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SUPPLEMENT ARTICLE

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Molecular diagnosis of *Leishmania* spp. in dogs of a subtropical locality of Argentina

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Abstract

Leishmaniosis is a tropical and subtropical vector-borne disease caused by hemoparasites of the genus *Leishmania*. The disease can infect humans, as well as domestic and wildlife animals. Dogs are the main reservoir for *L. infantum*, the aetiological agent of visceral leishmaniosis (VL) in America, and a domestic source of *L. braziliensis*, the most widespread aetiological agent of American tegumentary leishmaniosis. Infected dogs can develop a clinical syndrome called canine leishmaniosis (CanL), which presents with skin lesions, mild fever; additionally hepatomegaly and splenomegaly can be observed, although asymptomatic infections are frequent. Direct microscopic observation of the parasite in bone marrow, blood, skin scrapings and conjunctival swab samples is the gold standard of diagnosis and is usually complemented with serological tests, and to a lesser extent, molecular detection of the parasite. In Argentina, leishmaniosis is an emerging disease, with a growing number of human and canine clinical cases since 2006. Our study was carried out in Mercedes, a town located in the subtropical north-eastern area of Argentina, where dogs with positive parasitological test results for *Leishmania* spp. must be euthanized according to local regulations. We evaluated the presence of *Leishmania* spp. DNA in the blood of dogs (n = 166) from urban and peri-urban zones. Genomic DNA was extracted from whole blood using Chelex 100 resin and a conserved 116 bp region of the kinetoplastid DNA was amplified by conventional PCR. Clinical signs, age and gender were recorded. Our results showed that 120 out of 166 surveyed dogs (72%) were positive for *Leishmania* spp. DNA of which only seven were positive by parasitological and serological tests. No significant correlation between positive cases and gender or age groups was found. This report shows the high prevalence of this disease in Argentina and contributes to improve public health policy with regard to diagnosis, prevention and treatment of infected dogs.

KEYWORDS

Argentina, dogs, leishmaniosis, molecular diagnosis, PCR, subtropical region

1 | INTRODUCTION

Leishmaniosis is a group of zoonotic diseases caused by flagellated hemoparasites of the genus *Leishmania*, which are transmitted through the bite of female sand flies to humans and other mammals. The disease is endemic in 98 countries and affects 12 million people around the world (WHO, 2010). According to the World Health Organization, there are 350 million people at risk and 2 million new cases reported each year. There are two main clinical forms of leishmaniosis: cutaneous (CL) and visceral (VL). The main clinical signs for CL are mucocutaneous and skin lesions, while VL can cause fever, hepatomegaly, splenomegaly, anaemia and progressive cachexia being lethal in 90% of untreated cases (Gould et al., 2013). In the tropical and sub-tropical regions of the Americas, *L. (Viannia) braziliensis* is the main causative agent of CL and is transmitted mainly by the sand fly vectors *Lutzomyia neivai*, *Lu. cortelezzi* and *Lu. migonei* (Barroso et al., 2015; Carvalho, Filho, Falcão, Rocha Lima, & Gontijo, 2008; Oliveira et al., 2011; Quintana, Salomón, Guerra, De Grosso, & Fuenzalida, 2013; Rosa et al., 2012). Its wildlife reservoirs are mainly small rodents and wildlife mammals (Dantas-Torres, 2007). Visceral leishmaniosis is mainly caused by *L. infantum* (syn. *chagasi*) and transmitted by *Lu. longipalpis* sand flies. Domestic dogs are very susceptible to this parasite and play an important role in the transmission of the disease to humans, since they act as reservoirs of infection in both rural and urban areas (Dantas-Torres, 2007). Dogs may suffer from a complex deadly syndrome referred to as canine leishmaniosis (CanL) that includes hair loss or scaling, weight loss, skin lesions, deformed and elongated nails (onychogriphosis), hepatomegaly, and splenomegaly. These clinical signs are caused both by *L. infantum* and *L. braziliensis* infections (Barroso et al., 2015; Dantas-Torres, 2009).

In Argentina, human VL emerged in 2006 in the subtropical north-eastern province of Misiones, and following this first case, dogs from surrounding areas tested positive for CanL by serology and microscopic observation of parasites (Salomon et al., 2008). By early 2015, 140 human cases had been registered in the subtropical provinces of north-west and north-east Argentina (Moya, Giuliani, Manteca Acosta, Salomón, & Liotta, 2015). Following identification of CanL in different cities of Misiones province until a latitude of 29° south, several clinical cases were identified in different locations of Corrientes province extending the distribution of leishmaniosis to a latitude of 28° south (Maidana, Llano, Báez, Cabrera, & López, 2011; Nevot, Rosa, & Eiras, 2013; Salomon et al., 2008). Lately, CanL cases due to *L. donovani* complex were reported in Mercedes, Corrientes (29°12′S/58°05′W). According to Mercedes city regulations, dogs with positive parasitological diagnosis of CanL must be euthanized. Since it is vital to know the dissemination of the disease in the city to adapt public policies to the current situation, the presence of *Leishmania* spp. DNA in the blood of dogs from Mercedes was evaluated in this study.

