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Research paper

Development of a quantitative PCR for the detection of *Rangelia vitalii*

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ABSTRACT

The aim of this study was to develop and validate a SYBR® Green qPCR assay to detect and quantify a fragment of the 18S rRNA gene of *Rangelia vitalii* in canine blood. Repeatability of the qPCR was determined by the intra- and inter-assay variations. The qPCR showed efficiency of $E = 101.30$ ($r^2 = 0.996$), detecting as few as one copy of plasmid containing the target DNA. Specificity of the assay was performed using DNA samples of *Babesia canis*, *B. gibsoni*, *Ehrlichia canis*, *E. ewingii* and *Leishmania* sp. No cross-reactivity was observed. Field samples consisting of blood from 265 dogs from Porto Alegre, Brazil were also tested. A total of 24 (9.05%) samples were positive for *R. vitalii*. Amplicons of 50% of positive samples were confirmed to be *R. vitalii* by Sanger sequencing. The positive samples had an average of 3.5×10^5 organisms/mL of blood (range: 1.27×10^3–1.88×10^6) based on the plasmid-generated standard curve. In conclusion, the SYBR® Green qPCR assay developed herein is sensitive and specific and can be used as a diagnostic tool for detection and quantification of *R. vitalii* in canine blood samples.

1. Introduction

Rangelia vitalii is the etiological agent of rangeliosis, also known as "nambiuvú" (blood dribbling down from the ears' margins) or "peste de sangue" (bleeding plague) (Loretti and Barros, 2004). This tick-borne protozoan was first described in 1910 as a distinct piroplasm capable of infecting dogs, primarily from rural and peri-urban areas in Southern Brazil (Pestana, 1910). Recently, the tick *Amblyomma aureolatum* was reported to be a vector of *R. vitalii* (Soares et al., 2012; França et al., 2014). Rangeliosis was currently diagnosed in dogs from others South American countries such as Argentina (Eires et al., 2014) and Uruguay (Soares et al., 2015). The disease causes extravascular hemolysis and external hemorrhage in dogs (Loretti and Barros, 2004; Fighera, 2007; Da Silva et al., 2011; Lemos et al., 2012; França et al., 2014). The taxonomy of *R. vitalii* is not completed understood but this parasite is known to belong to the Apicomplexa phylum, order Piroplasmorida (Loretti and Barros, 2005).

This piroplasm can be found inside erythrocytes (Fig. 1), neutrophils and monocytes in the blood circulation or within parasitophorous vacuoles in the cytoplasm of endothelial cells (Fighera et al., 2010; França et al., 2010). *R. vitalii* is frequently confused with *Babesia* spp. due to its morphological similarity (intraerythrocytic form), clinical signs, and response to treatment. On the other hand, the extraerythrocytic form of the parasite is similar to *Leishmania* spp. (Krauspenhar et al., 2003; Loretti and Barros, 2005; Soares et al., 2011). The diagnosis of the disease relies on clinical signs, observation of parasites in peripheral blood or endothelial cells and, more recently, by conventional PCR (Soares et al., 2011; Lemos et al., 2012; Soares et al., 2015). In order to accurately diagnose rangeliosis, more sensitive and specific techniques, such as molecular methods, are needed.

R. vitalii has been the subject of several morphologic studies and clinical reports, but has attracted little attention at the molecular level. Phylogenetic analysis based on fragments of the 18S rRNA and heat shock protein 70 (*hsp70*) genes suggest a close relationship to the genus *Babesia* (Soares et al., 2011). In this study, the genes were amplified by conventional PCR (cPCR) from blood samples of dogs

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naturally infected with *R. vitalii* in Southern Brazil; however, these assays were not validated to be used as a diagnostic tool.

SYBR® Green quantitative PCR uses fluorescent DNA binding dye to detect and quantify the amplification products during each cycle of PCR (Navarro et al., 2015). This is a sensitive technique with low cost compared to methods requiring fluorescent probes. Therefore, the aim of this study was to develop and validate a sensitive and specific quantitative SYBR® Green qPCR based on a fragment of the 18S rRNA gene of *R. vitalii* for detection and quantification of this organism in blood samples of dogs.

2. Material and methods

2.1. Experimental design

R. vitalii isolate was obtained from a dog experimentally infected with *R. vitalii* from a previous study (Da Silva et al., 2011). The dog was infected by intravenous inoculation of 2.0 mL of fresh blood collected from a dog naturally infected with *R. vitalii*. Before inoculation, the dog was confirmed clinically healthy and proven negative by cPCR for *R. vitalii Babesia* spp., *Hepatozoon* spp., and *Ehrlichia* spp. Infection was confirmed 7 days after inoculation by optical microscopy and cPCR assay for *R. vitalii* (Soares et al., 2011). This study was approved by the UFSM research ethics committee (protocol number 039/2012).

Clinical evaluation and evolution of parasitemia of the infected dog was performed at two-day intervals (Da Silva et al., 2011). Peripheral blood smears were performed with blood obtained from the large vein at tip of the dog's ear using a 25 gauge, hypodermic needle. The smears were stained by Romanovsky (Diff-Quick), and visualized under 1000x magnification by optical microscopy. Since no quantitative molecular techniques are available, an estimate of *R. vitalii* organisms was performed by calculating the average of 10 random oil immersion fields in blood smears (100x magnification by light microscopy).

