Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 157 (2008) 294–298
veterinary parasitology
www.elsevier.com/locate/vetpar

Short communication

First molecular characterization of *Babesia vogeli* in two naturally infected dogs of Buenos Aires, Argentina

Diego Fernando Eiras [a,b,*], Julia Basabe [a,b], María Mesplet [c], Leonhard Schnittger [c]

[a] *Laboratorio DIAP, Inca 109 (B1836BBC) Llavallol, Buenos Aires, Argentina*
[b] *Cátedra de Parasitología y Enfermedades Parasitarias, Departamento de Epizootiología y Salud Pública, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC 296 (B1900AVW) La Plata, Argentina*
[c] *Instituto de Patobiología, CICVyA, INTA-Castelar, Buenos Aires, Argentina*

Received 13 January 2008; received in revised form 22 July 2008; accepted 31 July 2008

Abstract

Large piroplasms (>2.5 μm) were detected by direct microscopical investigation in 34 out of 16,767 (0.20%) canine blood smears in the Southern region of Greater Buenos Aires. Genomic DNA was extracted from two parasitemic dogs and the hypervariable 18S RNA gene region of the pathogen was specifically amplified, sequenced, and aligned with corresponding gene sequences available in the GenBank. Phylogenetic trees were constructed and compared. 18S RNA gene sequences reliably segregated in three clearly distinguishable clades representing *Babesia canis*, *Babesia vogeli* and *Babesia rossi* isolates, respectively. The 18S RNA gene sequences of both *Babesia* isolates from Argentina affiliated to the *B. vogeli* branch. This finding represents the first molecular evidence of the existence of *B. vogeli* in Argentina.

Keywords: *Babesia vogeli*; Canine babesiosis; PCR; Argentina

1. Introduction

Canine babesiosis is a hemoprotozoal tick-borne disease characterized by fever, depression and anemia (Bicalho et al., 2004; Furlanello et al., 2005; Passos et al., 2005). Traditionally, *Babesia* sp. infection in dogs is identified based on the pear-shaped morphology of the intraerythrocytic stage of the parasite. *Babesia* sp. parasites with a large intraerythrocytic stage (>2.5 μm, ''large *Babesia*'') have been designated *Babesia canis*, whereas those with a small erythrocytic stage (<2.5 μm, ''small *Babesia*'') were named *Babesia gibsoni* (Brandão et al., 2003; Furlanello et al., 2005; Passos et al., 2005).

Although *B. canis* cannot be further subdivided by classical microscopical means, other characteristics such as vector specificity, geographical distribution, antigenic differences, clinical signs, and pathogenicity provide evidence for further subdivision into three subspecies (*B. canis canis*, *B. canis vogeli* and *B. canis rossi*) (Uilenberg et al., 1989). Based on comparative molecular studies of rRNA genes, a three species nomenclature (*B. canis*, *B. vogeli*, and *B. rossi*) has recently been recommended and will be used in the following, as proposed by others (Schetters et al., 1997; Zahler et al., 1998; Carret et al., 1999; Depoix et al., 2002; Passos et al., 2005).

Typically, *B. canis* infection presents mild to severe clinical signs, is transmitted by the tick *Dermacentor*

* Corresponding author. Tel.: +54 11 42986377; fax: +54 11 42986377.
E-mail addresses: diap@diap.com.ar, diegoeiras@diap.com.ar (D.F. Eiras).

0304-4017/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2008.07.037

reticulatus and is endemic throughout Europe and Northern Asia. In contrast, *B. rossi* exhibits a more restricted distribution confined to South Africa and Eastern Sudan, is transmitted by *Haemophysalis leachi* ticks, and the infection is often life-threatening or even fatal (Shaw et al., 2001; Furlanello et al., 2005; Oyamada et al., 2005; Garcia de Sá et al., 2006; Uilenberg, 2006). *B. vogeli* displays the most widespread distribution as it has been identified in South Africa, Eastern Africa, United States, South America, Japan and Australia and shows an overlapping distribution with *B. canis* in Southern Europe; it is transmitted by *Rhipicephalus sanguineus* ticks and the course of infection is usually asymptomatic or accompanied by mild clinical signs.

