

## SPECIES ASSIGNATION OF *LEISHMANIA* FROM HUMAN AND CANINE AMERICAN TEGUMENTARY LEISHMANIASIS CASES BY MULTILOCUS ENZYME ELECTROPHORESIS IN NORTH ARGENTINA

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**Abstract.** Sixteen *Leishmania* stocks, 15 isolated from patients with cutaneous (CL), mucocutaneous (MCL), or recurrent cutaneous leishmaniasis, plus one from a dog with CL in Salta and Corrientes Provinces, Argentina, were studied by multilocus enzyme electrophoresis. Thirteen of the stocks from humans were grouped in two zymodemes; nine termed as KMS 1, four as KMS 2, and assigned to *Leishmania (Viannia) braziliensis*. Two additional stocks from CL cases expressed a KMS 4 enzyme profile, corresponding to *L. (V.) guyanensis*. Although the parasites from the dog were also assigned to *L. (V.) braziliensis*, its zymodeme, KMS 3, was not expressed in any of the current human isolates. The characterization of *Leishmania* from a dog was done for the first time in Argentina. The importance of the intraspecific polymorphism in the induction of clinical forms and in the host-reservoir concept is briefly discussed, based on the zymodeme data of isolates from humans and dogs. The presence of *L. (V.) guyanensis* was confirmed in the country.

### INTRODUCTION

American tegumentary leishmaniasis (ATL) is a group of zoonotic diseases widespread in 22 countries from Texas (USA) to northern Argentina. It is caused by flagellar protozoa belonging to the genus *Leishmania*<sup>1</sup> in which a tremendous diversity or polymorphism has been found.<sup>2</sup> The diseases caused by different species are distinct in their clinical manifestations, drug responses, and prognosis. Therefore, it is necessary to characterize and identify specifically the parasites obtained in each endemic area where a survey is taken.<sup>3</sup> For this purpose, multilocus enzyme electrophoresis (MLEE) analysis has been recommended by the World Health Organization (WHO).<sup>4</sup>

In northern Argentina, some areas have been reported to be endemic for human and canine leishmaniasis. However, little is known about the causative agents, *Leishmania* spp., and the transmission cycles implicated there. Salta and Corrientes Provinces are endemic areas, where most of the clinical forms of human ATL showed single and multiple cutaneous (CL), mucocutaneous (MCL), or mucosal lesions (Borda CE and others, unpublished data).<sup>5,6</sup> Vegetant nonulcerated lesions with metastasis to the lymph nodes and viscus (spleen) have also been described.<sup>7</sup> As to the causative parasites, it was found that *L. (Viannia) braziliensis* was predominant in Salta Province, followed by *L. (Leishmania) amazonensis*, and *L. (V.) guyanensis*, though relatively few isolates were specifically characterized to date.<sup>2,8,9</sup>

Previously, we studied the clinical, parasitological, and serological characteristics of canine tegumentary leishmaniasis in Salta Province. Several observations from these works suggested that dogs might be considered as a susceptible host without having a principal role in the spread of the disease.<sup>10,11</sup> However, any *Leishmania* stock was not isolated from dogs in this area before.

This paper reports the species attribution and the examinations of the relationship between *Leishmania* isolates from different clinical forms of human and canine ATL in northern Argentina, based on MLEE data; the first description of ca-

nine *Leishmania* isolate in the country is herein provided. The relevance of the intraspecific polymorphism to the induction of different clinical forms, as well as its incidence in the host-reservoir concept, is briefly discussed.

### MATERIALS AND METHODS

**Parasites and study area.** Sixteen stocks of *Leishmania* were included in this study. Fourteen of them, from 11 humans, and one stock from a dog were isolated in Salta Province, northwest Argentina. The remaining one was obtained from a patient in Corrientes Province, located in the northeast of Argentina. Their denomination, the clinical data of the host, and a description of the geographical origin are shown in Table 1 and Figure 1. The samples were taken from patients who were accessed in the four institutions, viz., the Instituto de Investigaciones en Enfermedades Tropicales, Subse de Orán, Universidad Nacional de Salta; the Dermatology Ward of Hospital del Milagro; the Dermatology and Otorhinolaryngology Wards of Hospital San Bernardo of Salta City; and Centro Nacional de Parasitología y Enfermedades Tropicales, Universidad Nacional del Nordeste, Corrientes City. A part of the current isolates was obtained from humans and canine cases during house-to-house visits.

