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Canine infection and the possible role of dogs in the transmission of American tegumentary leishmaniosis in Salta, Argentina

A.M. Padilla [a], J.D. Marco [a], P. Diosque [a], M.A. Segura [a], M.C. Mora [a], M.M. Fernández [b], E.L. Malchiodi [b], M.A. Basombrío [a,*]

[a] *Laboratorio de Patología Experimental, Facultad de Ciencias de la Salud, Universidad Nacional de Salta, Calle: Buenos Aires 177, 4400 Salta, Argentina*

[b] *Instituto de Estudios de la Inmunidad Humoral (IDEHU), CONICET-UBA, Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 4to piso, 1113 Buenos Aires, Argentina*

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Abstract

Some *Leishmania* species affect humans in two principal forms: visceral and cutaneous leishmaniosis (CL). Several studies have identified dogs as the main reservoirs of the visceral leishmaniosis (VL) caused by *Leishmania infantum*. The purpose of this work was to carry out a survey of the canine population associated with human cases of American tegumentary leishmaniosis (ATL), in order to establish the clinical, parasitological, serological and immunological characteristics of the canine disease, in an endemic region for both ATL and Chagas' disease in the province of Salta, in northwestern Argentina. Two hundred and eight dogs from the endemic area were examined and 41 (19.7%) of them presented lesions compatible with leishmaniosis. In order to investigate the presence of antibodies against *Leishmania* spp. and *Trypanosoma cruzi*, sera were screened by ELISA using two complex antigens from these parasites and, because of cross-reactions between them, a specific antigen for diagnosis of *T. cruzi* infection. Sixty-two (29.8%) of 208 dogs were positive for the complex antigen F45 from *Leishmania* and 50 (24%) were positive for the complex antigen F105 from *T. cruzi*. Nine dogs (4.3%) were positive for the specific Ag163B6-cruzipain suggesting that these dogs were truly infected with *T. cruzi*. Furthermore, three of these nine dogs presented *Leishmania* sp. in their skin lesions and therefore were considered as infected by both, *T. cruzi* and *Leishmania* parasites. The prevalence of *Leishmania* infection detected by lesions and/or positive serology was 27.4% (57/208). On the basis of previous observations regarding the clustered appearance of human ATL, the dog population was divided into two groups: zone A, dogs living within a 100 m radius from houses with human cases, and zone B, dogs living beyond this

* Corresponding author. Tel.: +54-387-4255333; fax: +54-387-4255333.
E-mail address: basombri@unsa.edu.ar (M.A. Basombrío).

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limit. The prevalence of ATL in dogs was significantly higher in zone A (34.6%) than in zone B (7.3%), suggesting a strong correlation between canine and human cases. The average time required for a parasitological diagnosis by microscopy was six times longer for dog samples than human ones, and the average number of parasites per 100 microscopic fields was 14-fold lower in canine samples. The high prevalence of *Leishmania* infection and the close association with human cases, demonstrated that dogs are a very susceptible host for *Leishmania* infection, but the scarcity of parasites in their lesions suggests that they may not be the main reservoir of the parasite in this endemic area.

Keywords: Tegumentary leishmaniosis; Reservoirs; Transmission; Dogs

1. Introduction

Leishmania infection affects humans in two principal forms: visceral leishmaniosis (VL) and cutaneous leishmaniosis (CL). American tegumentary leishmaniosis (ATL) is caused by several parasites of the genus *Leishmania*, producing skin ulcerations which heal very slowly. The most serious complication of this disease is the involvement of mucosae and cartilage with destruction of nasal and pharyngeal structures. Dogs have been identified as the main reservoirs of VL caused by *Leishmania infantum* and parasites have been found in these animals in many internal organs, blood, skin and even saliva (Deane, 1956; OMS, 1990; Abranches et al., 1991). Dogs infected with ATL show similar features to the human disease, such as rounded and raised-edge ulcers that last long time without healing, enlargement of lymph nodes, alopecia and presence of parasites in the ulcers. These shared characteristics between infected humans and dogs may imply that dogs either play a role in the transmission of the disease or that they are simply as susceptible to infection as humans, without a fundamental implication in the transmission.