A novel large *Babesia* sp. was also reported in North America (Birkenheuer et al., 2004). Furthermore, a piroplasm that is pathogenic for dogs has been reported in Brazil. It seems to be different from *Babesia* and *Theileria* since it has an intraendothelial stage. Up to now the taxonomic status of this apicomplexan pathogen of dogs is not well known (Loretti and Barros, 2005).

In addition, a proposed new *B. canis* subspecies that infects felines has been named *B. canis* subspecies *presentii*. This subspecies shows a high molecular similarity of 18S RNA genes with *B. canis* but is markedly smaller in size (Baneth et al., 2004).

Apart from *B. gibsoni* genotype Asia (*B. gibsoni sensu stricto*), molecular studies carried out on small *Babesia* (and *Theileria*) have recognized other genetically distinct organisms parasites that infect dogs: *T. annae* (Zahler et al., 2000; Camacho-García, 2006) that is very similar to *B. microti* and *T. equi* (Criado-Fornelio et al., 2003), both reported in Spain and *B. conradae* described in California (Kjemptrup et al., 2006).

In South America, *B. vogeli* has been reported in Venezuela and Brazil (Passos et al., 2005; Criado-Fornelio et al., 2007). In Argentina it is still unknown whether this large *Babesia* species is present. In the study at hand we established the identity of the large *Babesia* parasites in two naturally infected dogs from Southern Greater Buenos Aires, Argentina, by sequencing the variable 18S RNA gene region.

2. Materials and methods

A total of 16,767 EDTA anticoagulated blood samples from owned household dogs from Southern Greater Buenos Aires were submitted by veterinary practitioners to DIAP Laboratory for diverse diagnostic purposes from October 2003 to May 2007. Giemsa-stained blood smears were examined by light microscopy (by observing 100 microscopic fields at 1000× magnification) for the presence of *Babesia* sp. merozoites. Genomic DNA was extracted from 2-ml aliquots of EDTA blood samples obtained from two *Babesia* sp. infected dogs using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Subsequently, the sequence of the variable region of the 18S RNA gene was independently amplified from each sample by using *Babesia*-specific PCR primers RLB-F (5′-GAGGTAGTGACAAGAAATAACAATA-3′) and RLB-R (5′-TCTTCGATCCCCTAACTTTC-3′) to generate an amplicon of about 415 bp-length using a PCR mix composition as described by Gubbels et al. (1999) and a touchdown temperature cycle reaction as reported by Matjila et al. (2004, 2005). Amplicons were run on an ethidium bromide-stained agarose gel and checked under ultraviolet light for expected size. The DNA sequence was analyzed by direct sequencing using RLB-F and RLB-R primers on an automated sequencer (Applied Biosystems). Analyzed sequences were deposited in the GenBank under accession numbers EU362993 and EU362994. Percent identity with a selected reference sequence was assessed with MatGAT (Matrix Global Alignment Tool; Campanella et al., 2004). The obtained 18S RNA gene sequences were aligned to 17 matching sequence regions currently available in the GenBank database (accession numbers are shown in Fig. 1) and the alignment visually inspected and trimmed so that sequences coincided in length (Tamura et al., 2007). Distance matrix based (UPGMA, Neighbor Joining) and character state based (maximum parsimony) algorithms were applied to infer phylogenetic trees that were rooted by using *B. microti* as an outgroup. The exemplified tree was constructed by using the Neighbor Joining method (Saitou and Nei, 1987). The confidence probability (multiplied by 100) that the interior branch length is greater than 50, as estimated using the bootstrap test (1000 replicates) is shown next to the branches (Dopazo, 1994; Rzhetsky and Nei, 1992). To emphasize the reliable portions of branching patterns the tree was condensed. Branches supported by a bootstrap value lower than 50% were reduced to 0 resulting in a multifurcated tree in which branch lengths are not proportional to the number of nucleotide substitutions. The evolutionary distances were computed using the Jukes–Cantor method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 368