All the referred patients, from whom *Leishmania* parasites were isolated from their lesions, were systemically treated for 25–30 days with 10–20 mg d<sup>-1</sup> kg body wt<sup>-1</sup> of pentavalent antimony for each cycle. In the cases of the incomplete clinical cure of lesion, another cycle of treatment or amphotericin B treatment was given.<sup>4</sup> The clinical controls and treatment protocols were exclusively conducted for local physicians. The patients voluntarily consented to participate in this study, and the Bioethical Commission of Health Ministry of Salta Province approved the procedures.

Six WHO reference strains were included; *L. (V.) braziliensis*, MHOM/BR/75/M2904; *L. (V.) panamensis*, MHOM/PA/71/LS94; *L. (V.) guyanensis*, MHOM/BR/75/M4147; *L. (L.) amazonensis*, MHOM/BR/73/M2269; *L. (L.) mexicana*,

TABLE 1  
Zymodemes and species assignment of 16 Argentinian *Leishmania* stocks and clinical-epidemiological aspects of the hosts

Zymodeme	Species	Designation	Clinical form, lesion features	Lesion locations	Evolution (days)	Sex	Age (years)	Geographical origin
KMS1	<i>L. (V.) br</i>	MHOM/AR/99/AZ3	CL, ulcer	Right wrist	30	M	20	Salta
KMS1	<i>L. (V.) br</i>	MHOM/AR/02/NSS3	MCL, ulcerative-infiltrative lesion	Naso-oropharynx, larynx	1080	M	65	Salta, Tabacal
KMS1	<i>L. (V.) br</i>	MHOM/AR/02/RLS6	CL, ulcer	Left shin	90	M	39	Salta, Embarcación
KMS1	<i>L. (V.) br</i>	MHOM/AR/02/MAS5a*	CL, incomplete healed infiltrative plaque	Forehead	85	M	60	Salta, Aguas Blancas
KMS1	<i>L. (V.) br</i>	MHOM/AR/02/MAS5b*	CL, incomplete healed infiltrative plaque	Forehead	209	M	60	Salta, Aguas Blancas
KMS1	<i>L. (V.) br</i>	MHOM/AR/03/AAS4	MCL, ulcerative-infiltrative	Nasopharynx	120	M	63	Salta, Chaguaral
KMS1	<i>L. (V.) br</i>	MHOM/AR/03/OLO1†	CL, scar with peripheral activity	Right hand	186	M	35	Salta, Pichanal
KMS1	<i>L. (V.) br</i>	MHOM/AR/03/MRO2a‡	CL, incomplete scarred ulcer	Left thigh	231	M	36	Salta, Río Bermejo
KMS1	<i>L. (V.) br</i>	MHOM/AR/03/MRO2b‡	CL, incomplete scarred ulcer	Right thigh	231	M	36	Salta, Río Bermejo
KMS2	<i>L. (V.) br</i>	MHOM/AR/03/HNO3a	CL, ulcer	Right cheek	15	M	47	Salta, Río Blanco
KMS2	<i>L. (V.) br</i>	MHOM/AR/03/HNO3b	CL, enlarged lymph node	Behind right ear	14	M	22	Salta, Río Blanco
KMS2	<i>L. (V.) br</i>	MHOM/AR/03/FDO4	CL, ulcer	Right wrist	14	F	22	Salta, Pichanal
KMS3	<i>L. (V.) br</i>	MHOM/AR/03/CFO5	RCL, ulcer	Skin nose	11	M	49	Salta, SRN Orán
KMS4	<i>L. (V.) gu</i>	MHOM/AR/02/LFO1	CL, ulcer	Snout	365	M	2	Salta, SRN Orán
KMS4	<i>L. (V.) gu</i>	MHOM/AR/98/LBC1	CL, ulcer	?	?	M	?	Corrientes
KMS4	<i>L. (V.) gu</i>	MHOM/AR/99/JDM1	CL, ulcer	Right ear	60	M	26	Salta, Río San Francisco

KMS = Koochi Medical School; *L. (V.) br* = *Leishmania (Viannia) braziliensis*; *L. (V.) gu* = *L. (V.) guyanensis*; CL = cutaneous leishmaniasis; MCL = secondary mucocutaneous leishmaniasis; RCL = recurrent cutaneous leishmaniasis; SRN Orán = San Ramón de la Nueva Orán.

