



## Detection and molecular characterization of a canine piroplasm from Brazil

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### ABSTRACT

In the beginning of the 20th century, a new canine disease was reported in Brazil under the name “nambiuvú”, whose etiological agent was called *Rangelia vitalii*, a distinct piroplasm that was shown to parasitize not only erythrocytes, but also leucocytes and endothelial cells. In this new century, more publications on *R. vitalii* were reported from Brazil, including an extensive study on its ultrastructural analysis, in addition to clinical, pathological, and epidemiological data on nambiuvú. However, a molecular analysis of *R. vitalii* has not been performed to date. In the present study, we performed molecular phylogenetic analyses of *R. vitalii* based on fragments of the genes 18S rRNA and the heat shock protein 70 (*hsp70*), amplified by PCR performed on blood samples derived from five clinical cases of dogs presumably infected with *R. vitalii* in southern Brazil. In addition, we examined Giemsa-stained thin blood smears from these same dogs. DNA sequences (604-bp) of the 18S rRNA gene obtained from the five dogs were identical to each other, and by Blast analysis, this sequence shared the highest degree of sequence identity (95%) with *Babesia* sp. China-BQ1. DNA sequences (1056-bp) of the *hsp70* gene obtained from the five dogs were identical to each other, and by Blast analysis, this sequence shared the highest degree of sequence identity (87%) with *Babesia bigemina*. Phylogenetic analyses inferred from either of the two genes resulted in the newly genotype being placed in the *Babesia* spp. *sensu stricto* clade with very high bootstrap support (95–100%) in three analyses (Neighbor-Joining, Maximum parsimony, and Maximum likelihood). Giemsa-stained thin blood smears from the dogs were shown to contain piroplasm organisms within erythrocytes, monocytes and neutrophils (individual forms), and schizont-like forms within neutrophils, in accordance with literature reports of *R. vitalii*. Based on these results, we conclude that *R. vitalii*, the etiological agent of “nambiuvú” in southern Brazil, is a valid species of piroplasm. Further studies are required to evaluate the validity of the genus *Rangelia*.

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### 1. Introduction

Parasites of the genus *Babesia* are intraerythrocytic tick-transmitted Apicomplexa protozoa found in a wide

variety of mammal and some avian hosts (Hunfeld et al., 2008). Before the wide use of molecular techniques for genetic identification and characterization of *Babesia* parasites during the last two decades, taxonomic identification of these parasites relied chiefly on morphological analysis of intracellular forms detected in blood smear examinations (Hunfeld et al., 2008; Irwin, 2009). In Brazil, only two species, namely *Babesia canis vogeli* (= *Babesia vogeli*) and

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*Babesia gibsoni*, have been confirmed by both morphological and molecular methods to infect dogs (Passos et al., 2005; Trapp et al., 2006). The brown dog tick, *Rhipicephalus sanguineus*, is the primary vector of *B. canis vogeli*, and a suspected vector of *B. gibsoni* in the country (Dantas-Torres, 2008).

In 1908, a new canine disease was reported in Brazil under the name “nambiuuvú”, a Guarany Indian word that means “bloody ears”, which is a classical clinical sign of acutely infected dogs (Carini, 1908; Carini and Maciel, 1914). Two years later, this disease was officially attributed to a new piroplasm, named *Piroplasma vitalii* (Pestana, 1910). Its generic classification was based on its similarities with other piroplasms, namely *Piroplasma canis* (a former synonym of *B. canis*), to induce fever, hemorrhage, anemia, splenomegaly, and jaundice in infected dogs, and because of its classical intraerythrocytic forms similar to *B. canis* in the peripheral circulation. However, its status of a new species relied on its additional intracellular presence within macrophages and endothelial cells, which has never been reported for any other canine piroplasm. Because of this particularity, in 1914 the parasite was transferred to a new genus, *Rangelia vitalii* (Carini and Maciel, 1914; see review by Loretto and Barros, 2004).

