Parasitology International 63 (2014) 729–734

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Short communication

First report of *Rangelia vitalii* infection (canine rangeliosis) in Argentina

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ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form 10 June 2014
Accepted 13 June 2014
Available online 23 June 2014

Keywords:
Canine
Piroplasm
Rangelia vitalii
Rangeliosis
Nambiuvú
Argentina

ABSTRACT

A 12-year old mixed breed neutered bitch from Misiones, Argentina, was presented with a history of fever and epistaxis. Blood, bone marrow, and lymph node samples were collected for hematology and cytology. Mild regenerative anemia was recorded and large, round, poorly stained piroplasms (>2.5 μm) were found within erythrocytes in blood and lymph node smears. Nested PCR-RFLP on blood and bone marrow samples was positive for piroplasm DNA. The 18S rRNA gene of piroplasms was targeted. A restriction pattern of a previously unreported piroplasm was observed. The PCR product was sequenced, and the sequence obtained had 99% identity with the *Rangelia vitalii* sequences from Brazil when compared by BLAST analysis. Further characterization of the detected piroplasm consisted of nearly full-length sequencing (1668 bp) of the 18S rRNA gene of this organism. Those sequences were deposited in GenBank. A phylogenetic analysis indicated that they clustered together with *R. vitalii* from Brazil but separately from large *Babesia* species of dogs such as *Babesia canis*, and from species of *Theileria* of dogs as well. This is the first report of *R. vitalii* infection in Argentina, and the first case of canine rangeliosis diagnosed outside Brazil.

1. Introduction

Rangelia vitalii is a large piroplasm described in dogs in the south and southeastern Brazil at the beginning of the 20th century [1–4]. *R. vitalii* is the causal agent of canine rangeliosis which is popularly referred to as *nambiuvú* (bloody ears). Based on field observations, it has been suggested that this disease would be naturally transmitted by the ixodids ticks *Amblyomma aureolatum* and *Rhipicephalus sanguineus* as well [3,5]. The life cycle of *R. vitalii* consists of a blood stage in erythrocytes and leucocytes and a tissue stage in the cytoplasm of capillary endothelial cells [5–7]. On blood smears *R. vitalii* zoites are found in erythrocytes, monocytes, neutrophils, and free in the plasma. The intraerythrocytic form of this parasite is most often seen in blood samples collected when there is fever in the acute stage of the disease. Clinical signs of canine rangeliosis include fever, anemia, jaundice, splenomegaly and lymphadenomegaly, hemorrhage in the gastrointestinal tract and persistent bleeding from the nose, oral cavity and tips, margins and outer surface of the ear pinnae. Laboratory findings include severe regenerative anemia, spherocytosis, thrombocytopenia, icterus and bilirubinuria. Erythrophagocytosis and other hematologic findings consistent with an immune-mediated hemolytic anemia may also be observed [3,5–8].

A presumptive diagnosis of canine piroplasmosis is based on the history, clinical signs, and hematological and serum biochemical findings. However, a definitive, etiological diagnosis requires identification of the piroplasm species and cells parasitized by examination of blood smears by light microscopy and identification of these pathogens by PCR [3,6,9–11].

The present report describes a case of natural *R. vitalii* infection in a dog from Misiones Province, Argentina, a previously unreported disease in this country, by using hematological and molecular methods.

2. Materials and methods

2.1. History

A 12-year old spayed mixed breed female dog from Misiones Province, a subtropical region in the northeast of Argentina, was presented to DIAP (a small animal diagnostic laboratory located in Buenos Aires, Argentina) in October 2011. A canine vector-borne disease (CVBD) was suspected based on clinical signs and the dog location (an area with increasing detection of vector borne pathogens). The animal had no history of traveling outside the country. Six months before being examined by a local veterinary clinician, the dog had moved from the southern area of Greater Buenos Aires, Argentina, to a suburban area of Leandro N. Alem Department, Misiones Province, Argentina, which is located approximately 30 km from the border of the State of Rio Grande do Sul, Brazil. The dog had not been treated prophylactically

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http://dx.doi.org/10.1016/j.parint.2014.06.003
1383-5769/

with ectoparasiticides and tick infestation was not observed by the owner.

