected by *B.ovis*. Sequence determination and analysis of amplified fragments confirmed specificity of the PCR. The PCR was sensitive enough to detect parasite DNA from 5µl of blood samples with a parasitemia of 0.000005%. These results suggest that the PCR-based diagnostic assay for *B.ovis* is highly specific and sensitive.

Key words: *Babesia ovis*, PCR, 18S rRNA, ATP-binding protein

Introduction

Babesiosis, caused by infection with intraerythrocytic parasites of the genus *Babesia*, is a well-recognized disease of veterinary importance (Duh 2003). *Babesia ovis* (*B.ovis*) is one of the most pathogenic protozoa and babesiosis has been considered as a major problem to efficient sheep and goats production in Iran (Hashemi-Fesharki 1997). Epidemiological studies have been done to show the prevalence of *Babesia sp.* in small ruminants in Iran (Hashemi-Fesharki 1991 and 1997, Razmi 2002).

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Babesia sp. is identify based on morphological parameters of the intraerythrocytic forms visible in stained blood smears from infected animal. However, there are several copmlications with this identification. First, different parasites in the same hosts may be morphologically similar (e.g., *Plasmodium* and some *Babesia* sp.). The second complicating factor is that the same parasite may has different microscopic appearances in different hosts. The third is that the classification of *Babesia* sp. on the basis of host specificity appears to be less useful than once thought, since certain extensively studied species such as *B. microti* have been shown to have a broad host specificity (Brandt *et al* 1977, Etkind *et al* 1980, Moore & Kuntz 1981, Spielman 1981). The difficalties associated with conventional detecting methods for *Babesia* sp. Developed a new technique based on comparison of the nucleic acid sequences that is more objective than visible characteristics (Persing & Conrad 1995) and is independent of the host's immune response schedule. Therefore, much earlier detection of the parasite is possible. PCR and other DNA based methods now applicable to detection of *Babesia* sp. (Allsopp *et al* 1994, Ellis *et al* 1992, Fahrimal *et al* 1992, Figueroa *et al* 1992, Persing 1992, Reddy & Dame 1992, Carret *et al* 1999, Conrad *et al* 1992, Hitoshi *et al* 2001). However, no approach has been applied on the use of PCR for diagnosis of ovine babesiosis in Iran. In this study, we used PCR to detect the 18S ribosomal RNA and ATP-binding protein genes of *B.ovis* from sheep blood DNA.

Material and Methods

Parasite. Splenectomized lambs were experimentally infected with a local srain of *B.ovis.* Blood samples were collected at peak of parasitemia and preserved in cryopreservative media at -70°C in our laboratory. Babesiosis due to the stablished isolate of *B.ovis* was diagnosed and confirmed on the basis of noting parasite inclusions in erythrocytes on peripheral blood smears and occurrence of marked clinical symptoms.

DNA extraction. DNA was obtained from 1000µl of whole blood and treated by standard proteinase K digestion and phenol/chloroform extraction (Sambrook *et al* 1989). Briefly, blood was mixed with 0.8ml of 1Xsodium salt buffer complete containing 0.5% sodium dodecyl sulfate (Merck, Germany) and 5µl proteinase K (20mg/ml) (Fermentas, Germany) and incubated for an hour at 55°C. After incubation, 120µl of phenol/chloroform/isoamyl alcohol (CinnaGen, Iran) was added and spun at 12000rpm for 2min. DNA was precipitated from aqueous phase by ethanol (Flucka, Germany). After washing and drying the pellet, DNA was resuspended by adding 200µl of TE buffer and used for PCR.

PCR procedure. The primers Babl (5'-GAC CTA AAC CCT CAC CAG AG) and Bab 2 (3'-CCT TAG TAA TGG TTA ATA GGA ACG) (TIB Molbiol, Germany and CinnaGen, Iran), which amplify fragment 286 base pair (bp) nucleotides in the small subunit of 18S ribosomal RNA gene of *Babesia* sp. and, the primers Bab 3 (5'-GCT CAA AAC ACA CCT GGT CG) and Bab 4 (3'-GCG ACG AAT CCT TGT GGC AC) (TIB Molbiol, Germany and CinnaGen, Iran), which amplify fragment 453 nucleotides in the ATP-binding protein of *B.ovis*. These primers were designed according to the nucleotide sequence in data bank (GenBank accession no. AF373333 and U44919). PCR was performed in 20µl of a mixture containing about 1µg of genomic DNA, 20pM of each primer, 200µM of dNTPs (Fermentas, Germany) and 0.5U *Taq* DNA polymerase (CinnaGen, Iran) in 1X PCR buffer (10mM Tris-HCl pH8.3, 1.5mM $MgCl_2$ and 50mM KCl). PCR amplification was performed using a programmable thermal cycler (Techgene, Techne, UK) following with the conditions: after denaturation at 94°C for 2min; 35 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1min and extension at 72°C for 1min followed by a final extension at 72°C for 5min. The amplified PCR products were checked on 2% agarose (Roche, Germany) gel electrophoresis and stained with ethidium bromide (Roche, Germany). Positive and negative controls were included in all tests.