Through the following decades of the 20th century, while only a few more cases of nambiuuvú were reported in Brazil (Braga, 1935; Carini, 1948; Rezende, 1976), different authors doubted on the validity of *R. vitalii*, arguing its misidentification with *Toxoplasma gondii* or *Leishmania* spp. (for the extraerythrocytic forms) or *B. canis* (for the intraerythrocytic forms) (Moreira, 1938; Paraense and Vianna, 1948). In fact, *R. vitalii* was considered to be a synonym of *B. canis* in books of protozoa (Levine, 1973; Peirce, 2000). In this new century, more publications on *R. vitalii* were reported from southern Brazil (Krauspenhar et al., 2003; Figuera, 2007; Fischer et al., 2009), including an extensive study on its ultrastructural analysis, in addition to clinical, pathological, and epidemiological data of nambiuuvú (Loretto and Barros, 2005). However, a molecular analysis of *R. vitalii* has not been performed to date.

Recently, a series of seven clinical cases of dogs naturally infected with *R. vitalii* was described in southern Brazil (França et al., 2010). Blood samples from four of these cases were available for the present study, plus another new case, in which we performed molecular phylogenetic analyses of *R. vitalii*. In addition, we provide morphometric data for the intracellular forms found in blood smears from some of these dogs.

## 2. Materials and methods

Whole blood samples collected in ethylenediaminetetraacetic acid (EDTA) from five dogs naturally infected by *R. vitalii* were provided for this study. Four of these dogs were referred to a local University Veterinary Hospital (Federal University of Santa Maria, State of Rio Grande do Sul, Brazil), and a fifth dog was referred to private veterinary clinics in Cachoeira do Sul Municipality, State of Rio Grande do Sul. The diagnosis of nambiuuvú was based on general clinical data, namely fever, apathy, jaundice, anemia, and bloody ears (Fig. 1). In addition, clinical pathological data,

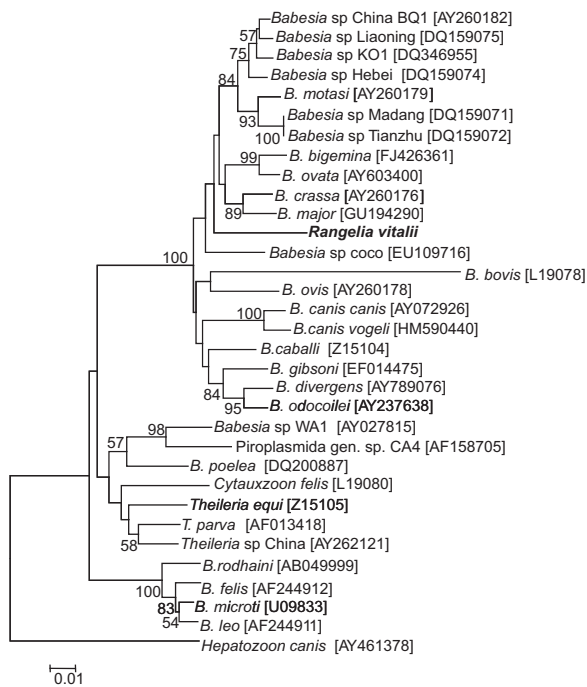


Fig. 1. One of the dogs sampled in the present study. Note bloody ears.

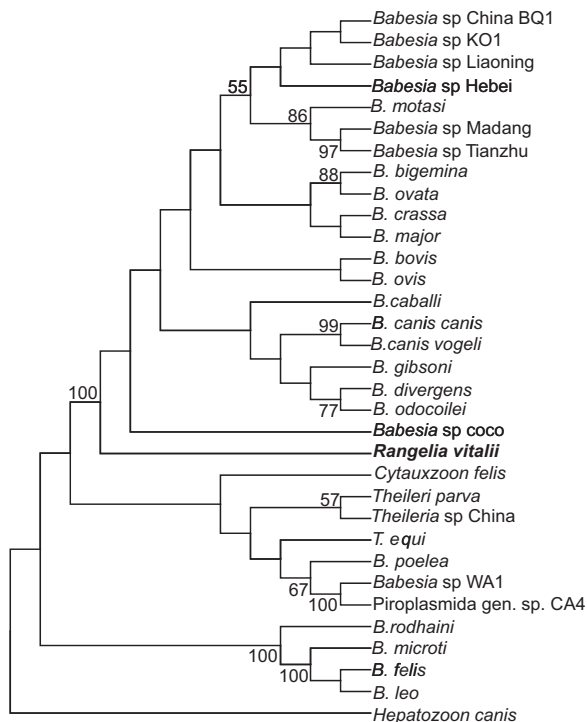
and the presence of piroplasm-like intracellular organisms inside monocytes, erythrocytes, and neutrophils in Giemsa-stained thin blood smears were reported for the four dogs from Santa Maria, as detailed by França et al. (2010).

