Frequency of *Babesia vogeli* in domestic dogs in the metropolitan area of Piura, Peru

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Abstract

The aim of this study was to assess the presence of piroplasms in dogs in the metropolitan region of the city of Piura, Peru. Two hundred and twelve canine blood samples were randomly collected. The deoxyribonucleic acid was extracted from each blood sample and was tested using the polymerase chain reaction, restriction fragment length polymorphism and sequence analyses. The study showed the occurrence of *Babesia vogeli.* For the first time, this approach revealed the presence of canine babesiosis caused by *B. vogeli* in Peru. This highlights the need to test for pathogens that might be responsible for causing canine babesiosis, through using proper molecular tools.

Piroplasmids, PCR, RFLP, sequence analyses, Latin America

Piroplasmosis is an important tick-borne protozoal disease worldwide, caused by intraerythrocytic protozoa of the genera *Babesia* and *Theileria* (Vannier and Krause 2012). The clinical disease in dogs is variable, and the haemolytic type of babesiosis may range from subclinical to severe and fatal (Yamane et al. 1993; Lobetti 1998). The severity of clinical signs, mostly nonspecific, varies with the species or subspecies of *Babesia*, along with age, host immune response and presence of concomitant infections (Schetters et al. 1997; Boozer and Macintire 2003). The large *Babesia* spp., previously considered to be *B. canis*, currently include *B. canis*, *Babesia rossi* and *Babesia vogeli* as distinct species (Solano-Gallego et al. 2016).

Furthermore, there are other piroplasms, apart from *B. canis* and *B. gibsoni,* which infect dogs: *Theileria annae* (Zahler et al. 2000) reclassified as *Babesia vulpes* (Baneth et al. 2015), *B. conradae* (Kjemtrup and Conrad 2006), a new species of *Babesia*, described in North Carolina, USA, named *Babesia* sp. (coco) (Birkenheuer et al. 2004) and *Rangelia vitalii* infecting dogs of rural and urban fringe areas (Lemos et al. 2017)

The geographical distribution of piroplasm species is directly related to the distribution of their tick vectors (Sollano-Gallego et al. 2008). In dogs, *Rhipicephalus sanguineus* is the primary vector of *B. vogeli*; *Dermacentor reticulatus* is the vector of *B. canis*; and *Haemaphysalis elliptica* is the vector of *B. rossi* (Uilenberg et al. 1989). Moreover, *B. vogeli* has an extensive geographical range, considering that many reports have described its presence around the world (Uilenberg et al. 1989; Duh et al. 2004; Gülanber et al. 2006; Criado-Fornelio et al. 2007; Cardoso et al. 2008; M'Ghirbi and Bouattour 2008; Sollano-Gallego et al. 2008; Beck et al. 2009; Hamel et al. 2009).

In South America, Passos et al. (2005) reported *B. vogeli* for the first time in Brazil through molecular methods, and Sá et al. (2006) did the first report in Rio de Janeiro. The protozoan *B. vogeli* has also been reported in other South American countries: in Venezuela it was first amplified through molecular analyses in dogs by Criado-Fornelio et al.

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(2007); in Argentina it was described by Eiras et al. (2008); and in Colombia, the DNA of this haemoparasite was detected by Vargas-Hernández et al. (2012). More recently, in southern Brazil, Trapp et al. (2006) reported *B. gibsoni* for the first time in South America and confirmed the report with PCR.

In the state of Rio Grande do Sul, Brazil, Loretti and Barros (2005) used immunohistochemical techniques to detect the piroplasm *Rangelia vitalii* in dogs. In the southeast region, Lemos et al. (2012) were the pioneers in the molecular detection of *Rangelia vitalii* by PCR and RFLP, and confirming diagnosis with sequencing. Some years later, this protozoan was detected for the first time in dogs in Argentina (Eiras et al. 2014) and Uruguay (Soares et al. 2015).

The Peruvian coastal zone is characterized by arid areas. However, the cold Humboldt marine current provides the central zone of the coast, in the Piura area, with a climate consisting of an average temperature of 31.2 °C (maximum) and 17.7 °C (minimum), with 83% air humidity in the mornings and 51% at night (Senamhi 2011).