\* Two stocks isolated from the same lesion: MAS5a, 5 days after finishing two cycles of pentavalent antimony treatment; MAS5b 17 days after finishing the third cycle.

† Isolated 29 days after finishing one cycle of treatment.

‡ Two stocks isolated from different ulcers of the same patient 7 days after finishing three cycles of treatment.

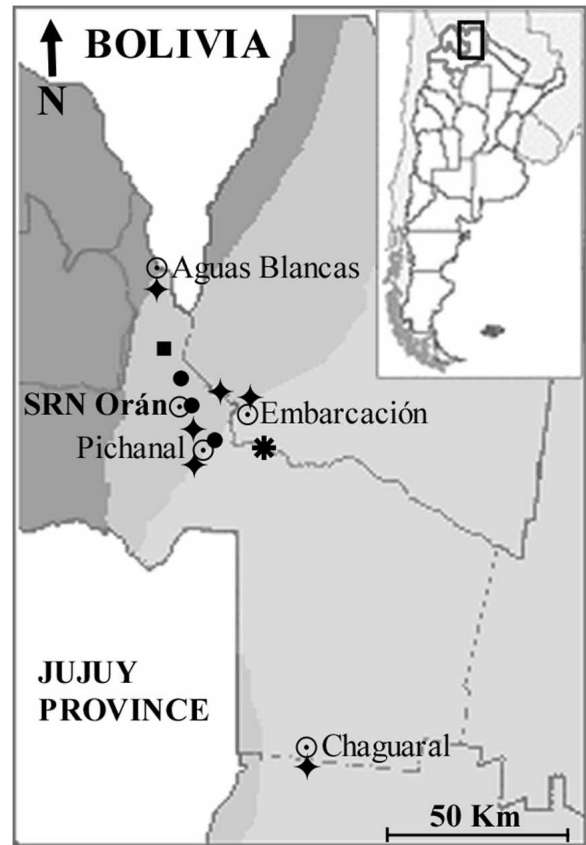


FIGURE 1. Map of the north of Salta Province, Argentina, showing phytogeographical locations of the zymodemes. *Leishmania (Viannia) braziliensis*: ♦ KMS 1, ● KMS 2, and ■ KMS 3. *L. (V.) guyanensis*: \* KMS 4. ■ Selva y Bosque Montano: Subtropical humid forest within 900–3000 m above sea level (m.a.s.l.). Annual precipitations (ann. prec.): 700–2000 mm. Annual mean temperature (AMT): 20°C. □ Selva de Transición: Subtropical humid forest within 340–700 m.a.s.l. Ann. prec.: 700–1070 mm. AMT: 20°C (Comité MAB Argentino, unpublished data). □ Chaco Occidental: Xerophytic lowland plain and semiarid climate within 300 m.a.s.l. (max). Ann. prec.: 550 mm. AMT: 23°C (Gasparri I and others, unpublished data).

MNYC/BZ/62/M379; and *L. (L.) chagasi* (syn. *Infantum*), MHOM/BR/74/M2682.

**Isolation and mass cultivation.** Thirteen stocks were obtained by aspiration of the CL or MCL lesion edges of humans or dogs, using a syringe with 0.5 mL of sterile proline balanced salts solution (PBSS) containing 100 U/mL penicillin and 50 µg/mL streptomycin (P E). The material aspirated was inoculated into Difco blood agar (USMARU) medium containing 20% of defibrinated rabbit blood.<sup>12,13</sup> One milliliter of PBSS with P E was added after 4 days of incubation at 23°C. A final volume of liquid phase of 2 mL was reached after 4 days more. One stock (JDM1) was obtained by an inoculation of the aspirated material into a footpad of a golden hamster (*Mesocricetus auratus*). Two months after the inoculation, the footpad tissue was aseptically removed, homogenized, and inoculated into five culture tubes containing 2 mL of sterile liver infusion tryptose medium supplemented with P E and 20% heat-inactivated fetal bovine serum (hiFBS).<sup>12</sup> The remaining two stocks (AZ3, LBC1) were isolated by skin biopsy of the cutaneous lesion edges and transferred to Novy-Nicolle-Mac Neal (NNN) medium.