2.2. Clinical signs, hematology and serum biochemistry

Clinical signs included epistaxis, hyperthermia (39.8 °C), anorexia, weight loss, and dry, scaly skin. Blood was sampled from the cephalic vein. Bone marrow was aspirated from the sternum. Samples were collected by aspiration from a popliteal lymph node.

Complete blood cell count was performed in an automated cell counter (Abacus, Diatron, Austria). Blood, bone marrow and lymph node smears stained with May-Grünwald-Giemsa were examined by light microscopy. Serum biochemical analyses were done on an automated analyzer. Serum protein electrophoresis was conducted in a cellulose acetate membrane.

There was an improvement in the clinical picture of the animal over the next several months without any therapy. The dog was re-evaluated in December 2012 when a second blood sample was collected and hematology, serum biochemistry and molecular studies were done again. Any clinical signs were recorded at this time point. In this opportunity the owner refused bone marrow sampling of his dog. Since there was no client consent to this procedure these samples were not collected this time.

2.3. Molecular diagnosis (PCR)

DNA was extracted from blood, bone marrow, and a control by using the commercial Wizard® Genomic DNA Purification Kit (Promega, USA), according to the manufacturer's instructions.

A fragment of the 18S rRNA gene from each of several canine piroplasms (*i.e. Babesia* spp. and *Theileria* spp.) was amplified by nested PCR as previously described [10] with external primers BTF1 and BTR1 and internal primers BTF2 and BTR2. Positive and negative controls were used for each PCR analysis. The internal PCR products (around 800 bp) were subjected to restriction fragment length polymorphism (RFLP) analysis with restriction enzyme HinfI, and double digestion with HinCII–BslI (Fermentas, USA) [10]. PCR products were purified and sent for sequencing at the Genetic Diagnosis Unit, Domestic Animal Services, College of Veterinary Medicine, National University of La Plata (UNLP), La Plata, Buenos Aires Province, Argentina.

2.4. Amplification, cloning and sequencing of the piroplasm 18S rRNA gene

Nearly the full-length of the 18S rRNA gene of the piroplasm was amplified by using primers 5-22F and 1661R as previously described [9]. The protocol used was an initial step of 94 °C for 4 min followed by 40 amplification cycles (94 °C for 40 s, 58 °C for 1 min, and 72 °C for 1 min) and a final extension step of 72 °C for 5 min. The PCR was conducted in a Personal Mastercycler (Eppendorf, Germany) using Taq polymerase from Stratec Molecular GmbH, Germany. A PCR product of about 1700 bp was purified and cloned into plasmids with the TA cloning kit (Invitrogen, USA) as described previously for *Sarcocystis* spp. 18S rRNA gene amplicons [12]. Plasmid DNA was extracted and samples of each plasmid DNA and primers M13-F, T7Prom-R, BTF2 and BTR2 were sent for sequencing to Lightrun service of GATC Biotech (http://www.gatc-biotech.com/lightrun) as previously described [12].

2.5. Sequences analysis

Sequences were analyzed with the Chromas Lite version 2.01 software and assembled with the free version 5.5.6 of GENEIOUS program (http://www.geneious.com), and the consensus sequences obtained were compared with available data in GenBank using the BLASTN 2.2.26 program (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis for the obtained sequences was conducted from a performed global multialignment with a similarity cost matrix of 70% (GENEIOUS) using the longest available 18S rRNA gene sequences in GenBank for several canine piroplasm species (*Babesia canis canis* AY072926 and HQ662634: 1542 bp; *Babesia canis rossi* DQ111760: 1546 bp, JN982343 and JN982352: 1529 bp; *Babesia canis vogeli* AY371195: 1502 bp, DQ297390 and DQ439545: 1538 bp; *Babesia conradae* AF158702: 1717 bp; *Babesia equi* DQ287951: 1583 bp; *Babesia gibsoni* AB478320 and AB478330: 1545 bp; *Babesia microti* AB219802: 1773 bp; *Babesia microti* like AF188001: 1712 bp and EU583387: 1654 bp; *Babesia* sp. coco AY618928, EU109716 and EU109717: 1666 bp; *Babesia (Rangelia) vitalii* HQ150006: 604 bp and JN880428: 591 bp) and the 18S rRNA gene sequence of *Hepatozoon canis* (GenBank DQ439540) as out-group (tree function of the GENEIOUS program). A neighbor-joining method was applied with a Tamura-Nei-model genetic distance calculation and with 1000 bootstrap replicates using 50% of support threshold. Additionally, the alignment was analyzed by the program MrBayes (Plugin from GENEIOUS) for Bayesian inference of phylogeny and by Maximum Likelihood tree building (PhyML, GENEIOUS).