Results

PCR amplification and DNA sequencing of 18S rRNA and ATP-binding protein genes of *B.ovis* were identified in the blood specimens of experimentally infected sheep. From a *Babesia* sp. gene sequence coding for an 18S ribosomal RNA and *B.ovis* gene sequence coding for ATP-binding protein, two sets of primers were designed for the PCR assays. Based on the PCR assays, all of the eight isolates of experimentally infected lamb blood samples showed the predicted PCR fragments sizes for *B.ovis* in presence of the two primer pairs, 286 and 453bp for genus and species specific respectively. The results of electrophoresis are shown in figure 1.

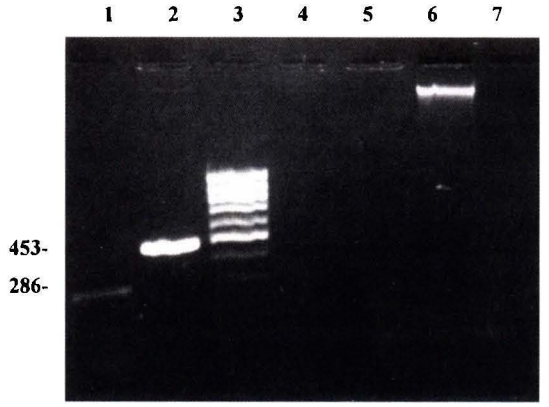

Figure 1. *Specificity of the PCR method. Ethidium bromide stained agarose gel electrophoresis of the PCR products from Babesia genus (lane 1), Babesia ovis (lane 2), 100bp DNA ladder marker (lane 3), Leishmania major (lane 4). Theileria annulata (lane 5), normal sheep blood DNA (lane 6) and negative control (lane 7)*

Specificity of the PCR. To confirm the specificity of the PCR method, the PCR products were sequenced. Results of the nucleotide homology searches conducted using the "Blast n". Analy[illegible] sequenced PCR products showed very high degree of identity (99%) to *B.ovis* ATP-binding pro[illegible] U44919) and (98%) to *B.ovis* 18S rRNA (AF373333) genes. As shown in figure 1 the expected fragments were amplified only from *B.ovis* DNA. No DNA was amplified from uninfected blood, nor from other hemoparasites such as *Theileria annulata* or *Leishmania major*.

Sensitivity of the PCR. *B.ovis*-infected erythrocytes with 5% parasitemia were subjected to 10-fold serial dilutions using normal sheep erythrocytes, and DNA was extracted from each diluted sample for testing the sensitivity of the PCR method. As shown in figure 2 detection limit of the sample at the lowest parasitemia was estimated as 0.000005%.

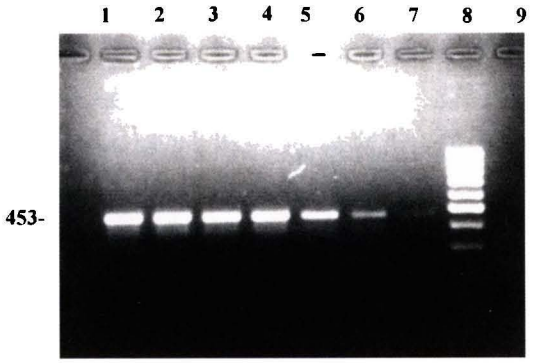

Figure 2. *Sensitivity of the PCR method. Ethidium bromide stained agarose gel electrophoresis of the PCR products from 10-fold serial diluted samples. Lanes 1-7, dilutions of 10-1 to 10-7 with 5% parasitemia, lane 8, 100bp DNA ladder marker and lane 9, negative control*

Nucleotide sequence GenBank accession numbers. The determined sequences for 18S rRNA (286bp) and ATP-binding protein (400bp) were sequensed by SEQLAB (Sequence Laboratories Gttingen GmbH) and deposited in GenBank and may be accessed under accession no. AY362829 and AY383087.