The high prevalence of infection among dogs from endemic zones, the close relationship between human and dog cases and the identity of parasites infecting humans and dogs, suggest that this disease possibly involves these animals as reservoirs (Aguilar et al., 1989; Lainson et al., 1994; Falqueto et al., 1986, 1991; Yoshida et al., 1990). There is evidence of the role of dogs as reservoirs of CL, supported mainly by the correlation between cases in human and dogs detected by clinical and serological analysis; but the actual role of dogs in the transmission remains unclear (Reithinger and Davies, 1999).

In northwestern Argentina several outbreaks of ATL have been observed, mainly in areas of deforestation of autochthonous jungle. Mazza (1926) in Salta and Romaña et al. (1949) in Tucumán described many human cases, which occurred in association with canine cases, but because of low proportion (5.1%) of dog lesions that presented parasites, the role of this animal as reservoirs could not be convincingly established. In the province of Salta, a remarkable increase of ATL cases has been observed after 1978. This has been attributed to the extensive deforestation performed in the endemic foci.

The purpose of this work was to investigate the occurrence of canine infection in areas where human ATL occurs, and to establish the clinical, parasitological, serological and immunological characteristics of canine ATL.

2. Materials and methods

2.1. Study area

This work was performed in the localities of Colonia Santa Rosa, Pichanal and Orán, Province of Salta, Argentina. This area is included within the biogeographic "Yungas" jungle and was deforested in recent decades to allow agriculture and human settlement. An urban epidemic outbreak of ATL was detected in Pichanal in 1985. Since then, new cases have been recorded every year. This geographic region is also an endemic area for Chagas' disease.

2.2. Sample collection

A house to house survey was carried out, selecting dwellings where sanitary agents had reported human cases of ATL. Transmission sites were identified through questionnaires addressed to patients and doctors. All dogs of affected houses were studied and data on habitants (persons and animals) were recorded.

2.3. Clinical examination of the dogs

The entire skin surface was carefully inspected in search for lesions or scars. Particular attention was paid to the limbs, ears, nose and scrotum, since ulcerous lesions were most often found in these areas. Palpation of liver, spleen and popliteal-prescapular lymph nodes was attempted in every dog and the eventual enlargement of these organs was recorded. External signs of VL such as dermatitis, onychogryphosis and conjunctival lesions were recorded. The criteria used to define a lesion as "compatible with leishmaniosis" were: usually rounded shape, raised, indured edges and slow or no healing. Lesions clearly caused by trauma, or surrounded by flat, irregular and smooth edges were considered as not due to *Leishmania* infection.

2.4. Serological methods

As the surveyed area is endemic for leishmaniosis and Chagas' disease, we used a specific antigen for *Trypanosoma cruzi* in the ELISA test in order to determine the Chagasic dogs and avoid false positive results by cross-reaction. Sera from two hundred and eight dogs living in the area of study were analyzed. Indirect enzyme linked immunosorbent assays (ELISA) for antibody detection were used. Briefly, polystyrene plates (NUNC, Roskilde, Denmark) were sensitized with three different antigens: 4 μg/ml of the complex antigenic fractions F105 of *T. cruzi*, F45 of *Leishmania mexicana* (Malchiodi et al., 1994), or 1 μg/ml of the purified Ag163B6-cruzipain from *T. cruzi* (Carbonetto et al., 1990; Malchiodi et al., 1993). This antigen does not react with anti-*Leishmania* antibodies. Sera were analyzed at 1/1000 dilution. Peroxidase-conjugated rabbit immunoglobulins to dog IgG (whole molecule) (Sigma) was used as the second antibody at 1/25,000 dilution. Sera having an absorbance higher than the mean absorbance of normal sera, from dogs living in non-endemic area, plus three standard deviations were considered positive.