In Peru, ticks that are competent vectors of *B. canis* and *B. gibsoni* including *Rhipicephalus sanguineus*, are highly prevalent (Estares et al. 1999). However, there are no reports describing the presence of these two piroplasms in Peru.

Therefore, the aim of this study was to detect and characterize piroplasm species in dogs in northern Peru using molecular biology methods, such as the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and sequence analyses.

Materials and Methods

A survey was conducted in the metropolitan region of Piura, Peru (4° 59' 24. 00" S and 80° 24' 36. 00" W), in which two hundred and twelve canine blood samples were collected from dogs in different urban neighborhoods of this region. The selection criteria did not take age, sex, breed or clinical status into account. The animals' owners were asked to sign an informed consent form prior to data collection. All procedures were approved by the Ethics Committee for Animal Use (CEUA) of the Universidade Federal Fluminense, under protocol 759.

The DNA was extracted from 300 μl of whole blood from each sample using a commercially available kit (Illustra Blood Genomic Prep Mini Spin Kit, GE Healthcare, Chalfont St. Giles, UK), in accordance with the manufacturer's instructions. For DNA detection, we used the primers PIRO A (5'-AAT ACC CAA TCC TGA CAC AGG G-3 ') and PIRO B (5'-TTA AAT ACG AAT GCC CCC AAC-3') to amplify a fragment of 400 bp of the 18S rRNA gene, which is present in almost all species of piroplasms (Carret et al. 1999). The final concentrations of the reagents involved were 200 μM of each dNTP, 200 nM of each primer, 1.625 mM of MgCl$_2$, 1.25 U of GoTaq® Flexi DNA Polymerase (Promega®, Madison, WI, USA) and 5 μl of the DNA sample, in a total of 25 μl. The amplification program started with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. After this, a final extension step was applied for 5 min at 72 °C (Carret et al. 1999).

Samples that tested positive for piroplasmids by PCR were then evaluated by means of the RFLP technique, to differentiate *Babesia canis, Babesia rossi* and *Babesia vogeli* by means of enzymatic cleavage with Hinf I and Taqα I (Biolabs®, New England, USA) at specific sites depending on the species (Carret et al. 1999). The reaction was performed with 10 μl of amplified PCR products along with 10 units (1 μl) of Hinf I and 10 units (1 μl) of Taqα I, and their respective buffers, in different reaction tubes, thus resulting in a 20 μl volume of final solution. Enzymatic digestion was performed in a programmable thermocycler (Applied Biosystems®;Veriti®), Foster City, CA, USA) at 65 °C with the enzyme Taq I, and in a "dry block" (Solab SL20®, Piracicaba, SP, BRA) at 37 °C for digestion with the enzyme Hinf I, both for one hour.

The PCR and enzymatic restriction products were tested by means of electrophoresis on 1 and 2% agarose gel, respectively. Both of them were stained with 5 μl Gel Red® (Biotium, Hayward, CA, USA) to evaluate the DNA fragment sizes obtained.

To confirm the validity of our PCR results, samples positive for *B. vogeli* and *Rangelia vitalii* were used as positive controls. For the negative control, distilled water (UltraPure™ DNase/RNase-Free, Gibco/Invitrogen, Carlsbad, USA) was used instead of the target DNA. Furthermore, and to ensure that the negative results were reliable, all the samples were tested for the presence of amplifiable DNA through using the primer set GAPDH - F (5'- CCTTCATTGACCTCAACTACAT - 3 ') and GAPDH - R (5'- CCAAAGTTGTCATGGATGACC - 3'). This provided amplification of a 399-base pair fragment of the gene encoding the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme, which is present in all mammals (Birkenheim et al. 2003).