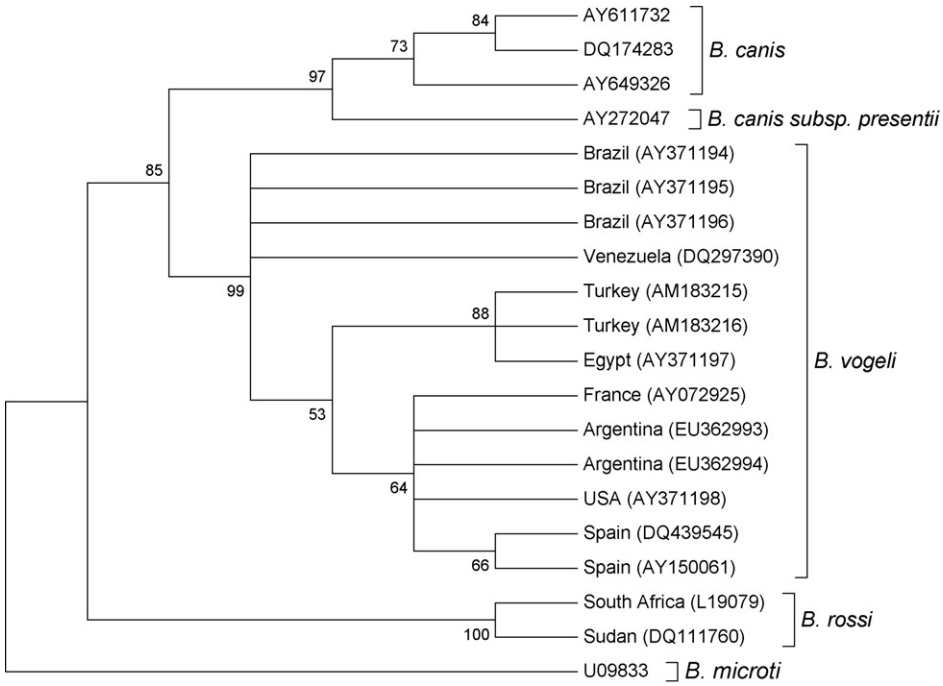

Fig. 1. Phylogenetic tree based on hypervariable 18S RNA gene sequences of large canine *Babesia* sp. Accession numbers EU362993 and EU362994 designate sequences of isolates from Argentina. To root the tree, *B. microti* was used as an outgroup. The tree was condensed: branches supported by a bootstrap value lower than 50% were reduced to 0 resulting in a multifurcated tree in which branch lengths are not proportional to the number of nucleotide substitutions. A bootstrap test (1000 replicates) was done and values are given at the nodes. Accession numbers for *B. canis*, *B. vogeli*, *B. rossi* isolates and the outgroup species *B. microti* are given in the figure.

positions in the final dataset. Alignments and phylogenetic analyses were conducted using the MEGA 4 software package (Tamura et al., 2007). The BioEdit Sequence Alignment Editor was used to carry out the restriction map analysis (Hall, 1999).

3. Results

Large piroplasms (>2.5 μm) were detected by microscopical investigation in 34 out of 16,767 blood smears (0.20%) of dogs living in the Southern region of Greater Buenos Aires.

From two parasitemic dogs the variable region of the parasite 18S RNA gene was amplified, sequenced, and, after alignment with corresponding 18S RNA sequences available from the GenBank, used to determine the *Babesia* species by phylogenetic analysis. All algorithms used, as outlined in Section 2, resulted in essentially similar tree topologies and *B. canis*, *B. vogeli* and *B. rossi* 18S RNA sequences segregated recurrently into three distinct clades. The Neighbour Joining tree shown in Fig. 1 demonstrates the phylogenetic relationships between isolates. The percentage of trees in which associated taxa cluster together after performing 1000 bootstrap replicates is shown at branch nodes and support with high confidence three different *B. rossi, B. canis* and *B. vogeli* taxa. The 18S RNA gene sequences of both *Babesia* isolates segregated into the *B. vogeli* branch providing evidence that they are representatives of this species. Both analyzed 18S RNA sequences of the isolates were found to be 100% identical to each other and exhibited an identity of 99.2% to the respective sequence of a *B. vogeli* reference strain (AY371197) (Uilenberg et al., 1989). The BioEdit Sequence Alignment Editor of Hall (1999) was used to confirm the presence of 2 TaqI and the absence of 1 HinfI diagnostic restriction sites within the variable 18S RNA gene region of *B. vogeli* that have been reported by Carret et al. (1999).

4. Discussion

In the present study, the microscopic examination of blood smears of an extensive panel of canine blood samples from a Southern urban area of Greater Buenos Aires, Argentina, revealed the presence of large *Babesia* sp. piroplasms. The piroplasm 18S RNA gene sequence

contained in two of these blood samples was analyzed and a phylogenetic tree was constructed with corresponding sequences available in the GenBank database. In the inferred tree, the 18S RNA sequences under analysis segregated into a common *B. vogeli*-clade providing the first molecular evidence for the existence of *B. vogeli* in Argentina.