Blood sample from each dog was subjected to DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA samples were tested individually in two PCR assays targeting overlapping portions of the 18S rRNA gene of *Babesia* spp: primers BAB-33-57 (5'-GCC AGT AGT CAT ATG CTT GTC TTAA -3') and BAB-432-409 (5'-TTC CTT AGA TGT GGT AGC CGT TTC-3'), designed to amplify a ≈370-bp fragment, as previously reported (Spolidorio et al., 2009); and primers BAB143-167 (5'-CCG TGC TAA TTG TAG GGC TAA TAC A-3') and BAB694-667 (5'-GCT TGA AAC ACT CTA RTT TTC TCA AAG-3'), designed in this study to amplify a ≈500-bp fragment. DNA samples were also tested individually in two other PCR assays targeting overlapping portions of the heat shock protein 70 gene (*hsp70*) of *Babesia* spp. and *Theileria* spp. For this purpose, the *hsp70* sequences of different species of *Babesia* and *Theileria* available in Genbank, including those parasites of dogs (Yamasaki et al., 2007), were aligned, and the following primers were designed for the present study: primers *hsp70* F1 (255–275) (5'-CAT GAA GCA CTG GCC HTT CAA-3') and *hsp70* R1 (881–903) (5'-GCN CKG CTG ATG GTG GTG TTG TA-3'), designed to amplify a ≈740-bp fragment; and primers *hsp70* F2 (740–759) (5'-GGA TCA ACA AYG GMA AGA AC-3') and *hsp70* R2 (1348–1369) (5'-GBA GGT TGT TGT CCT TVG TCA T-3'), designed to amplify a ≈720-bp fragment.

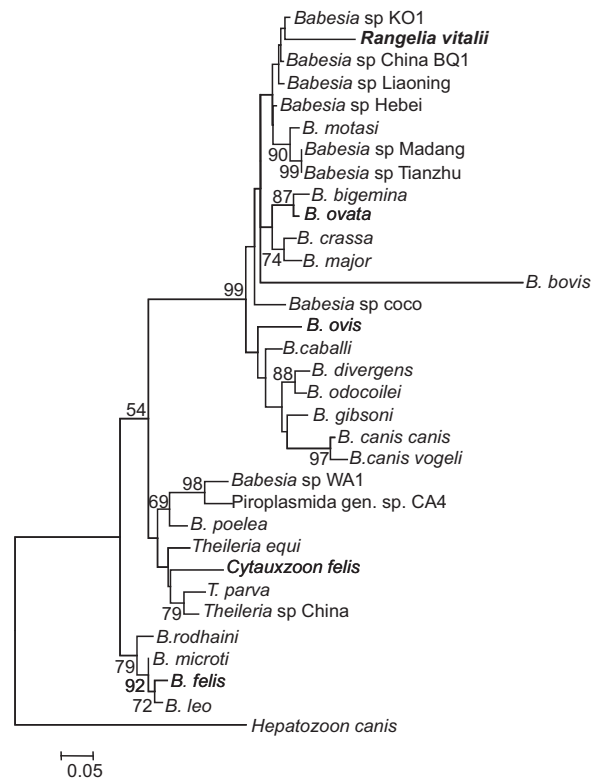
PCR products were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide, and examined by UV transillumination. Amplicons of the expected size were purified with ExoSap (USB) and sequenced in an automatic sequencer (Applied Biosystems/PerkinElmer, model ABI Prism 310 Genetic, Foster City, CA) according to the manufacturer's protocol. The two overlapping sequences of each gene (18S rRNA or *hsp70*) were aligned using the program SeqMan (DNASTar, Lasergene, Madison, WI), to form a consensus partial sequence of each gene. The sequences obtained were submitted to BLAST analysis (Altschul et al.,



**Fig. 2.** Neighbor-joining phylogenetic tree of 18S rRNA partial sequences (604-nt) of *Rangelia vitalii* and other piroplasms. Numbers on the nodes indicate bootstrap support from 1000 replications. Only bootstrap values >50 are shown. The scale bar represents the number of substitutions per nucleotide. Numbers in brackets are GenBank accession numbers.