The PCR products of the expected amplicon size (PIRO A/PIRO B) were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Heathcare Life Sciences, Buckinghamshire, UK), in accordance with the manufacturer's instructions, and then were sequenced using Big Dye Terminator Cycle Sequencing Standard Version 3.1 (Life Technologies, Carlsbad, CA, USA) com polymer POP7, in accordance with the manufacturer's instructions, in the sequencer 3130/3130X/Genetic Analyzer Applied Biosystems (Hitachi, USA). The sequences were aligned using the ClustalW software, version 2.1 (Larkin 2007), and the alignment gaps were removed. Subsequently, phylogenetic analysis was performed through the Phylogeny Inference Package (PHYLIP) (Felsenstein 2005), using maximum parsimony analysis with 1000 bootstraps, and a consensus tree was generated (the software applied was Seqboot, Dnapars and Consense). The graphical processing of the tree was performed using the Mega software, version 6.06.

The sequences used in the comparison were *Babesia rossi* (GenBank accession number L19079), *Babesia canis* (AY072926) dog-Croatia, *Babesia* sp. (AY077719) dog-Japan, *Babesia vogeli* (AY072925) dog-France. These were chosen because they align group on the same side of *B. vogeli* in the phylogeny proposed by Lack et al. (2012). Furthermore, the sequences *Babesia gibsoni* (AF205636) dog-Oklahoma, and Babesia sp. Coco (AY618928) dog-North Carolina were used as comparisons for other species of piroplasms. Likewise, for comparison purposes, we used sequences that have been reported in different places in South America: *Babesia vogeli* (DQ297390) dog-Venezuela (Criado-Fornelio et al. 2007); *Babesia vogeli* (EU362993) dog-Argentina (Eiras et al. 1998); *Babesia vogeli* (KT333456) dog-Brazil (Moraes et al. 2015); and *Babesia vogeli* (JN368081) dog-Colombia (Vargas-Hernandéz et al. 2012). The sequence *Neospora caninum* (L24380) was chosen as an outgroup because it belongs to a clade that differs from the *Babesia* species evaluated (Fig. 1). The sequences of this study were uploaded to Gen Bank and received the following access codes: KY349101, KY349102, and KY349103.
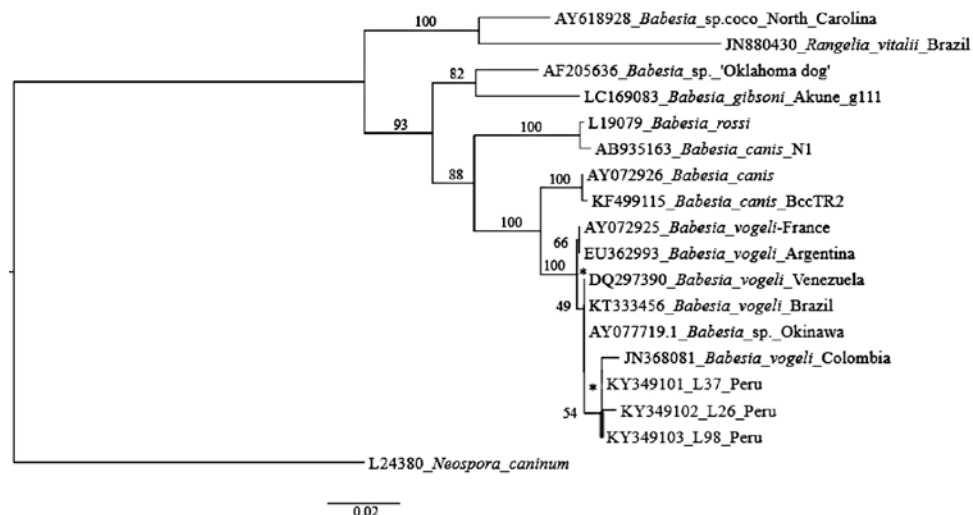

Fig. 1. Phylogenetic tree based on partial sequence of the 18S rRNA gene from samples positive for *B. vogeli*, in the metropolitan region of Piura, Peru, and related sequences obtained from the GenBank, showing the bootstrap values obtained after 1000 replications. The sequences labelled as "Peru" are samples from this study positive for *B. vogeli* on PCR-RFLP, namely L37_Peru, L26_Peru, L98_Peru (The sequences of this study uploaded to GenBank). The tree was produced by the PHYLIP's PhyML algorithm using JC69 substitution model. Sequence names are preceded by Genebank IDs and 0 length branches are marked with *.