2 | MATERIALS AND METHODS

Mercedes urban and peri-urban area cover ~9.4 km^2 with a human population of 33,551 and around 15,000 dogs according to the census of the year 2011 (Sarmiento et al., 2011). The study area has a wet subtropical climate and is located at 96 metres above sea level (m.a.s.l.), with an average temperature of 19.7°C and an annual rainfall of 1,396 mm.

The survey included 166 domestic and stray dogs from urban and peri-urban areas of Mercedes. Sampling took place in the summer season (December–February) of 2015–2016 and 2016–2017. Two sets of four mL of blood were collected through venipuncture and stored at −20°C in tubes with and without EDTA until use. One mL bone marrow was collected from all dogs for parasitological diagnosis. The sampling location, clinical signs, age and gender were recorded. All procedures followed the protocol of the National Program for Leishmaniosis (MSAL, 2010).

Parasitological diagnosis was carried out by microscopic observation of amastigotes in Giemsa stained smears. Blood aliquots without EDTA were centrifuged, and serum was separated and analysed using the Kalazar Detect™ Rapid Test, Canine (InBios) which specifically detects antibodies against the rK-39 protein of the *L. donovani* complex including *L. infantum*, *L. donovani* and *L. archibaldi*.

Genomic DNA was extracted with Chelex®100 resin (Bio-Rad) using an optimized protocol previously standardized in our laboratory (Ascencio, Florin-Christensen, Schnittger, & Rodriguez, 2017). Briefly, 400 µl of 5 % Chelex®100 suspension was added to 40 µL of blood and mixed thoroughly. Samples were incubated for 10 min in a water bath at 100°C and then centrifuged at 13,600 × *g* for 10 min. The supernatant was collected and used as PCR template. *Leishmania* spp. DNA was detected by PCR using the 13A (5′-GTGGGGGAGGGGCGTTCT-3′) and 13B (5′-ATTTTACACCAACCCCCAGTT-3′) primers previously described by Reale et al. (1999), which amplify a 116 bp region of the kinetoplast minicircle. PCR conditions were similar to those described by Rodgers, Popper, and Wirth (1990). Briefly, reactions were carried out in a final volume of 12.5 µl, containing 1 × Green GoTaq buffer (Promega), 1.8 mM $MgCl_2$ (Fermentas), 0.2 mg/ml bovine seroalbumin, 0.2 µM of each dNTP, 0.28 µM of each primer, 0.025 U/mL of GoTaq DNA polymerase (Promega) and 1 µl of genomic DNA. One µl DNA from a positive dog as determined by serological and parasitological diagnosis was used as a positive control, and distilled water was used as a negative control. Amplification was carried out using the following conditions: initial denaturation for 3 min at 94°C, 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 10 min. Amplification products were run on a 1.8% agarose gel in the presence of ethidium bromide and visualized under UV light. GeneRuler 100 bp® DNA Ladder (Promega) and 1Kb Plus® DNA Ladder (Thermo Scientific) were used as DNA markers.

Statistical significances were determined by chi-square test.

3 | RESULTS

Twenty of the 166 dogs (12%) showed clinical signs consistent with leishmaniosis. Most common clinical signs included skin lesions in the ears, nose, periorbital areas together with peeling, hair loss, emaciation and onychogryphosis. Seven of these dogs tested positive for

Leishmania spp., by serological and parasitological determinations, while these tests were negative for all asymptomatic dogs.

Molecular diagnosis showed that 120 out of 166 dogs were positive for *Leishmania* spp. DNA. Positive cases were randomly distributed across the sampled area (Figure 1). The infection was not associated with the age of the dogs ($p > 0.05$). The percentage of positive animals were 74%, 67% and 79% for dogs of 0–3, 3–7 and older than 7 years of age, respectively. In addition, gender was not associated with infection ($p > 0.05$). Of the sampled dogs, 70% of males and 75% of females were determined positive. PCR-positive animals included 16 (13%) dogs with clinical signs of CanL and 104 (87%) asymptomatic animals. On the other hand, conventional PCR was unable to detect the parasite in the blood of four animals with clinical signs of leishmaniosis.

4 | DISCUSSION

At the beginning of this study, more than 20 *Leishmania* seropositive dogs had been reported in Mercedes as the result of a continuous containment strategy carried out by the local municipality and INTA. The current plan implies vector blocking through fumigation, data recording and sampling of stray and house dogs, direct observation of parasites and culling of *L. infantum*-positive dogs. Diagnosis of CanL is often difficult since pathological anomalies, clinical signs and biochemical parameters show a wide range, of which none is pathognomonic for the disease (Rodriguez, Estévez, Nevot, Barrios, & Florin-Christensen, 2018). Against this background, clinical examination needs to be complemented by laboratory diagnosis. Parasitological diagnosis remains the gold standard of *L. infantum* detection. However, since a bone marrow sample is required, owner's consent is difficult to obtain. Immunochromatographic tests based on rK39 detect antibodies against *L. donovani complex* (*L. infantum* is the only species within the complex that has been reported previously in America) with high specificity and sensitivity. However, their high cost hampers their application in epidemiological studies (Benitez, 2013). Molecular diagnosis is considered a complementary confirmatory test (Cortes, Rolão, Ramada, & Campino, 2004; Dantas-Torres et al., 2017; Gomes et al., 2007).

