Research Note

Braz. J. Vet. Parasitol., Jaboticabal, v. 23, n. 1, p. 105-108, Jan-Mar 2014
ISSN 0103-846X (Print) / ISSN 1984-2961 (Electronic)

Optimization of a molecular method for the diagnosis of canine babesiosis

Otimização de método molecular para diagnóstico da babesiose canina

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Received August 21, 2013
Accepted November 12, 2013

Abstract

Babesiosis is a hemolytic disease caused by protozoans of the genus *Babesia* (Apicomplexa). This disease occurs worldwide and is transmitted by ticks to a variety of mammals, including humans. The objective of the present study was to optimize a molecular approach for the detection of a fragment of 18S rDNA of *Babesia canis*, *Babesia vogeli*, *Babesia rossi* or *Babesia gibsoni* based on a single semi-nested Polymerase Chain Reaction (PCR), and compare the efficiency of this approach with that of a simple PCR protocol. To this end, 100 blood samples collected from dogs with suspected hemoparasite infections were analyzed. A comparison of the results of simple PCR and semi-nested PCR indicated a highly significant difference (p value = 0.0000). While only five (5%) of the samples tested positive using the simple protocol, 22 (22%) were positive using the snPCR technique. The results of this study reinforce the findings of previous studies, which have demonstrated the greater sensitivity of tests based on nested or semi-nested PCR. Therefore, to avoid false-negative results due to low levels of parasitemia, we suggest the preferential use of this protocol in epidemiological studies of canine babesiosis, particularly those that require reliable estimates of the prevalence of infection.

Keywords: *Babesia*, molecular diagnosis, semi-nested PCR, eastern Amazonia.

Resumo

A babesiose é uma doença hemolítica de ocorrência mundial, causada por protozoários do gênero *Babesia* (Apicomplexa), que são transmitidos por carrapatos a diversos mamíferos, incluindo o homem. O objetivo deste estudo foi otimizar um método molecular para a detecção de fragmento do 18S rDNA de *Babesia canis*, *Babesia vogeli*, *Babesia rossi* ou *Babesia gibsoni* com base em uma única semi-*nested* (snPCR), comparando sua eficiência com um protocolo de PCR simples. Para isso, 100 amostras de sangue de cães com suspeita de hemoparasitoses foram analisadas e, enquanto o protocolo de PCR simples indicou somente 5% (5/100) de amostras positivas, o protocolo de snPCR, com 22% (22/100) de amostras positivas, apresentou maior sensibilidade (p valor = 0,0000). Este resultado está de acordo com outros estudos que mostram a maior sensibilidade de detecção dos testes baseado em *nested* ou snPCR. Assim, como uma forma de prevenir resultados falso-negativos devido à baixa parasitemia, sugere-se que este protocolo seja preferencialmente usado nos estudos epidemiológicos de babesiose canina, em especial naqueles que tratam da sua prevalência.

Palavras-chave: *Babesia*, diagnóstico molecular, *semi-nested* PCR, Amazônia oriental.

Introduction

Babesiosis is a hemolytic disease caused by protozoans of the genus *Babesia* (Apicomplexa). This disease occurs worldwide and is transmitted to a range of different mammal species (BOOZER; MACINTIRE, 2003). Typical clinical signs include fever, depression, and anemia (PASSOS et al., 2005). Four protozoan species – *Babesia canis*, *Babesia vogeli*, *Babesia rossi* and *Babesia gibsoni* – are known to cause canine babesiosis or piroplasmosis. The first three species are recognized based on the vector (tick) species, virulence, and geographic distribution. *Babesia canis* is transmitted by *Dermacentor reticularis*, a tick species found in Europe (BOOZER; MACINTIRE, 2003), while the vector of *Babesia vogeli* is the red tick, *Rhipicephalus sanguineus*, which is found in the United States and in tropical and subtropical regions

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around the world, including Brazil (PASSOS et al., 2005). *Babesia rossi*, the most virulent of the three species, is transmitted by *Haemaphysalis leachi*, a tick found in South Africa (LOBETTI, 1998). In Brazil, canine babesiosis is typically caused by *B. vogeli*, although there are some reports of *B. gibsoni* in the south of the country (JOJIMA et al., 2008).

Procedures used for the diagnosis of babesiosis include direct detection, which is based on the presence of merozoites in the red cells of blood smears stained with Giemsa, Field's or Wright's solutions. This is a specific approach, which is adequate for the acute phase of the disease, although it has low sensitivity and certain limitations, given that the characteristics and duration of the parasitemia may vary considerably (GREENE, 2006). Indirect diagnosis is based on serological tests such as the Indirect Immunofluorescence Reaction (IIR), which is relatively sensitive but of low specificity due to cross reactions among the different *Babesia* species (VIDOTTO; MANDUCA, 2004). Diagnosis by IIR may also be ineffective in the early phase of the infection, when antibody concentrations may be too low to be detected. Both direct and indirect approaches thus present certain limitations, which may hamper diagnosis and lead to either false-negative or false-positive results.