3. Results

3.1. Hematology and serum biochemistry

Hematological and biochemical findings are summarized in Table 1. There was mild normocytic regenerative anemia with anisocytosis and mild polychromasia (2–3 polychromatic cells per field at 1000×). AST activity was increased, and the albumin/globulin (A/G) ratio was low. Few, large, round, poorly stained piroplasms, >2.5 μm in diameter were found inside erythrocytes on blood smears (Fig. 1) and on lymph node smears as well, but not on bone marrow smears. Parasitemia was <0.001%.

No hematological and biochemical changes were observed in the blood sample collected in December 2012, and piroplasms were not found on blood smears examined by light microscopy.

Table 1
Hematology, serum biochemistry and protein electroforesis findings from the dog with rangeliosis during parasitemia and fever in October 2011.

Parameter	Units	Findings	Reference[a]
Hematocrit	%	27.1	37–55
Hemoglobin	g/dl	12.4	12–18
Red blood cell count	$\times 10^6$/μl	4.06	5.5–8.5
MCV	fl	67	60–77
MHC	pg	30.5	19.5–24.5
MCHC	g/dl	45.6	32–36
Reticulocyte index	–	1.55	–
Leucocyte count	/μl	10,200	6000–18,000
Platelets	$\times 10^3$/μl	170	150–500
Urea	mg/dl	73.4	15–50
Creatinine	mg/dl	1.53	<1.5
ALT	U/l	65	10–60
AST	U/l	347	10–60
ALP	U/l	167	<200
Total proteins	g/dl	6.56	5.4–7.7
Albumin	g/dl	2.4	2.4–3.6
A/G ratio	–	0.4	0.6–1.1
Alpha 1 globulins	g/dl	0.1	0.2–0.5
Alpha 2 globulins	g/dl	0.43	0.3–1.1
Beta globulins	g/dl	1.1	1.2–2.2
Gamma globulins	g/dl	2.53	0.8–1.8

References: MCV = mean corpuscular volume; MHC = mean hemoglobin concentration; MCHC = mean corpuscular hemoglobin concentration; ALT = alanine transaminase; AST = aspartate transaminase; ALP = alkaline phophatase; A/G ratio = albumin/globulin ratio.

[a] Normal reference values were established with data obtained from clinically healthy dogs at DIAP laboratory and by comparing classical veterinary laboratory literature (Meyer DJ Harvey JW. Veterinary Laboratory Medicine: Interpretation and Diagnosis. 3rd ed., St. Louis: Saunders; 2004, p. 345–346.).

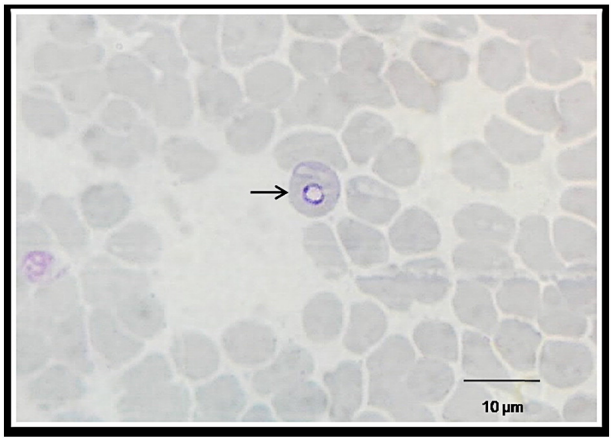

Fig. 1. May Grunwald-Giemsa stained blood smear from the Argentinean dog infected with *Rangelia vitalii*. Note one round merozoite (>2.5 µm) within an erythrocyte (arrow). 1000×.

3.2. Molecular diagnosis

Blood and bone marrow samples collected during the first visit of the animal to the laboratory tested positive for piroplasms by nested PCR. Lymph node samples were not tested.

