PARASITOLOGICAL AND MOLECULAR CHARACTERIZATION OF A VENEZUELAN ISOLATE OF *Babesia canis*

Caracterización parasitológica y molecular de un aislado venezolano de *Babesia canis*

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ABSTRACT

Babesiosis is one of the most important tick-borne hemoprotozoan diseases that affects dogs worldwide. In the last years, there has been a sharp increase in positive cases of *Babesia canis* infection in the Falcon State, Venezuela. Although recent studies conducted in South America suggest that *B. canis vogeli* might be the species present in Venezuela, no data on the pathogenesis and molecular characteristics of Venezuelan piroplasms are available at this moment. By this reason the present work was undertaken to describe the evolution of an experimental infection induced in dogs, as well as the first molecular characterization of the 18S rRNA gene of the Venezuelan *B. canis* isolate used for inoculating the experimental animals. Experimental infection was carried out in four dogs, two of them splenectomized. The animals were inoculated with 1 x 10^6 *B. canis* infected-red blood cells. Parasitemia, packed cell volume and body temperature were measured daily. Prepatent period of the infection oscillated between 2-6 days. All of the inoculated dogs developed a mild-type disease. Symptoms of canine piroplasmosis were moderate, and main clinical findings consisted in low parasitemia, short febrile period and slight anaemia. Imidocarb dipropionate showed to be the treatment of choice, since it cleared parasites from blood, as demonstrated by PCR assays. The partial sequence of the ssu rRNA gene of the Venezuelan piroplasm showed 100% identity with a Brazilian isolate of *Babesia canis vogeli*, which is in full concordance with the clinical signs caused by this subspecies in the experimental animals, as well as those reported previously by other authors.

Key words: *Babesia canis*, Venezuela, molecular characterization.

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RESUMEN

La babesiosis, causada por un hemoparásito trasmitido a través de las garrapatas, es una de las enfermedades más importantes que afectan a los caninos mundialmente. En los últimos años se ha observado un aumento abrupto de los casos positivos a *Babesia canis* en el estado Falcón, Venezuela. Aunque los estudios recientes realizados en Suramérica sugieren que *Babesia canis vogeli* pudiera ser la especie presente en Venezuela, no existen datos disponibles sobre la patogénesis y las características moleculares de los piroplasmas venezolanos. Por esta razón se realizó el presente estudio, a fin de describir la evolución de una infección experimental inducida en caninos, así como realizar la primera caracterización molecular del gen 18S ARNr del aislado venezolano de *Babesia canis* utilizado para inocular los animales experimentales. La infección experimental se llevó a cabo en cuatro caninos, dos de ellos esplenectomizados. Los animales fueron inoculados 1 x 10^6 eritrocitos infectados con *Babesia canis*. La parasitemia, el hematocrito y la temperatura corporal fueron evaluados diariamente. El período prepatente de la infección osciló entre 2 y 6 días. Todos los caninos inoculados desarrollaron una enfermedad leve. Los síntomas de la babesiosis fueron moderados y los principales hallazgos consistieron en baja parasitemia, período febril corto y anemia leve. Se demostró, a través de las pruebas de PCR, la efectividad del dipropionato de imidocarb como tratamiento de elección para la eliminación de los parásitos sanguíneos. La secuencia parcial de la ssu rARN del piroplasma venezolano mostró 100% de identidad con un aislado brasileño de *Babesia canis vogeli*, lo que coincide plenamente con los síntomas causados por esta subespecie en los animales experimentales, así como por lo reportado previamente por otros autores.

Palabras clave: *Babesia canis,* Venezuela, caracterización molecular.

INTRODUCTION

Babesiosis in dogs is caused by hemoprotozoans such as *B. canis* and *B. gibsoni*. In Venezuela, *B. canis* is sometimes accidentally found in routine blood analysis but *B. gibsoni* has not been reported yet in the country. Otherwise, babesiosis is not a concern for veterinarians, probably due to existence of effective treatments against the parasite or to misdiagnosis. A significant increase in the levels of canine babesiosis has been observed in the Diagnosis Unit of Francisco de Miranda University, at Coro City (Falcón State). The prevalence observed between 2000 and 2004 was 1.6%, compared with no detection of piroplasms between 1993 and 1999 [13]. Even higher levels of parasitization by canine piroplasm (5.3%) were found by Pérez and Rey [12] in five localities from the Falcón State after performing a one-month random sampling. In the same study, they showed that 78% of the dogs infested with ticks had been treated by their owners with drugs showing no acaricidal effect at all. Whenever the parasite is diagnosed, imidocarb propionate is used most times for treatment. This product is apparently effective in healing the treated animals, but it is uncertain whether they remain as carriers or not after chemotherapy.