Results

All 212 samples were tested with the GAPDH-F and GAPDH-R oligonucleotides. We obtained 400 bp bands indicating the presence of mammalian DNA in the samples. Among the 212 dogs evaluated in the city of Piura, Peru, from December 2014 to March 2015, three (1.4%) were positive for *Babesia* sp. By using PCR with the primers PIRO A and PIRO B, the bands were viewed at approximately 400 bp.

The positive samples were exposed to restriction enzyme digestion (RFLP). Use of the Hinf I enzyme did not produce any cleavage, and the only fragment visible was approximately 400 bp. The use of the Taq I enzyme produced cleavage in all samples, into two visible fragments: one of 175 bp and 210 bp. The cleavage pattern of these samples was compatible with *B. vogeli* (Carret et al. 1999).

Discussion

This was the first report of *Babesia vogeli* infecting dogs in Peru, with data obtained through the use of PCR, RFLP, and sequencing. *Babesia vogeli* was the only species diagnosed in naturally infected dogs in different urban neighborhoods in the metropolitan region of Piura, Province of Piura, Peru, between December 2014 and March 2015.

Compared with positive sample sequences of *B. vogeli* from studies conducted in other regions of Latin America, the samples L26 Peru and L98 Peru showed 99% identity to EU362993 *Babesia vogeli* Argentina (370bp), JN368081 *Babesia vogeli* Colombia (411bp), KT333456 *Babesia vogeli* Brazil and DQ297390 *Babesia vogeli* Venezuela. In addition, they demonstrated 99% identity to positive sample sequences of *B. vogeli* from studies conducted on other continents: AY072925 *Babesia vogeli* France and AY0777191 *Babesia vogeli* Okinawa.

Compared with other piroplasm species, the L98 Peru, L26 Peru and L37 Peru sequences possessed 95, 95 and 93% identity to AY072926 *B. canis* Croatia and KF499115 *Babesia canis* Turkey. Compared with *Babesia* sp. Coco isolated in North Carolina, USA (AY618928), the L98 Peru, L26 Peru and L37 Peru sequences possessed 89, 89 and 90% similarity, respectively.

In South America, molecular studies determined the presence of *B. vogeli, B. gibsoni* and *Rangelia vitalii,* as aetiological agents for canine piroplasmosis. In Venezuela and Colombia, studies on naturally infected dogs reported that the prevalence of *Babesia* infection was 2.24% (3/134) and 5.4% (5/91), respectively, (Criado-Fornelio et al. 2007; Vargas-Hernández et al. 2012), whereas Eiras et al. (2008) reported the first molecular evidence of *B. vogeli* in Argentina. In the state of Rio de Janeiro, Brazil, Lemos et al. (2012), found 6.8% (7/103) of positive dogs for *Babesia* spp by 18S rRNA gene-based PCR assay.

The three *B. vogeli*-positive dogs described in the present study did not show any clinical signs compatible with canine babesiosis. It must be noted that many "carrier" dogs with chronic infections would not present any clinical signs unless their immune system is compromised. Solano-Gallego et al. (2016) suggested that the presence of clinical signs for babesiosis could be attributed to a weak immune system in chronic infected "carrier" dogs. Similar observations have also been reported in adult Greyhounds and Pit Bull Terriers, which were clinically healthy despite being seropositive for *B. vogeli* and *B. gibsoni*, respectively. (Taboada et al. 1992; Birkenheuer et al. 2003; Taboada and Lobetti 2006). In general chronic infections result from the inability of the host's immune system to eliminate infection. Pathogens establish themselves and can reproduce with more intensity causing clinical signs when the immune system is debilitated (Solano-Gallego and Baneth 2011).

However, other studies with larger numbers of animals need to be conducted to confirm whether this is the only species of piroplasm that parasitizes dogs in this region at this moment.

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