The RFLP pattern of the PCR product was similar to the one described for *B. gibsoni* by Hinf digestion [10] (321, 270, 102, 81, 18 bp) but different in the double digestion (~405, ~205, ~102, ~80 bp). This last restriction pattern could not be matched with any piroplasm of dogs reported previously in the original paper where this same technique is described [10] (Fig. 2). A blood sample collected during the second visit of the animal to the laboratory was tested for piroplasms by nested PCR, and results came negative.

3.3. 18S rRNA gene amplification, cloning and sequencing

PCR products of approximately 1700 bp were obtained from blood and bone marrow from the first set of samples after the amplification with primers 5-22F and 1661R. Products obtained from the bone marrow were then cloned into plasmids. Plasmid DNA was extracted from 3 of the positive clones and 2 sequences of 1668 bp were obtained and deposited in GenBank (accession numbers KF218605 and KF218606). These sequences were 99.6% identical to each other; 7 single-base sequence differences were found.

3.4. Sequences analysis

Sequences obtained from the nested PCR products were compared by BLAST and showed a 99% sequence identity with *R. vitalii* 18S rRNA gene sequences from Brazil (GenBank HQ150006 and JN880428–JN880432) and only 92% sequence identity with other *Babesia* spp. 18S rRNA gene sequences (GenBank AY260179–AY260180 from *B. motasi*, and EU109716 and AY618928 from *Babesia* sp. coco).

The 18S rRNA gene sequences obtained after cloning into plasmids and deposited in GenBank also showed the highest sequence identity (98–99%) with Brazilian *R. vitalii* sequences with low sequence coverage on BLAST (36–47%).

By using Bayesian inference of phylogeny (MrBayes program) both sequences were placed in the same branch of a consensus phylogenetic tree together with *R. vitalii* (GenBank HQ150006 and KF218605) and *Babesia* sp. coco sequences (GenBank AY618928 and EU109716–17) with 100% branch support (Fig. 3). A similar construction and branch consensus support were obtained by the Neighbor-joining and Maximum Likelihood (PhyML program) analyses (obtained trees not shown).

4. Discussion

Canine rangeliosis is a CVBD that affects dogs from rural and suburban areas in the south and southeast of Brazil. Infected dogs have fever, anemia, jaundice, and marked hemorrhage along the margins of the ears, from the nares, and inside the intestinal tract [5,13,14]. This disease must be differentiated from other CVBD which have similar clinical, hematological, and pathological findings [7].

In the present study, few round large piroplasms were found inside erythrocytes on blood smears and on lymph node smears as well. By nested PCR-RFLP, an unexpected restriction pattern was detected when this sample was compared to those from other piroplasmids [10]. Further investigation of this unusual finding led to the first diagnosis of canine rangeliosis in Argentina. The amplified fragment showed a 99% sequence identity with the *R. vitalii* 18S rRNA gene sequences from Brazil [4,6]. Based on these findings, we conclude that our dog from Argentina was affected by the same pathogen that affects dogs in Brazil causing canine rangeliosis [7,13].

Parasitemia in our dog was low as reported by other authors for canine rangeliosis [6,13]. Only erythrocytes were parasitized by *R. vitalii* as opposed to the Brazilian cases in which on blood smears these organisms have been found not only inside red blood cells but also inside white blood cells and free in the plasma [5–8,13]. In one experimental