2.5. Parasitological examination

Lesion sites were locally anesthetized with 2% xylocaine–epinephrine and disinfected with alcohol. Four different methods were compared regarding their sensitivity for parasite detections: touch print (TP), obtained by touching a slide with a skin biopsy obtained with a steel punch from skin of the edges of the lesion; scraping (S), which consisted of scraping the ulcer margin with a woodstick and smearing the obtained material onto a slide; exudate (E), which consisted of collecting blood and exudates from the ulcer bed with a glass capillary tube, spinning down the fluid and smearing the leukocyte-rich interface on a slide and aspirate (A), obtained by injecting 0.1–0.4 ml of buffered saline solution plus penicillin–streptomycin into the edges of lesion, aspirating the fluid and smearing it on a slide.

The obtained material from biopsies and aspirates was cultured in LIT (Liver Infusion-Tryptose plus 10% Fetal Bovine Serum) or Schneider's medium.

2.6. Delayed-type hypersensitivity reaction

Leishmanin was prepared as a suspension of phenol-inactivated (WHO, 1996), LV 135 strain of *Leishmania pifanoi* promastigotes and 0.1 ml containing 25×10^6 inactivated parasites was injected intradermally in the hairless abdominal skin of the dogs. After 24–48 h, the induration was measured with a millimeter ruler after palpation and delineation of the infiltrate with a ball point pen. Thirty-five dogs were analyzed by this method.

2.7. Human samples

To compare the concentration of parasites between human and canine ulcers, sample smears were taken from seven *Leishmania* infected humans living in the region where the canine survey was carried out. Smears of human ulcers were made in a way similar to dog samples. Material was obtained from the ulcer margin with a woodstick and smeared onto a slide, afterwards the slides were stained by Giemsa.

2.8. Statistical analysis

Frequencies were paired in 2×2 tables and the probability of finding significant differences was calculated with the Fisher's exact test using the Epi Info6 Software.

3. Results

3.1. Clinical observations

Out of 208 examined dogs, 41 (19.7%) presented lesions compatible with ATL. Localization of the lesions was as follows: 28 in the ears, 18 on croups or legs, 10 on the face, 6 on the scrotum, 4 on the tail and loin, and 3 in the nose (muco-cutaneous) with destruction of the nasal septum. Twenty dogs presented more than one lesion. Swelled lymphatic nodes or external signs of VL were not observed.

Table 1
Percentages of *Leishmania* detection with each parasitological method

Method[a]	Positive test	Total tests[b]	%Positives
TP	8	10	80.0
S	15	24	62.5
E	2	7	28.6
A	0	8	0.0

[a] TP: touch print; S: scraping; E: exudate and A: aspirate. See Section 2.5 for description of each method.
[b] Total tests carried out in lesions compatible with leishmaniosis.

3.2. Serology

In order to investigate the presence of antibodies against parasite antigens, sera were analyzed by ELISA. Sixty-two (29.8%) out of 208 dogs from the endemic area were positive for the complex antigen F45 from *Leishmania*; 50 (24%) were positive for the complex antigen F105 from *T. cruzi*. Nine dogs (4.32%) were positive for the specific antigen Ag163B6-cruzipain suggesting that these dogs were infected by *T. cruzi*. The positive dogs for the purified antigen Ag163B6 were considered as chagasic and were not included in the leishmaniosis group, although eight of them were positive for complex antigen F45. Since three of these seven positive dogs for Ag163B6 presented parasites in their skin lesions, they were considered as doubly infected by *T. cruzi* and *Leishmania*.

3.3. Parasitological diagnosis

Microscopical observation of stained material from lesions showed that intact parasites were very scarce in dog lesions, disregarding size and time of evolution. Parasites were detected in only 14 (45.2%) out of 31 lesions studied by one or more methods. Table 1 summarizes the percentages of detection of *Leishmania* with each of the methods. The most practical and efficient were S and TP. Both extra and intracellular amastigotes were found more often in TP than in S, although S was easier to perform. All cultures were negative, even from material confirmed as positive in smears. Consequently, we were unable to isolate and characterize parasites from canine lesions, the main reason for this was contamination of the cultures with bacteria or fungi due to superinfection of these dogs lesions.