Molecular tests, particularly those based on the Polymerase Chain Reaction (PCR), appear to represent a promising tool for the diagnosis of many parasitic diseases (GASSER, 2006), given that they are both sensitive and specific (BÖSE et al., 1995). The sensitivity of this approach can be further enhanced by the application of nested (nPCR) or semi-nested (snPCR) techniques, which can be especially useful when parasitemia is low. Birkenheuer et al. (2003) recommended a semi-nested PCR approach for the amplification of DNA fragments from *B. gibsoni*, *B. canis*, *B. vogeli*, and *B. rossi*, with a specific snPCR protocol for each taxon. The objective of the present study was to optimize a molecular method for the detection of the DNA of these four *Babesia* species based on a single snPCR.

Materials and Methods

One hundred samples were selected randomly from those collected from dogs with clinical signs of hemoparasitosis treated at the Veterinary Hospital of the Federal Rural University of Amazonia (HOVET-UFRA), Belém (Pará State) between August and October 2011. The blood samples were drawn into tubes containing EDTA.

The total DNA of each sample was extracted from a 300 µl aliquot of the blood using the standard phenol-chloroform procedure described by Sambrook et al. (1989). DNA quality control was checked by electrophoresis in agarose gel, followed by quantification using a Qubit fluorometer (Invitrogen). The molecular diagnosis was based on the PCR protocol proposed by Duarte et al. (2008), which involves the amplification of a segment of the 28S rDNA gene. During the present study, a snPCR protocol was optimized based on the simple PCR protocol developed by Kordick et al. (1999) and Martin et al. (2006), which amplifies a fragment of the 18S rDNA gene of the *Babesia* species. This involved conducting an initial reaction in a total volume of 25 µL containing 10-20 ng of the DNA template, 1.5 mM of $MgCl_2$, 2.5 mM of each dNTP, 10 mM of Tris-HCl, 50 mM of KCl, 5 µM of each primer (*Bab-f* [MARTIN et al., 2006] + *Babesia* common [KORDICK et al., 1999]), and 1 U of *Taq* DNA polymerase (Invitrogen). The second reaction was also conducted in a total volume of 25 µL, containing 1 µL of the product of the first reaction, 1.5 mM of $MgCl_2$, 2.5 mM of each dNTP, 10 mM of Tris-HCl, 50 mM of KCl, 5 µM of each primer (*B. canis*/*Babesia* common [KORDICK et al., 1999]), and 1 U of *Taq* DNA polymerase (Invitrogen). The amplification protocol of both reactions consisted of 35 cycles of 1 minute at 94 °C, 2 minutes at 65 °C, and 1 minute at 72 °C, preceded by 3 minutes at 94 °C and followed by 5 minutes at 72 °C. The DNA of peripheral blood sample from one dog (from Belém) treated at HOVET-UFRA and showing large intraerythrocytic piroplasms morphologically compatible with *Babesia vogeli*, which was examined by light microscopy after panoptic staining, was used as the positive control, while sterile bi-distilled water was used as the negative control.

All the PCR products were visualized after electrophoresis in 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer, using GelRed™ Nucleic Acid stain (Biotium) and an ultraviolet transilluminator. The samples that produced fragments of 590 bps for the BAB1/BAB4 primers (DUARTE et al., 2008) and 394 and 208 bps for the first and second reactions of the snPCR, respectively, were considered to be positive. A 100-bp molecular marker (Invitrogen DNA ladder) was used to estimate the size of each amplified fragment.

The amplicon from the positive control sample was purified with a GFX PCR DNA and gel purification kit (GE Healthcare) and ligated to the pGEM-T vector plasmid (Promega), which was then inserted into TOP 10 *Escherichia coli* (Invitrogen Life Technologies). The cloned fragment was obtained by PCR from the recombinant clones of the colonies using the M13F/M13R primers and sequenced automatically in a 3500xl Genetic Analyzer (Applied Biosystems), according to the manufacturer's specifications. The BioEdit program (HALL, 1999) was used to align the forward and reverse sequences with AY072925, obtained from Genbank (NCBI).

Sensitivities of simple and semi-nested PCR for the detection of *Babesia vogeli* were compared by McNemar's test using BioEstat 5.3 software (AYRES et al., 2007).

Results and Discussion

All the DNA samples showed high purity and integrity. The positivity of the control was confirmed based on a comparison of the nucleotide sequence of the fragment amplified with the *Bab-fl Babesia* common primers with sequence AY072925 obtained from Genbank (NCBI - www.ncbi.nlm.nih.gov).