Peripheral whole blood was collected into EDTA tubes during the peak of parasitemia (9 and 11 days post-infection, 5 parasites per 100x magnification field) for DNA extraction. DNA was extracted from 2 mL of EDTA whole blood from each sample using the Quick-gDNA™ Blood MidiPrep Kit (Zymo Research Corp., Orange, CA, USA) according to the manufacturer's instructions.

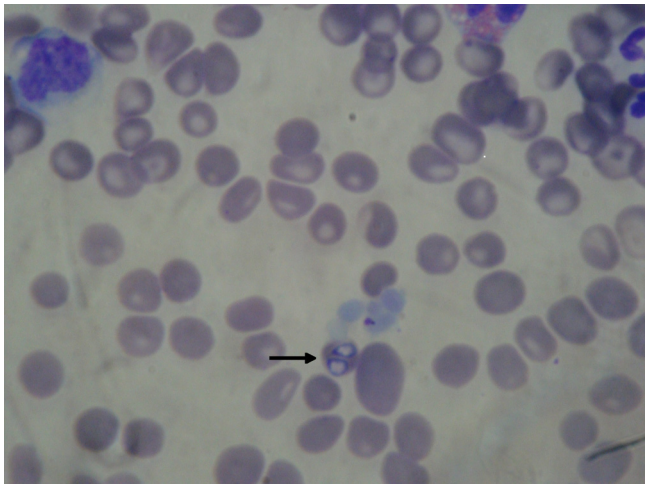

Fig. 1. Intra-erythrocytic form of *Rangelia vitalii* in blood smear (arrow) by light microscopy (modified Wright Giemsa stain).

2.2. Conventional PCR

Conventional PCR for amplification of the 18S rRNA gene was performed using DNA extracted from the blood sample of the dog experimentally infected with *R. vitalii* on day 7 after inoculation. Forward primer BAB143-167 (5′-CCG TGC TAA TTG TAG GGC TAA TAC A-3′) and reverse primer BAB-694-667 (5′GCT TGA AAC ACT CTA RTT TT CTC AAA G-3′) were combined to amplify a 544 bp fragment of the 18S rRNA gene (Soares et al., 2011). The PCR product was purified from agarose gel using the QIAquick Gel extraction kit (QIANGEN Inc., Valencia, CA, USA).

2.3. Cloning of Rangelia vitalii 18S rRNA gene

The fragment of 544 bp of the 18S rRNA gene of *R. vitalii* was cloned into a vector (pGEM®-T Easy Vector System II; Promega Corporation, Madison, WI, USA) according to the manufacturer's protocols. Extraction of plasmid containing the fragment of the 18S rRNA gene of *R. vitalii* was performed using QIAprep Spin Miniprep Kit, QIAGEN kit (QIAGEN). Plasmids were verified for the presence of the insert by cPCR (Soares et al., 2011). Positive plasmids were sequenced at the Genomics Core Facility at Purdue University, by the Sanger method (Sanger et al., 1977). Sequencing confirmed a fragment of ~544 bp with 99% identity with the 18S rRNA gene of *Babesia vitalii* (GenBank™ under the accession number: JN880432.1).

2.4. Primer design for qPCR

Primers were designed using Primer3 (v. 0.4.0) software (Koressaar and Remm, 2007; Untergrasser et al., 2012). Oligonucleotides properties were also analyzed using SMS (Sequence Manipulation Suite) PCR Primer Stats (Stothard, 2000). The forward RANGFW1 5′-CAA GAA ATA ACA ATA CAG GGC AAT A-3′ and reverse RANGRV1 5′- GCG AAA CCG ACA ATA CAC CA-3′ primer sequences were designed to amplify a 211 bp fragment of the 18S rRNA gene of *R. vitalii*.

2.5. Quantitative PCR

SYBR® Green qPCR assay was performed using a 7300 Real-Time PCR System (Applied Biosystems, Life Technologies Corporation, Grand Island, NY, USA). The reaction-mixture (total of 25 μL) contained 12.5 μL of 2x SYBR® Green Select Master Mix (Applied Biosystems), 0.75 μL of each primer (concentration of 0.3 μM), 6 μL of water, and 5 μL of DNA template. The thermocycler protocol consisted of one cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After the PCR run, dissociation was comprised by 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Nuclease-free water was used as negative control, and ten-fold serial dilutions of plasmids containing the fragment of the 18S rRNA gene of *R. vitalii* were used as template for the generation of a standard curve.

The data was analyzed using 7300 Real Time PCR System SDS software (Applied Biosystems). The cycle threshold (Ct) of plasmid dilutions were plotted against the logarithm of plasmid copies and used to determine the standard curve. All samples were performed in duplicate. Slope values and r^2 were calculated by linear regression analyses. The PCR efficiency (E) was calculated using the formula $E = 10^{(-1/\text{slope})} - 1$.

2.6. Standard curve

Plasmid concentration (copy number/μL) was determined by spectrophotometry (NanoDrop 2000; Thermo Scientific, Wilminghton, DE, USA) and ten-fold dilutions (10^9 to 1 copy of