According to the tree branch containing *B. vogeli* isolates, the isolates from Argentina are most closely related to isolates from Europe (France, Spain) and North America (USA). Given the long emigration from and close cultural relationship between Europe and the Americas (especially Argentina and USA) possibly including the migration of infected dogs between these continents/countries this is not surprising.

B. vogeli isolates from Argentina seem to be distantly related to *B. vogeli* isolates from other South American countries (Brazil, Venezuela). However, some of the tree branches discussed above are supported by rather limited bootstrap values. Furthermore, as only a very few isolates have been investigated and in the framework of our study only two parasitemic dogs were detected when molecular methods were available, the above statements are preliminary and need to be supported by a study of a larger number of samples.

Other aspects such as vectoring capacity should be also considered. Genetic divergence was reported between *R. sanguineus* from Brazil and Argentina and strong relationship was detected between European and Argentinean *R. sanguineus* populations. The Brazilian population was found related to African ticks (Szabo et al., 2005).

A diagnostic PCR-RFLP test has been developed distinguishing between *B. vogeli*, *B. canis*, and *B. rossi* species (Carret et al., 1999). In this method, *B. vogeli* is identified by the presence of 2 TaqI and the absence of 1 HinfI restriction site within the variable 18S RNA gene region. We ascertained *in silico* the presence of this restriction pattern in both *B. vogeli* isolates from Argentina underscoring the usefulness of this method in future epidemiological studies in the region.

B. gibsoni (genotype Asia) has been described in Brazil, (Dantas-Torres and Figueredo, 2006; Trapp et al., 2006). So far, small piroplasms have not yet been reported in dogs from Argentina and in the framework of our study, there was no microscopical evidence for their presence, but their existence cannot be excluded.

In this study, microscopical observation revealed the presence of large *Babesia* parasites in only 0.20% of the canine blood samples. However, preliminary serological investigations of canine babesiosis in the study region suggest a higher number of infected dogs (Eiras et al., unpublished data). Further studies on canine babesiosis are needed to allow a refined assessment of the current situation in Argentina.

Acknowledgments

We thank the DIAP Laboratory staff for technical support, and Dr. Monica Florin-Christensen, CICVyA, INTA and Dr. Darío Vezzani, EcoRVeP, FCEyN, UBA for critically reviewing this manuscript.

References

Baneth, G., Kenny, M.J., Tasker, S., Anug, Y., Shkap, V., Levy, A., Shaw, S.E., 2004. Infection with a proposed new subspecies of *Babesia canis*, *Babesia canis* subsp. *presentii*, in domestic cats. J. Clin. Microbiol. 42, 99–105.

Bicalho, K.A., Ribeiro, M.F., Martins-Filho, O.A., 2004. Molecular fluorescent approach to assessing intraerythrocytic hemoprotozoan *Babesia canis* infection in dogs. Vet. Parasitol. 125, 221–235.

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Vet. Parasitol. 124, 151–160.

Brandão, L.P., Hagiwara, M.K., Myiashiro, S.I., 2003. Humoral immunity and reinfection resistance in dogs experimentally inoculated with *Babesia canis* and either treated or untreated with imidocarb dipropionate. Vet. Parasitol. 114, 253–265.

Camacho-García, A.T., 2006. Piroplasma infection in dogs in northern Spain. Vet. Parasitol. 138, 97–102.

Campanella, J.J., Bitincka, L., Smalley, J., 2004. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinform. 4, 29.

Carret, C., Walas, F., Carcy, B., Grande, N., Precigout, E., Moubri, K., Schetters, T.P., Gorenflot, A., 1999. *Babesia canis canis*, *Babesia canis vogeli*, *Babesia canis rossi*: differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. J. Eukaryot. Microbiol. 46, 298–303.

Criado-Fornelio, A., Marinez-Marcos, A., Buling-Saraña, A., Barba-Carretero, J.C., 2003. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in sourthern Europe. Part I. Epizootiological aspects. Vet. Parasitol. 113, 189–201.

Criado-Fornelio, A., Rey-Valeiron, C., Buling, A., Barba-Carretero, J.C., Jefferies, R., Irwin, P., 2007. New advances in molecular epizootiology of canine hematic protozoa from Venezuela, Thailand and Spain. Vet. Parasitol. 144, 261–269.