**Fig. 3.** Maximum parsimony phylogenetic tree of 18S rRNA partial sequences (604-nt) of *Rangelia vitalii* and other piroplasms. Numbers on the nodes indicate bootstrap support from 1000 replications. Only bootstrap values >50 are shown. GenBank accession numbers for the sequences represented in this figure are the same as shown in Fig. 2.

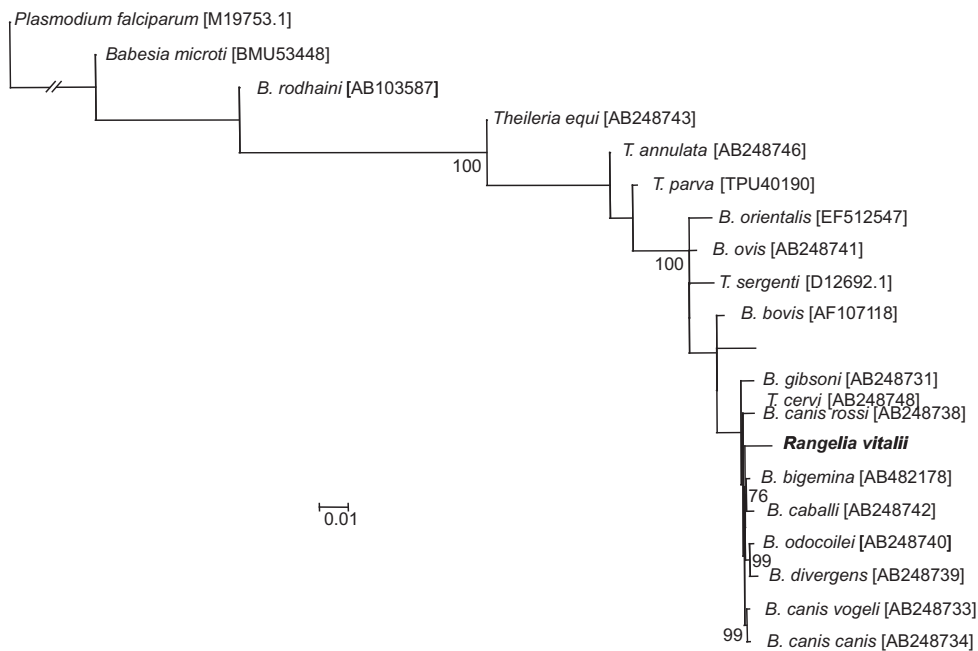


**Fig. 4.** Maximum likelihood phylogenetic tree of 18S rRNA partial sequences (604-nt) of *Rangelia vitalii* and other piroplasms. Numbers on the nodes indicate bootstrap support from 100 replications. Only bootstrap values >50 are shown. The scale bar represents the number of substitutions per nucleotide. GenBank accession numbers for the sequences represented in this figure are the same as shown in Fig. 2.

1990) to determine the closest similarities to corresponding sequences.

For phylogenetic analyses of each gene (18S rRNA and *hsp70*), the sequence of *R. vitalii* was aligned with homologous sequences retrieved from Genbank with *Hepatozoon canis* as an outgroup for the 18S rRNA analyses, and *Plasmodium falciparum* as an outgroup for the *hsp70* analyses (accession numbers in Figs. 2 and 5) using Clustal/W in Bioedit v. 7.0.5.3 (Hall, 1999). For each alignment, three trees were generated: Distance trees (Neighbor-Joining algorithm, 1000 bootstrap replicates; MCL and GTR+G+I models for the 18S rRNA and *hsp70*, respectively); Maximum likelihood trees (Heuristic Method, 100 bootstrap replicates; TN93+G+I and GTR+G+I models for the 18S rRNA and *hsp70*, respectively); and finally, Maximum Parsimony trees (min-mini heuristic algorithm, 1000 bootstrap replicates for both alignments) using PAUP\* 4b10 (Swofford, 2000) and Mega 4.1 (Tamura et al., 2007). Models were selected using the Akaike Information Criterion, Corrected, with ModelTest (Posada and Crandall, 1998).