Based on serological studies, differences in pathogenicity and vectors, Uilenberg *et al* [20] proposed three subspecies of *Babesia canis*: *B. canis rossi*, transmitted by the tick *Haemaphysalis leachi* and causing an usually fatal infection in domestic dogs even after treatment; *B. canis vogeli* transmitted by *Rhipicephalus sanguineus* and leading to a mild disease, usually under the form of an unapparent infection, and *B. canis canis,* transmitted by *Dermacentor reticulatus* and showing a more variable pathogenicity. Interestingly, some authors have indicated the convenience of assigning the status of species to the three subspecies of *B. canis* [21]. Of these three subspecies, it seems that *B. canis vogeli* is the sole representative present in South America, as judged by molecular data reported by Passos *et al.* [11] in Brazil. It is likely that the same pathogen is present in Venezuela as judged by the pathological findings observed in daily veterinary practice.

Although the pathogenic events occurring in dogs naturally infected by *B. canis* in South America have been described previously [2], it was found that there were no previous reports on the formal description of the experimental infection caused by Venezuelan isolates of *B. canis*. Likewise, no molecular characterization of canine piroplasms has been done in the country. By this reason, an experimental infection study was conducted, monitoring evolution of parasitemia in four experimental dogs. In addition, the piroplasm isolate used in this study was characterized by PCR and sequencing of the 18S rRNA gene. Finally, the use of molecular methods allowed to ascertain whether chemotherapy was effective in eliminating parasites from the bloodstream and to determine if treated animals can be considered as potential carriers of canine piroplasm.

MATERIALS AND METHODS

Animals

Four mixed-breed healthy dogs, of approximately eight months of age, were used in the experiment. Two animals underwent splenectomy as a part of surgery teaching practice in the School of Veterinary Medicine. All the animals were individually kept in tick-free conditioned facilities that had been previously cleaned and disinfected with sodium hypochlorite (4%). The dogs were fed daily with standard amount of dry food. Drinking water was supplied *ad libitum.* The dogs were cared according with The Bioethical and Biosafety Code ruled by the Venezuelan Science and Technology Ministry [9].

Splenectomy

Each of two dogs were anesthetized with xilazine (2 mg/Kg, intramuscular route), sodium thiopental (25 mg/ kg, intravenous route) y atropine sulphate (0.04 mg/kg, subcutaneous route). Surgery was made according to Alexander [1]. After surgery, dogs were treated with gentamicin (3 mg/Kg, every 8 hours during three days) and flunixin meglumine (1.1 mg/Kg every 12 hours, two days).

Parasite isolation

Microscopic examination of Hemacolor® (Merck)-stained blood smear of a stray dog captured in Coro, Falcon State, revealed the presence of parasites from genus *Babesia*. The parasites showed the morphology (pear-shaped parasites in pairs) and size typical of a large *Babesia* species, as *Babesia canis.* No other hemoparasites were detected in the sample; however, the dog was treated with *per os* with doxycycline (10 mg/kg, 21 days) [15]. Fresh blood from this dog with parasitemia of 0. 1% was used for experimental infection.

Experimental infection

Both splenectomized and non-splenectomized animals were inoculated by intravenous route with 10 ml of fresh blood containing aproximately 1 x 10^6 *Babesia canis*-infected red blood cells [16]. Before the infection, microscopic examination of Hemacolor® (Merck)-stained buffy coat smear of every animal was made to verify absence of parasites. The day that parasites were inoculated was considered as the “day 0". The experiment finished at day 30 post-infection.

Blood sampling and clinical examinations

Venous blood samples were obtained from experimental animals in a daily basis. Parasitemia was detected over Hemacolor®-stained thin blood smears, by counting at least 1000 red blood cells and noting the number that were infected with *Babesia.* The results were expressed in percentage [14]. Packed cell volume of each blood sample was also estimated. The early morning body temperature was observed everyday.