The cultures were examined regularly from the 4th to 30th day of inoculation. When promastigotes were not found, the culture was gently centrifuged and the used USMARU medium was replaced by a fresh one after washing the pellet twice in 5 mL of PBSS.

For mass cultivation, 1 mL of promastigotes-culture in logarithmic phase was transferred to 6 mL RPMI 1640 medium supplemented with 10% of hiFBS. The culture volume was duplicated once every 2 days to reach a volume of 70 mL and incubated for a maximum of 12 days. The promastigotes were harvested by centrifugation at 10,000 rpm for 15 minutes at 4°C, after washing (2,500 rpm, 20 minutes) 3 times with sterile 0.85% NaCl solution. The pellets were stored in a deep freezer (-80°C) until use.

**Sample preparation.** The soluble enzyme extracts were obtained by lysis of the pellets in a double volume of stabilizer solution containing 2 mM dithiothreitol, 2 mM  $\epsilon$ -aminocaproic acid, and 2 mM EDTA. After 3 cycles of freezing and thawing, the supernatants obtained by centrifugation at 27,000 rpm at 4°C were aliquoted and stored at -80°C.<sup>12</sup>

**Enzyme electrophoresis.** Electrophoresis and staining procedures were performed on cellulose acetate support (Sebiagel; Moulineux, France) following the methods described by Kreutzer and Christensen and by Evans, with slight modifications.<sup>12,14</sup> Pictures of gels were taken with a Kodak EDAS 290 digital camera. Each enzyme extract was analyzed at least twice by 11 enzyme systems for 12 putative enzymatic loci: alanine aminotransferase (E.C.2.6.1.2, ALAT), aspartate aminotransferase (E.C.2.6.1.1, ASAT), glucose-6-phosphate dehydrogenase (E.C.1.1.1.49, G6PDH), 6-phosphogluconate dehydrogenase (E.C.1.1.1.44, 6GPDH), glucose-phosphate isomerase (E.C.5.3.1.9, GPI), malate dehydrogenase (E.C.1.1.1.37, MDH), malic enzyme (E.C.1.1.1.40, ME), mannose-phosphate isomerase (E.C.5.3.1.8, MPI), nucleoside hydrolase (inosine) (E.C.2.4.2, NH<sub>1</sub> and NH<sub>2</sub>), phosphoglucomutase (E.C.2.7.5.1, PGM), and pyruvate kinase (E.C.2.7.1.40, PK).

**Data analysis.** The distance that each reproducible enzyme band migrated from the origin (anode) was measured. The bands were numerated from the fastest (the nearest one to the cathode) to the slowest for each enzyme gel or zymogram. The obtained set of bands defined an electrophoretic profile for each extract. Stocks with the same profile were grouped in

the same zymodeme<sup>2</sup>; the species attribution was made comparing the profiles with the WHO reference *Leishmania* strains. To examine the relationship between the zymodemes, Jaccard distances (JD) were obtained for all pairwise comparisons,<sup>15</sup> a distance matrix was built and transformed into a phenogram by the unweighted pair group method using the MEGA version 2.1 Software.<sup>16</sup>

## RESULTS

Fourteen stocks, included the isolate from the dog were assigned by MLEE analysis of 12 enzymatic loci to *L. (V.) braziliensis* and two to *L. (V.) guyanensis* (Table 1; Figure 2A). No *L. (L.) amazonensis* was isolated from any patient. For the *L. (V.) braziliensis* stocks, the isolates from humans were grouped in 2 zymodemes, KMS 1 and KMS 2, differing in 1 locus, MDH (Figure 2B). The stock from the dog was assigned to another zymodeme, KMS 3 that differed from KMS 1 only in 1 locus, ME (Figure 2C), and from KMS 2 in 2 loci, ME and MDH (Figures 2B and 2C).

Both *L. (V.) guyanensis* isolates were grouped in the same zymodeme, KMS 4, that included the reference strain. Two loci, G6PDH and 6GPDH, distanced them from the *L. (V.) panamensis* reference strain. The enzymatic profiles are shown in Table 2. The relationship between the zymodemes is shown in a phenogram (Figure 3). Over a JD of 0.9, 2 clusters, corresponding to the subgenera *Viannia* and *Leishmania*, can be distinguished. The zymodemes KMS 1 and KMS 2 were more closely related with each other (JD = 0.056) than with KMS 3 (JD = 0.126) and with *L. (V.) braziliensis* reference strains (JD = 0.161). The JD observed between KMS 4 and *L. (V.) panamensis* reference strains was 0.222.