Comparison was made between Giemsa stained positive slides (S) of 12 canine and 7 human samples of patients from the same area, measuring the time of microscopical observation needed to establish a positive diagnosis and the density of parasites (number of parasites per 100 microscopic fields). For human patients, these averages were 10.7 min and 3.85 parasites per 100 fields, respectively, whereas the canine samples averaged 65.6 min and 0.26 parasites per 100 fields.

3.4. Correlation between clinical, serological and parasitological findings

All dogs bearing lesions with confirmed parasites (14/14) and 11 out of 17 dogs without detectable parasites had positive serology. The association between positive serology and parasite detection is significant at the $P = 0.017$ level.

Table 2
Distribution of seropositivity in dogs by age

Age (years)	Seropositive dogs	Total dogs	%Seropositive
0.0–1.5	9	84	10.7
1.5–3.0	12	35	34.3
3.0–6.0	21	57	36.8
6.0–9.0	15	32	46.9
Total	57	208	

All dogs (41/41) presenting lesions compatible with ATL were also seropositive. The percentage of dogs with positive serological test without visible lesion was 28.1% (16/57). Most dogs not bearing lesions (151/167) were seronegative. The prevalence of *Leishmania* infection, as detected by lesions and/or positive serology was 27.4% (57/208). The association between positive serological tests and presence of lesions compatible with ATL was significant at the $P < 10^{-7}$ level.

3.5. Skin reactions

The leishmanin test was applied to 35 dogs. In five of nine seropositive dogs, the diameter of the induration exceeded 5 mm and in three dogs, it exceeded 3 mm. No induration was observed in normal seronegative dogs living in non-endemic area or in dogs without any *Leishmania*-compatible lesions and negative serology.

3.6. Distribution by sex and age

No significant difference in the prevalence of *Leishmania* infection in dogs was detected between sexes. Table 2 shows the percentages of seropositivity in dogs at different ages. Results indicate that cases of infection progressively increase as dogs grow older. The age interval where most lesions were found was 3–6 years (80.9%).

3.7. Distribution by areas

On the basis of previous observations regarding the clustered appearance of human ATL, the dog population was divided in two zones: zone A, dogs living within a 100 m radius from houses with human cases, and zone B, dogs living beyond this limit. The prevalence of ATL in dogs was significantly higher in zone A, 34.6% (53/153) as compared to zone B, 7.3% (4/55), indicating a strong correlation between canine and human cases. Only 7% (4/57) of the seropositive dogs were in zone B as compared to 93% (53/57) for zone A. There was strong association between seropositivity and zone A ($P < 10^{-3}$).

4. Discussion

The focal occurrence of ATL in human populations suggests that an unidentified reservoir may be maintaining the parasite and transmitting it to insect vectors. Several investigators

(Tolezano, 1994; Brandão-Filho et al., 1994; Yoshida et al., 1990; Coutinho et al., 1985; Deane, 1956) focused their studies on domestics animals in search of such reservoirs which, apparently, varied by country and region. The role of dogs in ATL transmission had been studied mainly in Brazil (Aguilar et al., 1987, 1989; Pirmez et al., 1988; Coutinho et al., 1985). In Argentina, these animals have not previously been studied systematically for ATL, although occasional descriptions of infected dogs (Mazza, 1926; Romaña et al., 1949) accompanied epidemiological studies of human cases. This work aimed to systematically survey hundreds of dogs using epidemiological, clinical, serological and parasitological approaches.

Since dogs are very often found infected with *T. cruzi* (Basombrío et al., 1993) and because the area under study is an endemic region for Chagas' disease, we decided overcome the serological cross-reactivity between these parasites by using three different antigens in ELISA tests. Besides, as previously reported in human cases by Chiaramonte et al. (1996), we found three dogs with amastigotes in their skin lesion having a positive test for Ag163B6, thus indicating the presence of a mixed infection by *T. cruzi* and *Leishmania*. The percentage of double infection in humans of this area was higher than in dogs.