Treatment

Dogs were treated with two doses of imidocarb dipropionate (6.6 mg/kg intramuscular route) with an interval of 15 days [3]. The splenectomized dogs were treated the day after the peak of parasitemia (FIG. 1). In non-splenectomized animals, a reduction of 50% of packed cell volume or the end of experimental trials (whichever occurred first) was the criteria of treatment. Dogs with fever were treated with dipirone (25 mg/Kg every 8 hours, intravenous route).

DNA isolation, amplification and sequencing

For parasite DNA analysis, blood samples were taken at the period of highest parasitemia. DNA was isolated from dog's blood with the aid of the Blood Spin kit (Mobio, Solana Beach, CA, USA). DNA amplification was performed with the following *Babesia* and *Theileria* primers: forward (BT-1F) = ggttgatcctgccagtagt; reverse (BT-1R): gcctgctgccttcctta [7]. This PCR assay should produce fragments of approximately 395 bp in *Babesia* spp.

Usual precautions to avoid DNA contamination (laminar flow hoods, separated work areas for reaction mixture preparation, DNA extraction, amplification and analysis of PCR products) were used in the laboratory to prevent carry over of amplified products [5]. The amplification mixture for single PCR contained 75 mM Tris, pH 9, 50 mM KCl, 2 mM $MgCl_2$, 20 mM $(NH_4)SO_2$, 200 μM each dATP, dCTP, dGTP and dTTP, 1 μM each primer and 1 unit of DNA polymerase (BioTools Labs, Madrid, Spain) in a final volume of 25 μl. Sample was overlaid with 50 µl of mineral oil to prevent evaporation. Amplification was carried out in an Perkin-Elmer 980 Thermal cycler (USA).

PCR diagnosis was performed with the following thermal cycling profile: hot start, 2 min 30 s at 96°C; 40 cycles of 50 s at 96°C, 1 min s at 60°C and 1 min at 72°C and a final extension of 6 min. at 72°C. Amplified products were separated in 5% polyacrylamide gels and visualized with ethidium bromide. The bands were recovered from 1.5% agarose gels with the Ultraclean 15 kit (Mobio, Solana Beach, CA, USA). Sequencing was carried out in an ABI 3130 (Applied Biosystems Inc.) automated sequencer. Products from at least two different amplifications were sequenced. DNA sequences were aligned using the CLUSTALW program [19]. The BIOEDIT program [8] was used to edit the sequences.

RESULTS AND DISCUSSION

Evolution of the parameters used to monitorize experimental infection

Parasitemia levels in experimentally-infected animals can be observed in FIG. 1. Five days after inoculation, parasites could be observed in blood smears from splenectomized dogs. The maximum level of blood parasites in these dogs was 2.3%, occurring 7 days after infection. In non-splenectomized animals, prepatent period oscillated between 2 to 6 days. The maximum parasitemia (3%) was detected three days after inoculation in a non-splenectomized dog, which also showed the shortest prepatent period.

The prepatent period observed in the experimentally-infected dogs (2-6 days) is in agreement with that observed for *Babesia canis* [17] although some authors reported a longer duration [18]. Parasitemia peaked around day 6 after inoculation in three of four dogs, which is in concordance with data published concerning other subspecies [16].

A moderate increase in body temperature was observed in splenectomized animals, starting from day 1 after inoculation (FIG. 2). The non-splenectomized animals presented intermittent fever during 9 days, starting from day 4. There was not a remarkable reduction in the PCV values in both groups of dogs (FIG. 3), with exception of a single animal from non-splenectomized group. The PCV average was lower in non-splenectomized group (33 ± 7.28) than in splenectomized one (34 ± 0.58).