In three different cases of the current study, two stocks each were isolated from the same patient (MHOM/AR/02/MAS5, MHOM/AR/03/MRO2, and MHOM/AR/03/HNO3); they expressed the same enzymatic profile for each pair, without demonstrating the possibility of co-infection with multiple zymodemes and/or species of *Leishmania* parasite (Table 1; Figure 3).

## DISCUSSION

*Leishmania* species identification is essential for clinical or epidemiologic surveys because of the diversity found among

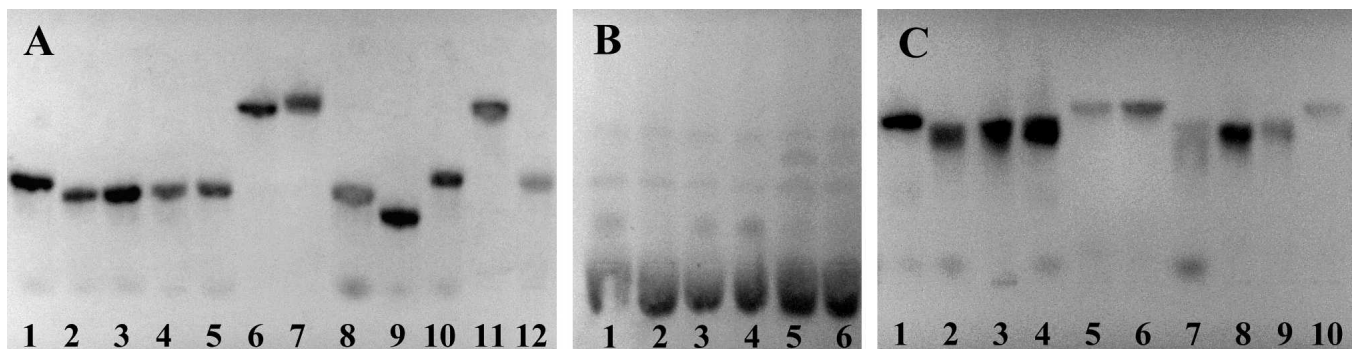


FIGURE 2. **A**, 6-Phosphogluconate dehydrogenase (6PGDH) zymogram. Lanes 1 and 12, KMS 4; Lane 2, KMS 3; Lanes 3 and 4, KMS 1; Lane 5, KMS 2; Lane 6, *Leishmania (Leishmania) mexicana* ref. strain; Lane 7, *L. (L.) amazonensis* ref. strain; Lane 8, *L. (Viannia) braziliensis* ref. strain; Lane 9, *L. (V.) panamensis* ref. strain; Lane 10, *L. (V.) guyanensis* ref. strain, and Lane 11, *L. (L.) chagasi* ref. strain. **B**, Malate dehydrogenase (MDH) zymogram. Lane 1, KMS 3; Lanes 2, 3, and 4, KMS 1; and Lanes 5 and 6, KMS 2. **C**, Malic enzyme (ME) zymogram. Lane 1, KMS 3; Lanes 2 and 3, KMS 1; Lane 4, KMS 2; Lane 5, *L. (L.) mexicana* ref. strain; Lane 6, *L. (L.) amazonensis* ref. strain; Lane 7, *L. (V.) braziliensis* ref. strain; Lane 8, *L. (V.) panamensis* ref. strain; Lane 9, *L. (V.) guyanensis* ref. Strain; and Lane 10, *L. (L.) chagasi* ref. strain.

TABLE 2  
Enzymatic profiles for Argentinian stocks and WHO reference strain zymodemes

Zymodeme	Enzymatic loci											
	ALAT	ASAT	G6PDH	6GPDH	GPI	MDH	ME	MPI	NH <sub>1</sub>	NH <sub>2</sub>	PGM	PK
KMS 1	4	2	2	3	1,2	1,4,5	3	3,4,5	1	2	2	2
KMS 2	4	2	2	3	1,2	1,2,4,5	3	3,4,5	1	2	2	2
KMS 3	4	2	2	3	1,2	1,4,5	2	3,4,5	1	2	2	2
KMS 4	3	1	1	2	1,2	1,6	3	4,5,6	2	1	3	1
KMS 5	1	3	3	1	4	2,5	1	1	2	3	1	3
KMS 6	2	4	2	1	3	4	1	1	2	–	2	3
KMS 7	4	2	2	3	1,2	2,5	3	3,4,5	1	2	2	2
KMS 8	3	1	2	4	1,2	1,6	3	4,5,6	2	1	3	1
KMS 9	2	3	3	1	3	3	2	2	2	–	3	2

ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; G6PDH = glucose-6-phosphate dehydrogenase, 6GPDH = 6-phosphogluconate dehydrogenase; GPI = glucose-phosphate isomerase; MDH = malate dehydrogenase, ME = malic enzyme, MPI = mannose-phosphate isomerase; NH<sub>1</sub> and NH<sub>2</sub> = nucleoside hydrolase (inosine); PGM = phosphoglucomutase; PK = pyruvate kinase; KMS = Kochi Medical School. KMS 1 (MHOM/AR/99/AZ3, MHOM/AR/02/NSS3, MHOM/AR/02/RLS6, MHOM/AR/02/MAS5 a and b, MHOM/AR/03/AAS4, MHOM/AR/03/OLO1, and MHOM/AR/03/MRO2 a and b in Table 1), KMS 2 (MHOM/AR/03/HNO3 a and b, MHOM/AR/03/FDO4, and MHOM/AR/03/CFO5) and KMS 3 (MCAN/AR/02/LPO1) correspond to *Leishmania (Viannia) braziliensis* from Argentina. KMS 4: *L. (V.) guyanensis* ref. strain and two current stocks (MHOM/AR/98/LBC1 and MHOM/AR/99/JDM1); KMS 5: *L. (Leishmania) mexicana* ref. strain; KMS 6: *L. (L.) amazonensis* ref. strain; KMS 7: *L. (V.) braziliensis* ref. strain; KMS 8: *L. (V.) panamensis* ref. strain; KMS 9: *L. (L.) chagasi* (syn. *infantum*) ref. strain.

this genus.<sup>3</sup> In this work, *L. (V.) braziliensis* was incriminated as a causative agent of three different clinical forms of ATL, viz., CL, MCL, and RCL (recurrent cutaneous leishmaniasis) and involved in failure of treatment with pentavalent antimony drug. *Leishmania (V.) braziliensis* was the predominant species in Salta Province, concordant with previous studies in which the samples were taken mainly from CL cases.<sup>8,9</sup>

Although many factors are responsible for the clinical expression of *Leishmania* infection,<sup>17</sup> it has recently been reported that intraspecific polymorphism in *L. (V.) braziliensis* species may be related to specific clinical forms of ATL.<sup>18</sup> In the current study, both of the *L. (V.) braziliensis* zymodemes, KMS 1 and KMS 2, were found to have an ability to metastasize to mucocutaneous tissues and lymph nodes. However, we could not exclude the possibility that they have a tropism

for different tissues, such as mucosa or skin and lymph nodes. Again, the main zymodeme, KMS 1, was isolated from both MCL and CL cases; the other zymodeme, KMS 2, is conformed for isolates that are able to infect the lymph node and induce recurrent cutaneous lesions far from the original scar.

*Leishmania (V.) guyanensis* (zymodeme KMS 4) was isolated from patients with CL in two different geographic regions, Salta in the northwest and Corrientes in the northeast of Argentina. The same zymodeme grouped these stocks and WHO reference strain, confirming the homogeneity previously reported for this species in other areas of different countries.<sup>2,19</sup> The current result confirms the presence of this species in Argentina, which was reported for the first time in 1987 from a patient in the same endemic area, Orán, Salta.<sup>2</sup>