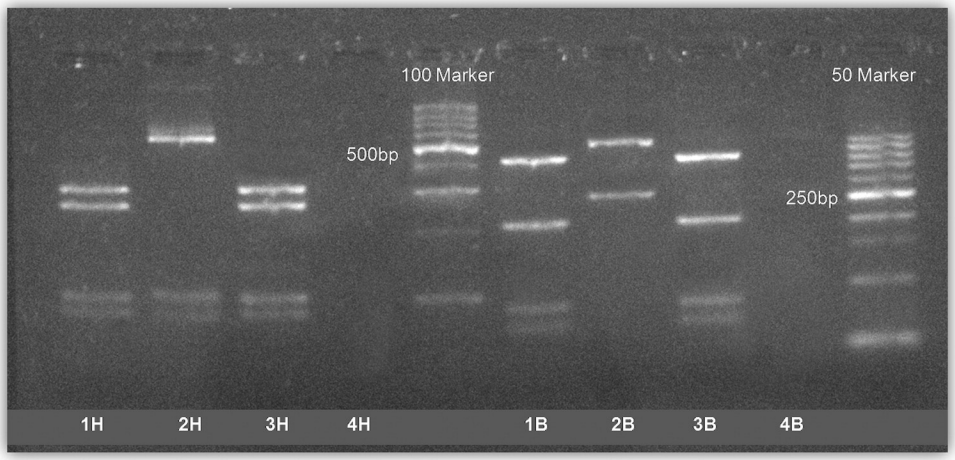

Fig. 2. Restriction fragment length polymorphism (RFLP) pattern of the nested-PCR product (≈800 bp) from the infected dog. References: (H) = shows single digestion with Hinf (37 °C for 1 h) and (B) = describes double digestion with BslI and HindII (37 °C for 1 h and 55 °C for 1 additional hour). Lines 1 and 3 show restriction of amplicons from blood and bone marrow samples of the Argentinean dog, respectively. Lines 2 show restriction of the reference control (*B. vogeli*). Lines 4 show negative control template.

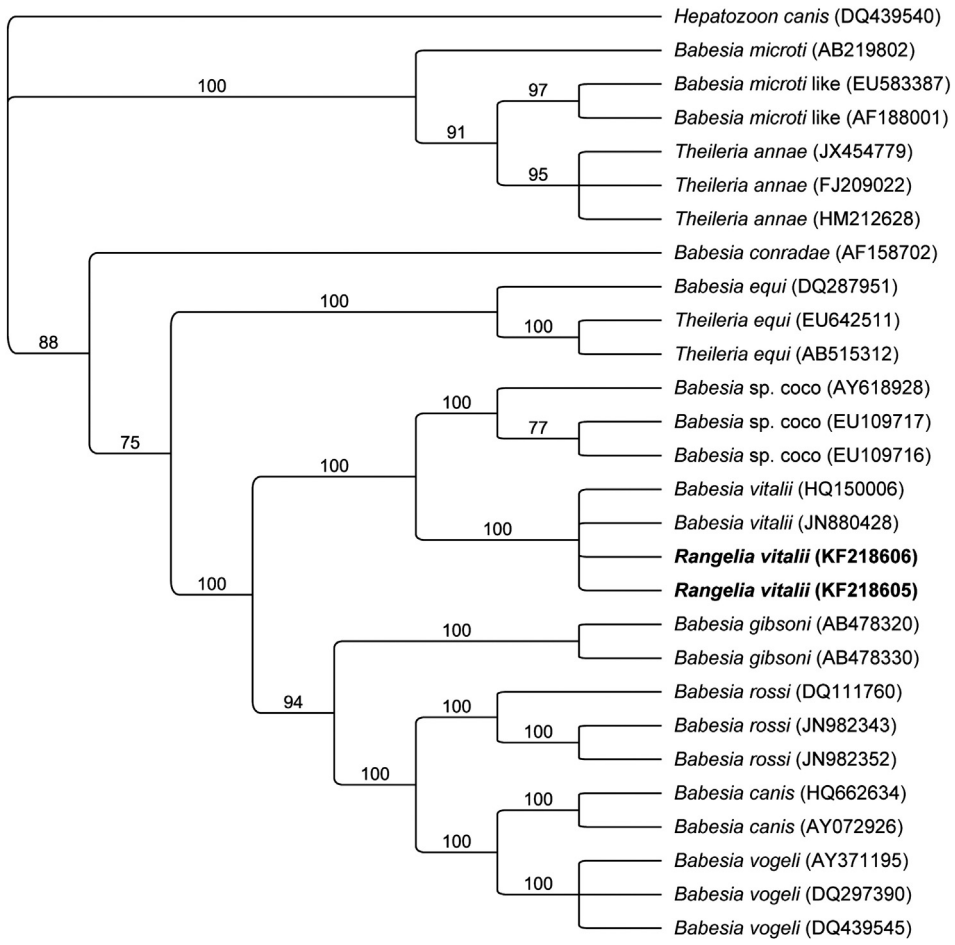

Fig. 3. Consensus phylogenetic tree from a multiple piroplasm 18S rRNA gene sequence alignment using Bayesian inference (MrBayes, GENEIOUS program). *Hepatozoon canis* sequence (GenBank DQ439540) was used as an out-group. Branch support is represented as probability (%) from 1000 bootstraps. *Sequences of R. vitalii from the* obtained sequences from the Argentinean dog are in bold. *Rangelia vitalii* sequences from Argentina were allocated together with the two *R. vitalii* sequences from Brazil. The latter was referred to as *B. vitalii* in this tree as these Brazilian sequences of *R. vitalii* appear as *B. vitalii* on GenBank.