It has been established that the pathology associated with *Babesia canis* in dogs varies with the infecting subspe-

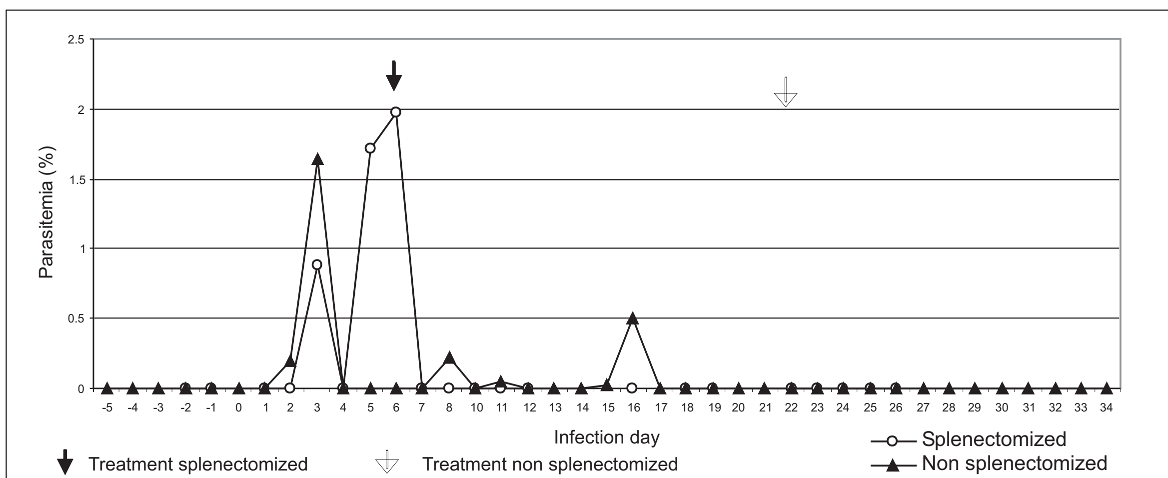

FIGURE 1. EVOLUTION OF PARASITEMIA IN DOGS INOCULATED WITH A VENEZUELAN ISOLATE OF *Babesia canis vogeli*. / EVOLUCIÓN DE LA PARASITEMIA EN CANINOS INOCULADOS CON UN AISLADO VENEZOLANO DE ***Babesia canis vogeli*.**

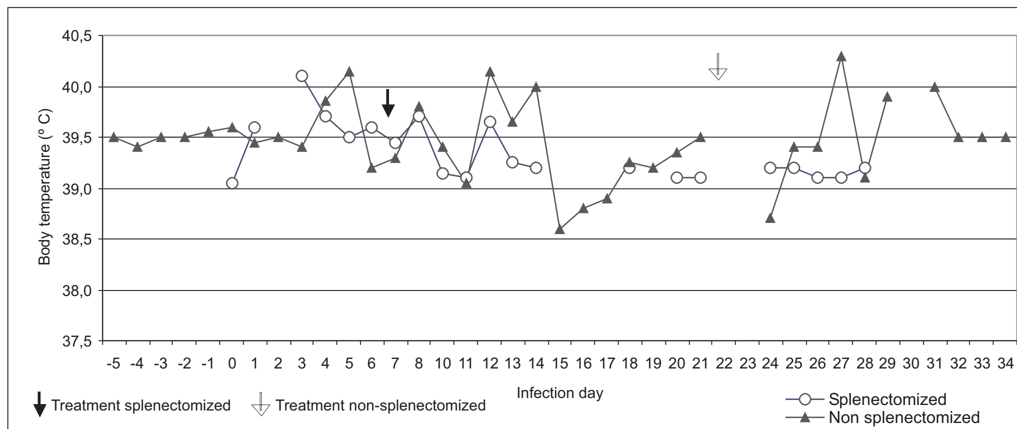

FIGURE 2. EVOLUTION OF BODY TEMPERATURE IN DOGS INOCULATED WITH A VENEZUELAN ISOLATE OF *Babesia canis vogeli* / EVOLUCIÓN DE LA TEMPERATURA CORPORAL EN CANINOS INOCULADOS CON UN AISLADO VENEZOLANO E *Babesia canis vogeli*.