Dantas-Torres, F., Figueredo, L.A., 2006. Canine babesiosis: a Brazilian perspective. Vet. Parasitol. 141, 197–203.

Depoix, D., Carcy, B., Jumas-Bilak, E., Pages, M., Precigout, E., Schetters, T.P., Ravel, C., Gorenflot, A., 2002. Chromosome number, genome size and polymorphism of European and South African isolates of large *Babesia* parasites that infect dogs. Parasitology 125, 313–321.

Dopazo, J., 1994. Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. J. Mol. Evol. 38, 300–304.

Furlanello, T., Fiorio, F., Caldin, M., Lubas, G., Solano-Gallego, L., 2005. Clinicopathological findings in naturally occurring cases of babesiosis caused by large form *Babesia* from dogs of northeastern Italy. Vet. Parasitol. 134, 77–85.

Garcia de Sá, A., De Mello Figueiredo Cerqueira, A., O'Dwyer, L.H., De Barros Macieira, D., Da Silva Abreu, F., Fernandes Ferreira, R., Müller Pereira, A., Bittencourt Velho, P., Pereira Almosny, N.R., 2006. Detection and molecular characterization of *Babesia canis vogeli* from naturally infected Brazilian dogs. Int. J. Appl. Res. Vet. Med. 4, 163–168.

Gubbels, J.M., de Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37, 1782–1789.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95–98.

Kjemptrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006. *Babesia conradae*, sp. Nov., a small canine *Babesia* identified in California. Vet. Parasitol. 138, 103–111.

Loretti, A.P., Barros, S.S., 2005. Hemorrhagic disease in dogs infected with an unclassified intraendothelial piroplasm in southern Brazil. Vet. Parasitol. 134, 193–213.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Vet. Parasitol. 122, 119–125.

Matjila, T.P., Nijhof, A.M., Taoufik, A., Houwers, D., Teske, E., Penzhorn, B.L., de Lange, T., Jongejan, F., 2005. Autochthonous canine babesiosis in The Netherlands. Vet. Parasitol. 131, 23–29.

Oyamada, M., Davoust, B., Boni, M., Dereure, J., Bucheton, B., Hammad, A., Itamoto, K., Okuda, M., Inokuma, H., 2005. Detection of *Babesia canis rossi*, *B. canis vogeli*, and *Hepatozoon canis* in dogs in a village of eastern Sudan by using a screening PCR and sequencing methodologies. Clin. Diagn. Lab. Immunol. 12, 1343–1346.

Passos, L.M., Geiger, S.M., Ribeiro, M.F., Pfister, K., Zahler-Rinder, M., 2005. First molecular detection of *Babesia vogeli* in dogs from Brazil. Vet. Parasitol. 127, 81–85.

Rzhetsky, A., Nei, M., 1992. A simple method for estimating and testing minimum evolution trees. Mol. Biol. Evol. 9, 945–967.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Schetters, T.P., Moubri, K., Precigout, E., Kleuskens, J., Scholtes, N.C., Gorenflot, A., 1997. Different *Babesia canis* isolates, different diseases. Parasitology 115, 485–493.

Shaw, S.E., Day, M.J., Birtles, R.J., Breitschwerdt, E.B., 2001. Tick-borne infectious diseases of dogs. Trends Parasitol. 17, 74–80.

Szabo, M.P.J., Mangold, A.J., João, C.F., Bechara, G.H., Guglielmone, A.A., 2005. Biological and DNA evidence of two dissimilar populations of the *Rhipicephalus sanguineus* tick group (Acari: Ixodidae) in South America. Vet. Parasitol. 130, 131–140.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. PNAS 101, 11030–11035.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Trapp, S.M., Messick, J.B., Vidotto, O., Jojima, F.S., Autran de Morais, H.S., 2006. *Babesia gibsoni* genotype Asia in dogs from Brazil. Vet. Parasitol. 141, 177–180.

Uilenberg, G., 2006. *Babesia*—a historical overview. Vet. Parasitol. 138, 3–10.

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Vet. Q. 11, 33–40.

Zahler, M., Schein, E., Rinder, H., Gothe, R., 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitol. Res. 84, 544–548.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. Vet. Parasitol. 89, 241–248.