Discussion

Generally, *Babesia* sp. is identified by demonstrating the organisms in blood smears under light microscope, serological examination and inoculation to splenectomized or laboratory animals (Conrad 1991). Recently, molecular techniques have been used for detection and identification of protozoa in different parts of the world. The genomic and extra chromosomal DNA analysis using PCR, RFLP-PCR and reverse line blot hybridization have been carried out successfully on the species. Caccio *et al*

(2000) found the beta-tubulin as an informative marker for species discriminative for *Babesia*. Carret *et al* (1999) differentiated the three subspecies of *B.canis* by RFLP analysis an amplifieds small subunit ribosomal RNA gene. Gubbles *et al* (1999) detected *Babesia* sp. simultaneously using reverse line blot hybridization. Salem *et al* (1999) used extra chromosomal DNA-based PCR test for diagnosing bovine babesiosis. Kjemtrup *et al* (2000) used 18S nuclear small subunit RNA gene in phylogenetic relationships of human and wildlife piroplasm isolates. We have focsed on 18S rRNA and ATP- binding protein genes. 18S rRNA gene is very conserved among the various piroplasms (Kjemtrup *et al* 2000), thus is suitable for using it as a genus specific gene (GenBank accession no. AF37333). ATP-binding protein was studied as species specific gene to detect *B.ovis* (GenBank accession no. U44919).

In another study on European ticks Duh *et al* (2001) used tick DNA for demonstrating the *Babesia* parasites with a PCR assay based on the nuclear small subunit rRNA gene. They collected adults and nymphal ticks in various parts of Slovenia and tested them for the presence of babesial parasites. The results revealed the genetic evidence of *B.microti* and *B.divergence*-like parasites in *Ixodes ricinus* ticks in Europe.

Hashemi-Fesharki (1997) and Razmi *et al* (2002) suggested that ovine babesiosis is caused most often by *B.ovis* and less frequently by *B.motasi* in Iran. Razmi *et al* (2002) in an epidemiological study showed that the infection rate for *B.ovis* was 24.6%. Here, we have evaluated the potential of using the combination of two genus and species specific genes as a molecular diagnostic approach. This study has permitted us to optimize our technique to test whether *Babesia* genus is present in the specimen and whether *B.ovis* is the infectious agent. Therefore, we used the primer sequences designed for *Babesia* sp. and *B.ovis* based on the published sequences. In this basic study for standardizing the method we used previously confirmed and established infected blood samples with *B.ovis* to demonstrate the sensitivity and specificity of the test. This report describes an efficient and rather

simple method for detecting and diagnosing of *B.ovis* infection. The procedure is adopted for routine usage due to its simplicity. According to the DNA amounts used in each assay, the PCR was sensitive to detect DNA from 5µl of blood sample with a parasitemia of 0.000005%. These finding are similar to those reported by Fukumoto *et al* (2001) who used the P18 gene to detect parasite DNA from blood samples of *B.gibsoni*-infected dogs by PCR. They found PCR was sensitive enough to detect parasite DNA from 2.5µl of blood samples with a parasitemia of 0.000002%.

In this study, no labeling and digestion of analyzed material is needed. Such analysis can be carried out directly from gel of the DNA and visualized with ethidium bromide staining. The experiments presented in this paper suggest that the DNA can be easily amplified by enzymatic polymerization. Oligonucleotide sequencing provides an efficient method for the identification and analysis of amplified product. Sequencing of the products was performed to confirm the specificity of the PCR. A comparison with the *B.ovis* sequence (GenBank accession no. U44919) revealed 99% identity. With this, we could confirm specific amplification from a minimal amount of infected blood sample. The results of nucleotide homology analysis demonstrated the presence of conserved regions in studied *B.ovis* gene sequences.

Here, we have shown that the specificity and high sensitivity of the PCR assay for the detection of *B.ovis* infection provide a valuable tool to apply in conducting molecular epizootiological studies. From our results, this PCR approach is useful to find the infection of bebesiasis in sheep with low parasitemia including carriers because of its high sensitivity. In conclusion our results represents the first study in Iran in which we developed a simple method to detect *B.ovis* infection in blood samples by PCR and subsequent sequence analysis.

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