Martin et al. (2006) used the BLAST program and observed that the primer *Bab-f* is highly specific to *Babesia* genus and also observed 100% specificity and sensitivity with results that were reproducible in later experiments, involving both sets of primers individually. Therefore, we simply checked the specificity of the protocol proposed here by obtain the sequence (GenBank accession

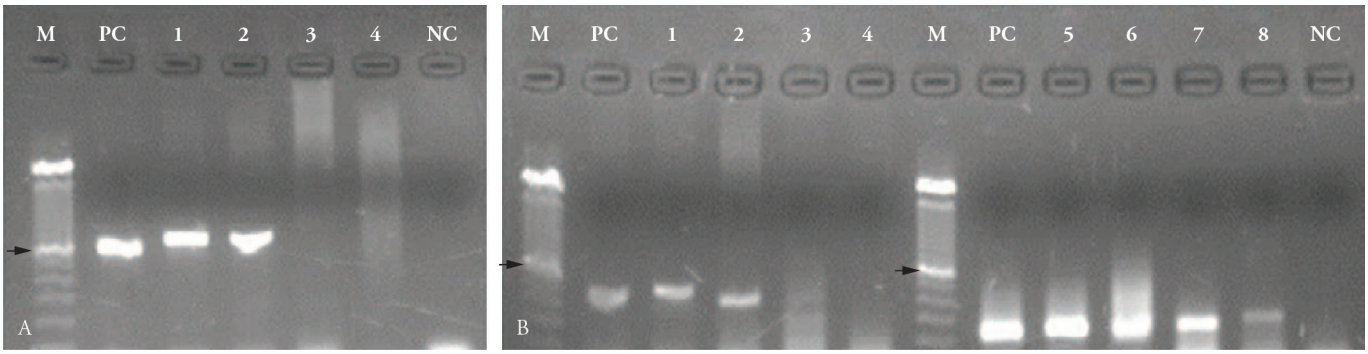

Figure 1. Detection of *Babesia vogeli* by: A) simple PCR based on the Bab1/Bab4 primers; B) semi-nested PCR based on the *Bab-fl/Babesia* common primers (lines 1-4) and *B. canis/Babesia* common (lines 5-8). M = 100 base-pair (bp) molecular marker, the arrows indicate the 600 bp fragments; PC = positive control; NC = negative control; reactions 1, 2, 3, and 4 included different samples.

number KF753247) of the product amplified from the positive control in the first reaction.

An identity of 100% was recorded between the nucleotide sequence obtained in the present study (positive control) and that of *B. vogeli* obtained from Genbank, which confirms, for the first time, the occurrence of this protozoan species in the metropolitan area of the city of Belém.

Both, the simple and the two semi-nested PCR, amplified fragments with lengths similar to those obtained for the positive control sample (Figure 1) were in agreement with the original descriptions (KORDICK et al., 1999; MARTIN et al., 2006; DUARTE et al., 2008). A comparison of the results of simple PCR and semi-nested PCR revealed a highly significant difference (p value = 0.0000). While only five (5%) of the samples tested positive using the simple protocol, 22 (22%) proved positive using the snPCR technique.

Calder et al. (1996) compared the clinical sensitivity of a serological test with a PCR-based molecular approach by analyzing cattle infected experimentally with *Babesia bovis*, and found that 30% of the samples presented false-negative results in the molecular test, particularly in cases of chronic infection, which are characterized by low levels of parasitemia. The most probable explanation for the discrepancy between that study and the present one is that the snPCR is more sensitive than the single PCR technique since, depending of the level of the parasitemia of the host, the number of copies of the target DNA may not be sufficient to be detected in a single reaction. In fact, in comparison with other diagnostic procedures, a number of studies have shown that nPCR or snPCR is much more effective for the detection of pathogens, including hemoparasites such as *Ehrlichia canis* and *Anaplasma platys* (CHANG; PAN, 1996; FERREIRA et al., 2007; MARTIN et al., 2005; McBRIDE et al., 1996; RAMOS et al., 2009; RUFINO et al., 2013).

Birkenheuer et al.'s study (2003) appears to be the only published report of the use of a semi-nested PCR protocol for the detection of *Babesia* protozoans. In this case, however, different primers were used for the detection of each of the following species *B. gibsoni*, *B. canis*, *B. vogeli*, and *B. rossi*. The protocol optimized in the present study thus represents a relatively cheap and effective test for epidemiological and prevalence studies, especially in areas where no information is available on the occurrence of the four different taxa.

A number of studies have reported the occurrence of canine babesiosis in Brazil and, according to them, *B. vogeli* is now known to occur in the south (JOJIMA et al., 2008; VIEIRA et al., 2013), southeast (COSTA-JUNIOR et al., 2009; LEMOS et al., 2012), mid-west (DUARTE et al., 2008; SPOLIDORIO et al., 2011), northeast (RAMOS et al., 2010; SILVA et al., 2012) and north of Brazil (SPOLIDORIO et al., 2013), while *B. gibsoni* has only been confirmed in the south (TRAPP et al., 2006). The present study is the first to identify the occurrence of *B. vogeli* in metropolitan Belém (eastern Amazonia).

Overall, given the zoonotic potential of this pathogen (KJEMTRUP; CONRAD, 2000; VANNIER; KRAUSE, 2012), the protocol optimized in the present study, which is clearly far more sensitive than simple PCR procedures, should be used for all future epidemiological studies of babesiosis caused by *B. gibsoni*, *B. canis*, *B. vogeli*, and *B. rossi*, especially those that aim to evaluate the prevalence of these protozoans.

Acknowledgements

We are grateful to FAPESPA (ICAAF 012/2012) for its financial support and to CAPES for masters scholarships granted to Pablo Moraes and Claudia Pinheiro Rufino. We are also indebted to the anonymous reviewers for their comments on the original manuscript.

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