study in dogs the first peak of parasitemia was observed from day 9 to day 11 day post infection (dpi) when parasites were seen inside red blood cells. Between 17 and 21 dpi piroplasms were also seen inside white blood cells [6]. In our case the dog had fever which is a clinical sign that has been observed in the acute stage of the infection [3,5,7]; therefore, we conclude that the dog was suffering acute rangeliosis. We presume that this animal became infected in Misiones Province where the dog had been living for 6 months before a diagnosis of *R. vitalii* infection was made. Hematological findings included mild regenerative anemia, a normal leukocyte count, a low packed cell volume, a low red blood count, and hemoglobin concentration within normal ranges. There was no obvious mechanism that would explain these changes but theoretically these could be due to increased erythrocyte fragility. In our case there was no evidence of hemolytic anemia such as erythrophagocytosis, auto-agglutination, or spherocytosis as observed in some cases of canine rangeliosis [3,5–8]. Elevated AST levels and normal ALT levels are both findings consistent with erythrocyte fragility. Increased activities in liver and muscle enzymes have also been reported in the acute stage of canine rangeliosis in experimental infected dogs [15].

Although canine rangeliosis is usually a severe disease and ultimately fatal if not treated accordingly and on time [7,13], our dog recovered without any treatment, and piroplasms were not found on blood samples a year later after *R. vitalii* infection was diagnosed by light microscopy and PCR. Instead of the episode of fever and epistaxis at initial diagnosis, the presentation of rangeliosis in this dog from Argentina, without the classical "bloody ears", was unusual based on available literature about clinical reports. Interestingly, a few additional cases of *R. vitalii* infection with an atypical clinical manifestation (*i.e.* without "bloody ears") have been diagnosed recently in Entre Rios province, Argentina (Eiras et al., unpublished data). Further studies are needed in order to characterize clinical manifestations of *R. vitalii* infection in Argentinean dogs.

Most veterinarians in Argentina are not familiar with nambiuvú (rangeliosis) in dogs. Therefore, we believe that because of this unawareness *R. vitalii* infection hasn't been diagnosed in our country. Possibly this disease is present since years as an undiagnosed condition in several Argentinean provinces as well as in other South American countries, especially those that border Brazil.

Nearly full-length of the 18S rRNA gene sequence of *R. vitalii* was amplified using generic primers previously used on phylogenetic studies of other piroplasms [9]. In order to obtain good sequencing results, the products obtained were cloned into plasmids as previously described for other apicomplexan species [12]. Sequencing of plasmid DNA samples resulted in 2 slightly different sequences (99.6% identity). The seven single base differences detected in a 1668 bp 18S rRNA gene sequence could be explained by a variant on repetitions of the sequences of this gene within one particular piroplasm species [16] but may be also due to the Taq polymerase that was used which lacks of 3′ to 5′ exonuclease proofreading activity. Both of our sequences showed the highest identity with those of *R. vitalii* from Brazil. Low coverage detected at BLAST comparison is explained by the shorter 18S rRNA previously reported sequences (between 604 and 800 bp) by other authors [4,6]. We compared our sequences with other sequences

of canine piroplasms by using Neighbor-joining, Maximum Likelihood, and Bayesian phylogenetic inferences. Both sequences were placed together in the *R. vitalii* branch with 100% bootstrap support. In agreement with a previous study published by Brazilian researchers in which smaller fragments of the *R. vitalii* 18S rRNA gene were sequenced, our 18S rRNA gene sequences were closely related to *Babesia* sp. coco sequences but separated from sequences of other large canine piroplasms such *B. canis* with a high bootstrap support [3].

The decision to keep *R. vitalii* in a single genus separate from the genera *Babesia* and *Theileira* is based on the results of our phylogenetic analysis and the results of other phylogenetic studies as well in which the sequences of this piroplasm, the causative agent of canine rangeliosis in Brazil and now Argentina, clustered separately from *B. canis* and *Theileria* spp. clades [3]. Additionally, these results reinforce the taxonomy based on the particular life cycle, hematological findings and clinical disease of *R. vitalii* infection.