Giemsa-stained thin blood smears from the four dogs from Santa Maria were examined for piroplasm organisms, which were found inside erythrocytes, monocytes, and/or neutrophils. Length (arbitrarily determined as the larger of the two measurements) and width of the organisms, and their nucleus, were determined using the Image-Pro Plus

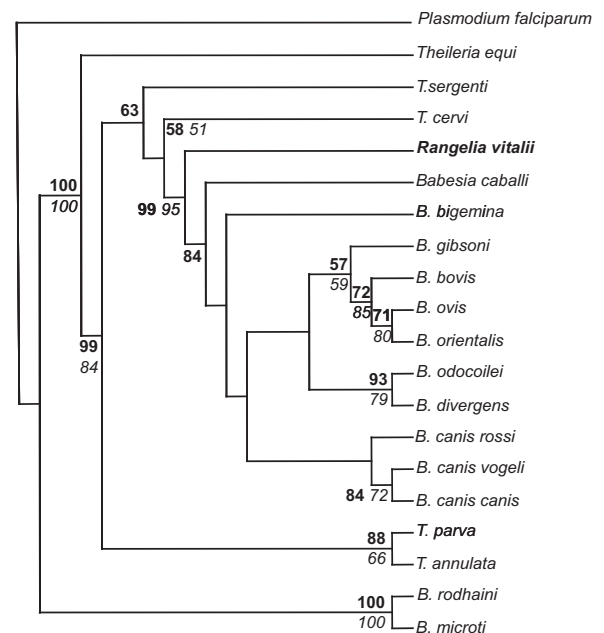


**Fig. 5.** Neighbor-joining phylogenetic tree of *hsp70* gene partial sequences (1056-nt) of *Rangelia vitalii* and other piroplasms. Numbers on the nodes indicate bootstrap support from 1000 replications. Only bootstrap values >50 are shown. The scale bar represents the number of substitutions per nucleotide. Numbers in brackets are GenBank accession numbers.

5.1 program for analysis of images and morphometry, fitted to an Olympus optical microscope. Measurements were compared to the values provided in the original description of *R. vitalii* by Pestana (1910) and with additional reports of Carini and Maciel (1914). All measurements are given in  $\mu\text{m}$ .