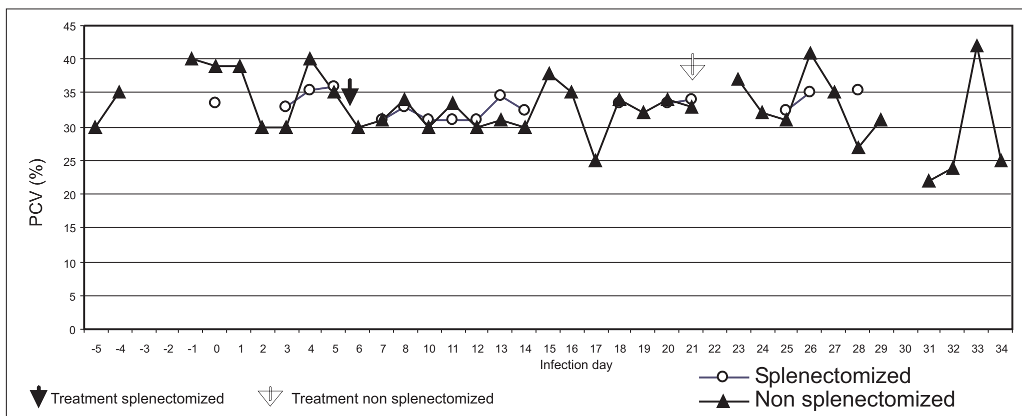

FIGURE 3. EVOLUTION OF PCV IN DOGS INOCULATED WITH A VENEZUELAN ISOLATE OF *Babesia canis vogeli*. / EVOLUCIÓN DEL HEMATOCRITO EN CANINOS INOCULADOS CON UN AISLADO VENEZOLANO DE *Babesia canis vogeli*.

cies. *Babesia canis vogeli*, transmitted by *Rhipicephalus sanguineus*, causes a mild, often clinically unapparent disease [21]. The experimental infected dogs, even the splenectomized, did not display severe clinical signs, as demonstrated by the low reduction of PCV values, short febrile periods and low parasitemia. One of the non-splenectomized dogs showed a shorter prepatent period, the lowest levels of haematocrit and the highest parasitemia, probably due to intrinsic features of this particular dog (breed, a weak immune status of the host through experimental trials or the presence of undetected concurrent disease). It can not be denied that the experimental animals would have been previously infected because blood obtained before inoculation in order to perform PCR assays was lost due to problems with the freeze-drying process. Moreover, there were no means whatsoever to check the serological status of the dogs in the laboratory. However, symptoms developed and prepatent period observed in experimental infections suggest that animals were negative before inoculation.

The mild symptoms observed in the four experimental dogs suggested that *B. canis vogeli* was the pathogen inoculated to the animals. Bicalho *et al.* [2], also reported low levels of parasitemia (0.01-2.34%) in capilar blood from dogs inoculated with 2.0 x 10^7 *B. canis* infected erythrocytes. Levels of parasitemia from venous blood were considerably lower (0.01-0.24%). Due to low parasitemia and absence of severe symptoms in the experimental dogs, they also suggested that the Brazilian isolates were *B. canis vogeli*.

Molecular characterization of the isolate

In Venezuela, two main parasite detection techniques are commonly used to diagnose *Babesia* infections in dogs, the microscopic examination of either thin full blood films or buffy coat thin smears [10]. The latter has the added advantage that the packed cell volume, and hence the level of anaemia, can be determined simultaneously in any animal diagnosed. Both techniques have also the disadvantage of a poor sensitivity. Moreover, subspecies of *B. canis* are morphologi-

cally indistinguishable by microscopy, and clinical features might be quite variable due to the different immunological/physiological responses observed among infected animals.

There is, however, a straightforward approach for a definitive diagnosis: the use of molecular methods [6, 7, 21]. A highly sensitive DNA-based test, the polymerase chain reaction, is not used yet in Venezuela for *Babesia* detection in canids. It can be assumed that many positive animals might be frequently misdiagnosed in the country.

Partial amplification of the 18S rRNA gene was obtained in the experimental dogs (FIG. 4). The amplified fragments (391 bp) were sequenced and BLASTN search in the GenBank database showed 100% identity to *B. canis vogeli* from Brazil (FIG. 5) (GenBank AY371196). The sequence of the Venezuelan isolate of *B. canis vogeli* was introduced in GenBank (accession number DQ297390).

The use of molecular diagnosis (PCR plus sequencing) demonstrated that the piroplasm used in the experimental infections was unequivocally *B. canis vogeli*, in agreement with data obtained by Passos *et al.* [11] in Brazil. The fact that *B. canis vogeli* isolates from Venezuela and other countries were identical in this partial sequence of the 18S rRNA gene indicates a highly-conserved region in this subspecies. Similar degree of sequence conservation among isolates has been found also in other piroplasm species like *T. annulata* [7].