Surprisingly, the 18S rRNA gene sequences of *R. vitalii* from Brazil—which is the piroplasm that causes "*nambiuvú*" (canine rangeliosis)—were submitted to GenBank as *B. vitalii* [4,6] which led to confusion and misidentification with the genus *Babesia* when sequences were compared by BLAST. Therefore, to avoid further problems, we submitted to GenBank our sequences from Argentina as *R. vitalii* instead of *Babesia vitalii*. That would avoid the software to include the genus *Babesia* in its automatic search. To the authors' knowledge, a nearly full-length of the 18S rRNA gene sequence of *R. vitalii* has not been submitted to GenBank to date.

Interestingly enough, our *R. vitalii* sequences formed a sister node with those of *Babesia* sp. coco on a consensus phylogenetic tree. *Babesia* sp. coco is a large piroplasm first reported and characterized by PCR on samples of an immunosuppressed dog from North Carolina (NC), USA [17]. The sequence of a fragment of the 18S rRNA gene of this species of *Babesia* from NC was allocated to a node separate from the *B. canis* group sequences, and was closely related with *B. bigemina* [17]. Apparently the species of *Babesia* from NC affect mainly immunosuppressed dogs. When cultured *in vitro*, this piroplasm had few ultrastructural differences compared to other species of *Babesia* [18,19]. According to our results, this large piroplasm from NC could be related to the genus *Rangelia*.

In Argentina, infestation of dogs with the tick *R. sanguineus* is widespread [20]. There are also a few reports of *A. aureolatum* infestation in the northeast of the country [21,22]. In a recent experimental study conducted by Brazilian researchers, *A. aureolatum* was capable of transmitting *R. vitalii* to dogs but *R. sanguineus* wasn't to transmit the same pathogen to these animals [23]. On the other hand, both *A. aureolatum* and *R. sanguineus* were found on the coat of dogs affected by *R. vitalii* and in the environment where these animals were located (*i.e.* in rural and suburban areas in southern Brazil) [5]. The hypothesis that there are vector-specific strains of *R. vitalii* in one specific geographic area that would be transmitted by one particular tick species should be investigated. Although the dog from Misiones Province was harboring *R. vitalii*, no ticks were found on the coat by the owner or clinicians. Possibly this dog, living in a suburban area, was exposed to and bitten by tick vectors at some point since this animal was not receiving any preventive treatment against these arthropods. Studies on the ability of *R. sanguineus* group [24] and *A. aureolatum* to transmit *R. vitalii* should be carried out in order to evaluate the epidemiology and risk factors of canine rangeliosis in Argentina. It has been speculated that in rural areas in Brazil a wild animal could have a role as a reservoir host for *R. vitalii* [5]. Recently in Brazil this protozoan parasite was found by PCR in the blood and a variety of tissues from the crab-eating fox *Cerdocyon thous* [25] which suggests that this wild dog might be involved in the life cycle of this protozoan parasite [5,25]. This canid is also present in north-eastern Argentina, and could potentially participate in the epidemiology of rangeliosis in this area.

In the present report, the dog that was infected with *R. vitalii* was living in Argentina in an area situated approximately 300 km away from the nearest area in Brazil where cases of canine rangeliosis have been diagnosed (in the city of Cruz Alta, State of Rio Grande do Sul) [3,5,7,13].

To the best of the authors' knowledge, this is the first case of *R. vitalii* infection diagnosed in Argentina and outside Brazil. Phylogenetic analysis of the 18S rRNA gene of *R. vitalii* showed that *R. vitalii* represents a single, distinct species separate from the genera *Babesia* and *Theileria*. Further investigation should be carried out in order to study the distribution, potential tick vectors, and clinical and pathological aspects of canine rangeliosis in dogs in other areas of Argentina.

Competing interests

The authors declare that they have no competing financial or non-financial interests.

Acknowledgments

We would like to thank Jorge Genini, owner of the infected dog, for his help. We also thank Dr. Gereon Schares from the Friedrich-Loeffler-Institut (Germany) for his collaboration and the working staff of the DIAP laboratory and laboratory of Immunoparasitology (FCV-UNLP, Argentina) for their daily support.

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