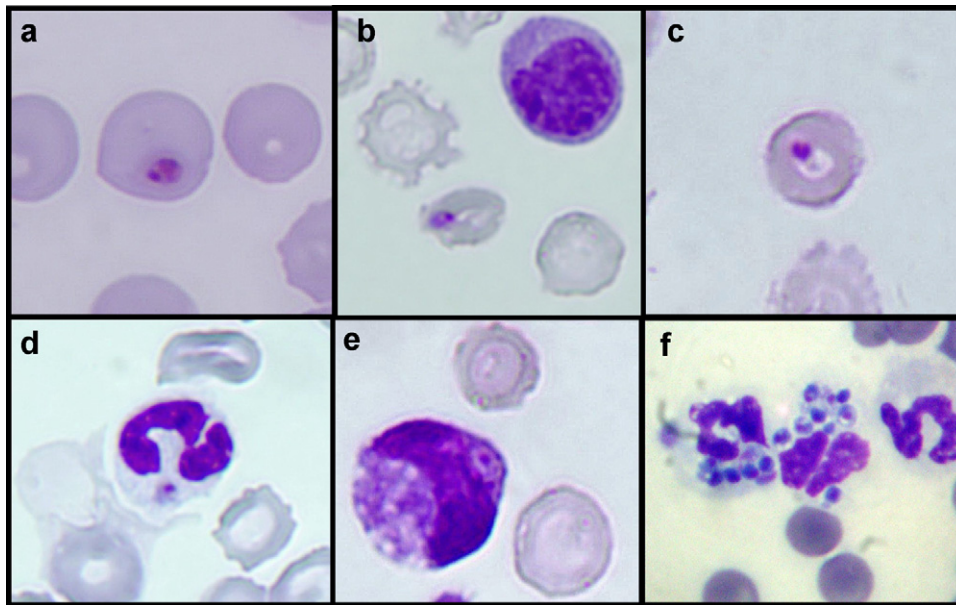
### 3. Results

Amplicons of the expected size were obtained from the five canine blood samples tested with the two 18S rRNA primer pairs and the two *hsp70* primers. For each gene, DNA sequences obtained from the five dogs were 100% identical to each other, and were merged through the overlapping regions to form a consensus sequence of 604-nt for the 18SrRNA gene, and 1056-nt for the *hsp70* gene. By Blast analysis, this 18SrRNA partial sequence shared the highest degree of sequence identity (95%) with *Babesia* sp. China-BQ1 (AY260182). When compared to *B. canis* and *B. gibsoni*, only 92–94% sequence identity was shared. The *hsp70* partial sequence shared the highest degree of sequence identity (87%) with *Babesia bigemina* (AB482178), and when compared to *B. canis* and *B. gibsoni*, only 82–86% sequence identity was shared. Phylogenetic analyses resulted in the newly genotype being placed in the *Babesia* spp. *sensu stricto* clade with very high bootstrap support (95–100%) in the three analyses for both 18S rRNA (Figs. 2–4) and *hsp70* gene (Figs. 5 and 6). However, the sequence of *R. vitalii* was basal to the *Babesia* spp. *sensu stricto* clade in at least three analyses, namely the Maximum parsimony trees for both 18S rRNA and *hsp70* genes, and the Maximum likelihood tree for *hsp70*



**Fig. 6.** Maximum parsimony (MP) phylogenetic tree of *hsp70* gene partial sequences (1056-nt) of *Rangelia vitalii* and other piroplasms. This tree topology was identical to the one generated by the Maximum likelihood (ML) analysis. Numbers on the nodes indicate bootstrap support from 1000 replications for MP (non italic numbers) and 100 replications for ML (italic numbers). Only bootstrap values >50 are shown. GenBank accession numbers for the sequences represented in this figure are the same as shown in Fig. 5.





**Fig. 7.** Piroplasms found in Giemsa-stained thin blood smears from dogs from Santa Maria, state of Rio Grande do Sul, Brazil. Individual forms within erythrocytes (a–c), neutrophils (d), monocyte (e), and multiple forms within neutrophils (f).

(Figs. 3 and 6). In all analyses the *Babesia* spp. *sensu stricto* clade (including the sequence of *R. vitalii*) was seen well separated from the *Theileria* species; the only exception was the Neighbor-Joining phylogenetic tree for the *hsp70* gene, in which the sequences of *Theileria sergenti* and *Theileria cervi* grouped within the *Babesia* spp. *sensu stricto* clade.

Giemsa-stained thin blood smears from the four dogs from Santa Maria were examined for piroplasm organisms. In each blood smear, at least 300 fields under 1000 $\times$  magnification were examined, with the following results: Dog 1 – piroplasms within 4 erythrocytes, 2 monocytes, 1 neutrophil, and one organism found outside cells; Dog 2 – piroplasm within 1 monocyte; Dog 3 (Fig. 1) – piroplasms within 1 neutrophil, and 1 monocyte; Dog 4 – no organism found. With these results, we calculated parasitemia levels varying from 0% to 0.0001%, considering all blood cells. Typically, only one oval or rounded cell was noted within each infected cell (Fig. 7a–e), however, groups of 6–33 organisms were found inside monocytes and neutrophils (Fig. 7f). The parasites had an eccentric magenta nucleus and a pale basophilic cytoplasm. The average length of 7 parasites found alone in 5 erythrocytes and 2 leucocytes was  $2.67 \pm 0.40$  (range: 2.00–3.35); the average width was  $1.94 \pm 0.22$  (range: 1.50–2.31). The average nucleus length of 7 parasites was  $1.06 \pm 0.17$  (range: 0.94–1.47); the average nucleus width was  $0.89 \pm 0.15$  (range: 0.69–1.22).

The GenBank nucleotide sequence accession number for the 18S rRNA and *hsp70* gene partial sequences generated in this study were HQ150006 and JF279603, respectively. The Giemsa-stained thin blood smears have been deposited in the Laboratory of Parasitic Diseases of the Faculty of Veterinary Medicine of the University of São Paulo.