Finally, there is an interesting application related to the use of molecular methods in hematozoan diagnosis: the confirmation of the curative effects of any chemotherapeutic assay.

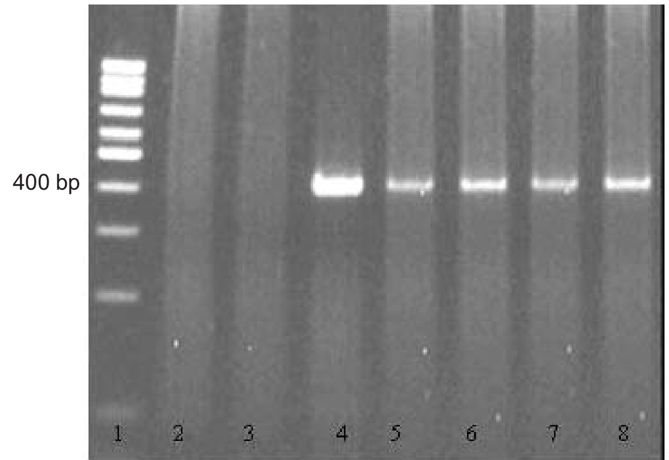

FIGURE 4. RESULTS OF PCR DIAGNOSIS IN 4 ANIMALS EXPERIMENTALLY INFECTED WITH A VENEZUELAN ISOLATE OF *Babesia canis.* LANE 1: MOLECULAR WEIGHT MARKER (100-1000 BP LADDER); LANES 2,3: NEGATIVE AMPLIFICATION CONTROLS; LANE 4, POSITIVE AMPLIFICATION CONTROL, *Babesia canis vogeli* SPANISH ISOLATE; LANES 5,6,7,8: DNA AMPLIFICATION FROM EXPERIMENTAL DOGS / RESULTADOS DEL DIAGNÓSTICO POR PCR EN 4 ANIMALES EXPERIMENTALES INOCULADOS CON UN AISLADO VENEZOLANO DE *Babesia canis.* CARRIL 1: MARCADORES DE PESO MOLECULAR (100-1000 PB); CARRILES 2,3: CONTROLES NEGATIVOS DE AMPLIFICACIÓN; CARRIL 4: CONTROL POSITIVO A *Babesia canis vogeli* AISLADO ESPAÑOL; CARRILES 5,6,7,8: AMPLIFICACIÓN DE ADN EN CANINOS EXPERIMENTALES.

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B. canis vogeli (Venez.)   GTTGATCCTGCCAGTAGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTAC 60
B. canis vogeli (Brasil)   GTTGATCCTGCCAGTAGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTAC 60
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B. canis vogeli (Venez.)   AAGCTTTTTACGGTGAAACTGCGAATGGCTCATTACAACAGTTATAGTTTATTTGGTATT 120
B. canis vogeli (Brasil)   AAGCTTTTTACGGTGAAACTGCGAATGGCTCATTACAACAGTTATAGTTTATTTGGTATT 120
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B. canis vogeli (Venez.)   CAGATTCAATGGATAACCGTGCTAATTGTAGGGCTAATACACGTTTGAGGTCTTTTGACC 180
B. canis vogeli (Brasil)   CAGATTCAATGGATAACCGTGCTAATTGTAGGGCTAATACACGTTTGAGGTCTTTTGACC 180
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B. canis vogeli (Venez.)   GCGTTTATTAGTTTGAAACCCGCCTTGGCTTTCGGTGATTCATAATAAACTGGCGAATCG 240
B. canis vogeli (Brasil)   GCGTTTATTAGTTTGAAACCCGCCTTGGCTTTCGGTGATTCATAATAAACTGGCGAATCG 240
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B. canis vogeli (Venez.)   CATTTAGCGATGGACCATTCAAGTTTCTGACCCATCAGCTTGACGGTAGGGTATTGGCCT 300
B. canis vogeli (Brasil)   CATTTAGCGATGGACCATTCAAGTTTCTGACCCATCAGCTTGACGGTAGGGTATTGGCCT 300
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B. canis vogeli (Venez.)   ACCGAGGCAGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAG 360
B. canis vogeli (Brasil)   ACCGAGGCAGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAG 360
                           ************************************************************