In this study, a direct PCR targeting kinetoplastid DNA (kDNA) was applied to gain insight into the spread of *Leishmania* spp. infection in the locality of Mercedes, Corrientes. This PCR has been reported to be highly sensitive, less expensive and easier to implement than other molecular, parasitological or serological determinations (Lachaud et al., 2002; Roura, Sanchez, & Ferrer, 1999). The application of an optimized protocol for DNA extraction using Chelex 100 resin allowed a reduced cost by a factor of 10 as compared to commercial DNA extraction kits (Ascencio et al., 2017). Surprisingly, most tested dogs in Mercedes were found to test positive for *Leishmania* spp. DNA. However, only a small fraction of PCR-positive animals were serologically and parasitologically positive for *L. infantum*. This result suggests co-existence of other *Leishmania* species in this area. Indeed, *L. guyanensis*, *L. amazonensis* and *L. braziliensis* have been detected in sand flies from Corrientes (Borda, Steindel, Rea, Campagnaro, & Miérez, 2013; Marco et al., 2005; Rea, Campagnaro, Borda, & Steindel, 2013), a city located 250 km from Mercedes. Further studies to identify the *Leishmania* species present in Mercedes dogs are underway in our laboratory.

With respect to vectors, *Lu. longipalpis* was the only sand fly species reported in Mercedes until 2013 (Miérez, Rea, Borda, & Mosqueda, 2013; Salomón et al., 2010; Sarmiento et al., 2011), but later, also *Nyssomyia neivai* and *Migonemyia migonei*, which can transmit the above mentioned *Leishmania* species, were reported in close locations (Berrozpe et al., 2017).

No significant correlation between PCR-positive dogs versus gender or versus age could be observed. A previous epidemiological study carried out in Spain with the same age groups showed higher infection rates among dogs younger than 3 and older than 7 years of age. Our results showed a similar, yet non-significant statistical, trend.

Regarding clinical signs, most (83%) of the PCR-positive dogs were asymptomatic. Similar results have been found in other epidemiological studies in South America (Padilla et al., 2002; Dantas-Torres et al., 2010; Oliveira et al., 2016). Although asymptomatic infections could be due to *Leishmania* species with lower pathogenicity, the occurrence of subclinical *L. infantum* infections has been reported (Michel, Pomares, Ferrua, & Marty, 2011; Porrozzi et al., 2007).

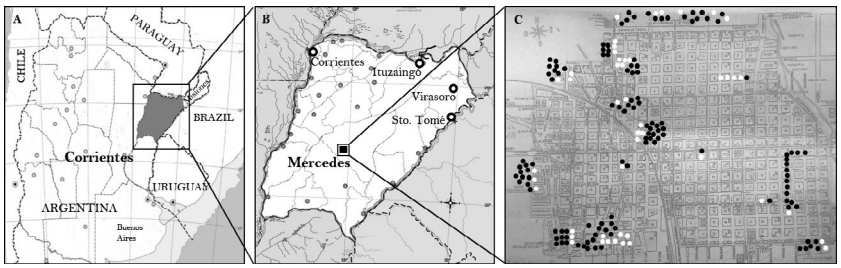

FIGURE 1 (a) Map of Argentina and bordering countries. The province of Corrientes is marked in grey and is crossed by the parallel 28° south. (b) Map of Corrientes province with the localizations of four cities where canine leishmaniosis has been reported: Corrientes (province capital), Ituzaingó, Virasoro (Gobernador Virasoro) and Sto. Tomé (Santo Tomé) marked by black rings. Mercedes city is marked by a black square dot. (c) Map of sampling area in and around Mercedes city showing the distribution of positive (black dots) and negative (white dots) canine leishmaniosis cases

This is the first molecular study of CanL in Mercedes, Corrientes. It contributes to the integrated plan of prevention and control of canine and human leishmaniosis in the city and surrounding regions, and supports the complementary use of molecular techniques in the screening diagnosis of dogs. Our study demonstrates that Mercedes is an area of *Leishmania* endemicity. Several studies have demonstrated the ineffectiveness of the euthanasia of *L. infantum*-positive dogs in regions where the disease is endemic (Marcondes et al., 2013; Otranto & Dantas-Torres, 2013; Solano-Gallego et al., 2017). Additional investigations are required to assess the necessity of culling of infected dogs as a preventive measure for canine and human leishmaniosis and to adapt public and veterinary health policies accordingly.

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ETHICAL STATEMENT

The authors declare that ethical statement is not applicable because sample collection or questionnaires from animals has been gathered.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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