Several observations of this work are relevant to the question whether dogs act as reservoirs, disseminating the infection or are just susceptible to *Leishmania* without having a major role in the spread of the disease.

We found a 27.4% prevalence of leishmaniosis by serology using ELISA and all the dogs presenting *Leishmania* compatible lesions were seropositive. These results are similar to those obtained by Santos Barboza et al. (1998), who found 25.5% of prevalence by immunofluorescence (IFA) in Brazil. We carried out IFA in 104 out of 202 dogs, finding that this test is highly specific (96%) but not sensitive (26%) (data not shown).

The distribution of lesions on the animal's skin in our study is similar to that described by Pirmez et al. (1988), e.g. lesions predominating in the inner side, edges and tip of the ears. In this study, the lesions found in the scrotum, although they were not very frequent (6/69), presented the typical aspect of a leishmanial lesion, rounded and limited by elevated, hard edges. In our survey, dogs showed a weak delayed type hypersensibility response to leishmanin. Thus, the skin reaction technique had a low efficiency, in spite of the high concentration of parasites used in the preparation.

Samples obtained from lesion in dogs showed very low amounts of parasites when compared to samples obtained from human patients, no matter what method was used. Therefore, the average time needed to reach a positive diagnosis was six times longer for dogs than for humans and the average number of parasites per 100 fields was 14-fold lower. In previous studies carried out in Brazil and Venezuela (Falqueto et al., 1986; Aguilar et al., 1989), parasite density in the samples from dogs, was also very low, even in recent lesions (Oliveira-Neto et al., 1988). This is not the case in visceral leishmaniosis, where abundance of infected dogs, coincidence of human and canine disease and abundance of parasites in skin or lesions are consistent with the role these animals play in parasite transmission (Abranches et al., 1991; Deane, 1956; Gradoni et al., 1988).

Although a few human cases of VL were described years ago in the area of the present study, the etiological agent was not identified (Salomón et al., 2001). Neither was canine Kala-Azar described. However, bearing in mind the expansive character of this zoonosis and

its current expansion to the south of Paraguay (Canese, 2000), every dog in this study was examined for its clinical signs. The clinical pattern (skin ulcers without apparent visceral compromise) was in all cases that of the tegumentary form, and not of the visceral form of the disease.

In the area under study, previous work showed that *L.* (*Viannia*) *braziliensis* is the causative agent of human leishmaniosis (Cuba et al., 1996; Sinagra et al., 1997; Segura et al., 2000). More recently, an extensive taxonomic study of the species in the area showed that 20% of the isolated parasites were *Leishmania amazonensis* (Malchiodi, personal communications). The remarkable similarity of lesions and the close association found between the presence of infected humans and dogs in the same house, strongly suggests that infection in dogs and humans is caused by a common agent; although we are unable to confirm this by identification of parasite isolates.

The cases of canine ATL observed in the present study occurred in close association with human cases and were limited to a close distance, since 93% (53/57) of the seropositive dogs belonged to zone A. In many semi-rural areas, infection of humans and dogs seemed connected to the habit of taking the dogs along to work in deforested areas. Although human and canine cases of ATL are closely associated, the low burden of parasites found in dogs may not be consistent with the possible role of these animals as reservoirs. In ATL, high seroprevalence rates in dogs are not sufficient to establish that these animals are an important source of infection for vectors (Reithinger and Davies, 1999; Vexenat et al., 1986).

Besides the abundance of parasites in lesions, other factors are important for transmission from animals to man, such as the preference of phlebotomine insects for feeding on particular species of animals or sites on the skin. Some experimental studies (Vexenat et al., 1986) showed that vectors become infected mainly by feeding on lesions. The long persistence of the ulcers, could balance the scarcity of parasites available to the vectors and its effect in the transmission would be further studied. Thus, according to our results, it is possible that the main direction of transmission may be from man to dog, rather than the other way around. The results presented in this work suggest that dogs are important indicators of the occurrence of human ATL in each house. However, these animals may not be the main reservoir disseminating the parasite but a highly susceptible host possibly acquiring infection from nearby human or sylvan reservoirs.

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