#### 4. Discussion

Based on our phylogenetic analyses, the 18S rRNA and *hsp70* sequences generated in this study showed to be at least 5% and 13%, respectively, distinct from any other available sequence, and was always placed within the *Babesia* spp. *sensu stricto* clade with excellent statistical support. These results suggest that the etiological agent of “nambiuú” in southern Brazil is related to the genus *Babesia*. However, because the sequence of *R. vitalii* was basal in the *Babesia sensu stricto* group in some analyses (Figs. 3 and 6), because only a few non-*Babesia sensu stricto* group sequences were available for analyses of the *hsp70* gene, and because we analyzed partial sequences of only two genes, further studies encompassing more species and additional genes are required to define the generic classification of *R. vitalii*.

Despite several disagreements among parasitologists, previous phylogenetic analyses of animal piroplasms have consistently separated the *Babesia* spp. *sensu stricto* clade from the *Theileria* spp/*Babesia microti* clade (Hunfeld et al., 2008; Irwin, 2009). This separation was also clearly visible in most of our analyses (Figs. 2–4 and 6). *Babesia* spp. *sensu stricto* clade has been known for their exclusive invasion of erythrocytes while infecting vertebrates. In contrast, many species of the *Theileria* spp/*Babesia microti* clade are known to initially penetrate a lymphocyte or macrophage in which the development of schizonts takes place, before the invasion of erythrocytes (Hunfeld et al., 2008). Interestingly, while *R. vitalii* grouped within *Babesia* spp. *sensu stricto* clade in our analyses, it seems that it also invades cell types other than erythrocytes. In the present study, we found piroplasm organisms within leucocytes, a condition that has been repeatedly reported for *R. vitalii* since its original description (Pestana, 1910; Carini and Maciel,

1914; Carini, 1948; Loretti and Barros, 2005; França et al., 2010). In addition, it has been reported that *R. vitalii* also invades and multiplies by schizogony within endothelial cells (Pestana, 1910; Carini and Maciel, 1914; Carini, 1948), which was recently demonstrated by ultrastructural analyses, immunohistochemistry, and in situ hybridization of *R. vitalii*-infected canine organs (Loretti and Barros, 2005). These facts reinforce that further studies are required to clarify the generic status of *R. vitalii*, in order to re-evaluate the paradigm that all organisms within the *Babesia* spp. *sensu stricto* clade multiply exclusively within erythrocytes while infecting vertebrates.

Our findings of very low parasitemia in the thin blood smears of acutely infected dogs are in agreement with previous studies, which reported that *R. vitalii* is only occasionally found in the peripheral circulation by thin blood smear examination; in all cases, parasitemia is always very low (Carini and Maciel, 1914; Loretti and Barros, 2005; Figuera, 2007). Our measurements values for the intracellular forms of *R. vitalii* are in agreement with Pestana (1910), who described the organisms having  $1.5 \times 2.0$  or  $2.0 \times 2.0 \mu\text{m}$ , and with Carini and Maciel (1914), who redescribed the organisms with length varying from 2.0 to 3.5 or 4.5  $\mu\text{m}$ .

The vector of *R. vitalii* is still unknown, although either *R. sanguineus* or *Amblyomma aureolatum* have been suspected (Loretti and Barros, 2005). Interestingly, during clinical examination in the present study, one of the dogs from Santa Maria was found infested by a *R. sanguineus* female, and the dog from Cachoeira do Sul was infested by two *A. aureolatum* males (data not shown). These results reinforce that further studies testing the vector competence of these ticks for *R. vitalii* are needed.

In conclusion, this report provides a molecular identification of *R. vitalii*, which is a genetically distinct piroplasm capable of infecting the domestic dog in southern Brazil. The synonym status of the taxon *R. vitalii* for *B. canis* (Levine, 1973; Peirce, 2000) must be declined, since both species are now considered valid species that infect dogs in Brazil. This present report further emphasizes the utility of molecular diagnostics for the accurate diagnosis of canine infection by *R. vitalii*. Possibly, the distribution area of this piroplasm is broader than currently known.

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