B. canis vogeli (Venez.)   ACGGCTACCACATCTAAGGAAGGCAGCAGGC 391
B. canis vogeli (Brasil)   ACGGCTACCACATCTAAGGAAGGCAGCAGGC 391
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FIGURE 5. DNA ALIGNMENT OF THE 18S rRNA GENE (PARTIAL SEQUENCES) OF *Babesia canis vogeli* FROM VENEZUELA (OBTAINED IN THE PRESENT WORK) AND BRAZIL (GENBANK ACCESSION NUMBER AY371196). THE CLUSTAL W SOFTWARE WAS USED IN THIS ANALYSIS / COMPARACIÓN DE LAS SECUENCIAS PARCIALES DEL GEN ARNr 18S DE *Babesia canis vogeli* DE VENEZUELA (OBTENIDA EN EL PRESENTE TRABAJO) Y BRASIL (GENBANK CON NÚMERO DE ACCESO AY371196) MEDIANTE EL PROGRAMA CLUSTAL W).

Imidocarb dipropionate was very effective in controlling parasitemia. PCR tests showed that parasites were absent from the blood of experimentally infected dogs one month after imidocarb treatment (FIG. 6). Similar results have been published by Birkenheuer *et al.* [4], although these authors treated a dog infected with *Babesia* spp. This has some interesting epizootiological consequences, since the application of chemotherapy leads unequivocally to a reduction in the number of carrier dogs (at least for some species of canine piroplasms).

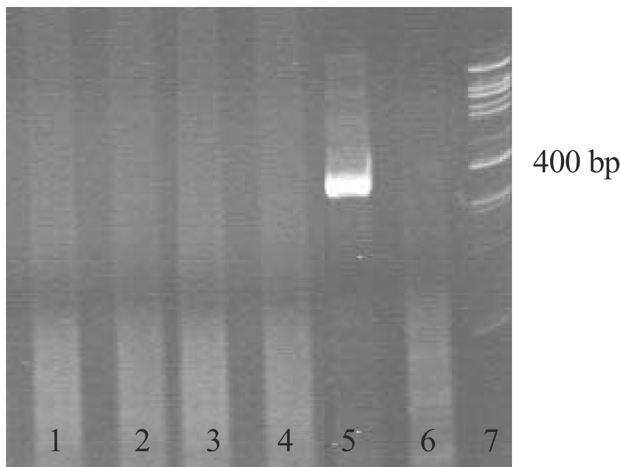

FIGURE 6. RESULTS OF PCR DIAGNOSIS IN 4 EXPERIMENTAL ANIMALS INOCULATED WITH A VENEZUELAN ISOLATE OF *Babesia canis* AND TREATED WITH IMIDOCARB DIPROPIONATE. LANES 1,2,3,4: AMPLIFICATIONS PERFORMED WITH DNA FROM THE TREATED ANIMALS; LANE 5: POSITIVE AMPLIFICATION CONTROL, *Babesia canis vogeli* SPANISH ISOLATE; LANE 6 NEGATIVE AMPLIFICATION CONTROL; LANE 7, MOLECULAR WEIGHT MARKERS (100-1000 bp LADDER). / RESULTADOS DEL DIAGNÓSTICO POR PCR DE LOS ANIMALES EXPERIMENTALES INOCULADOS CON UN AISLADO VENEZOLANO DE *Babesia canis* Y TRATADOS CON DIPROPIONATO DE IMIDOCARB. CARRILES 1,2,3,4: AMPLIFICACIONES REALIZADAS CON EL ADN DE LOS ANIMALES TRATADOS. CARRIL 5: CONTROL POSITIVO DE AMPLIFICACIÓN, *Babesia canis vogeli* AISLADO ESPAÑOL; CARRIL 6: CONTROL NEGATIVO DE AMPLIFICACIÓN; CARRIL 7: MARCADORES MOLECULARES (100-1000 PB).

CONCLUSIONS

Data obtained in the present report demonstrated the usefulness of PCR and sequencing to identify a single isolate of *B. canis vogeli*. Interesting parasitological data concerning evolution of canine piroplasmosis have been reported as well. However, the nature of the factors that caused the severe increase in prevalence detected in the Falcon State remain unknown, thus granting further epizootiological research in this region using molecular techniques.

ACKNOWLEDGMENTS

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