Recently, it has been proposed that *L. (V.) guyanensis* and

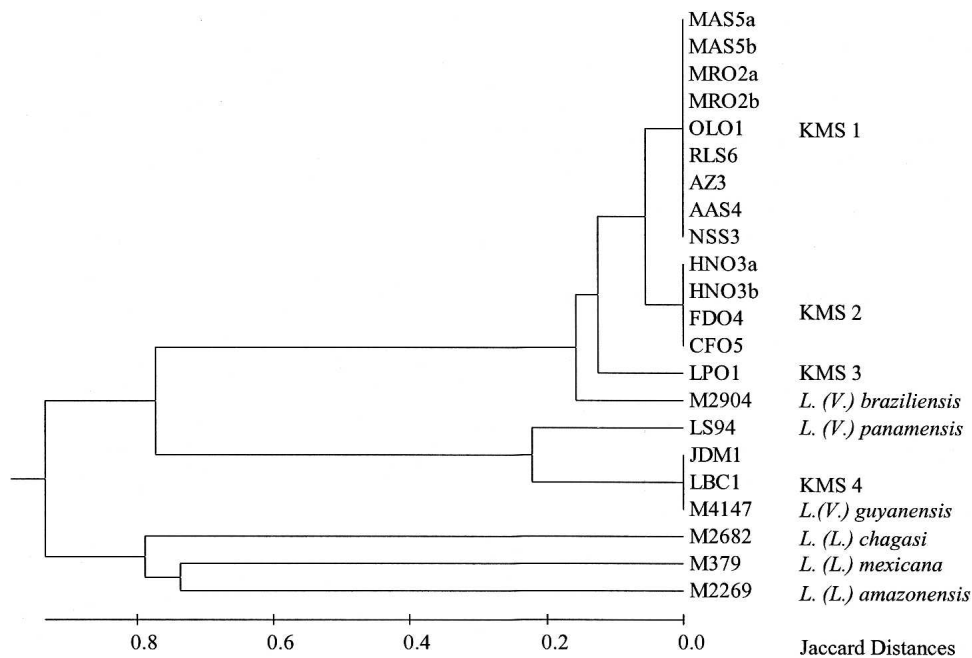


FIGURE 3. Relationship among 16 Argentinian *Leishmania* stocks from 12 patients (MAS5a, MAS5b, MRO2a, MRO2b, OLO1, RLS6, AZ3, AAS4, NSS3, HNO3a, HNO3b, FDO4, CFO5, JDM1, and LBC1; a, b mean different isolates from the same patient), a dog (LPO1), and WHO reference strains (M2904, LS94, M4147, M2682, M379, and M2269). The phenogram was built by the UPGMA method from the Jaccard distances calculated.



*L. (V.) panamensis* should be considered as a unique clade based on the discrete typing unit concept.<sup>15</sup> In this study, only two loci showed different characters between them, reaching a relatively small degree of distance (JD = 0.222). Such a difference may not be enough to separate them as distinct species. Nevertheless, the factors that control the species diversity within the genus *Leishmania* remain unknown. A better understanding of taxonomic units for *Leishmania* will come when we understand what controls their distribution.<sup>20</sup>

In a previous study of 208 dogs examined, 41 (19.7%) presented leishmanial lesions, and 14 (45.2%) out of 31 lesions studied intensively were positive for *Leishmania* amastigotes, but no parasite isolation *in vitro* was possible.<sup>11</sup> In this work, therefore, we are reporting for the first time the isolation and characterization of *Leishmania* from dogs. Although the species involved was *L. (V.) braziliensis*, its zymodeme was not expressed by any of the isolates from humans. To incriminate a reservoir host, the parasites isolated from the reservoirs must be indistinguishable from those of man.<sup>4</sup> This becomes especially important in the case of clades of parasites, such as *L. (V.) braziliensis*, in which their heterogeneity and plasticity have been demonstrated.<sup>17,19,20</sup> Although more isolates from dogs need to be analyzed, this observation adds a point of discussion on whether the animal has a principal role as a reservoir of this disease, even when its prevalence is relatively high in the endemic area. In Minas Gerais State, Brazil, an intraspecific variation among *L. (V.) braziliensis* from dogs was described.<sup>21</sup> On the other hand, in Três Braços, Brazil, two stocks isolated from dogs were shown to be indistinguishable from 15 isolates from humans by isoenzyme profiles.<sup>22</sup> Furthermore, although *L. (V.) braziliensis* was found infecting dogs and other domestic animals in the endemic areas of different countries, the stocks characterization had reached, analyzing a limited number of enzymes.<sup>23,24</sup> Under these circumstances, a generalization about the indistinguishableness among *L. (V.) braziliensis* isolated from humans and dogs cannot be done as has been suggested.<sup>25</sup> Therefore, in the future, it should be proved in each study area, examining enough materials (human and dog isolates) by suitable techniques for the analysis.

The geographic origin of the zymodemes in the current study suggests that both phytogeographical areas, Selva de Transición and Chaco Occidental, are possible transmission places for *Leishmania (V.)* spp. in Salta Province. Particularly the same zymodeme of *L. (V.) braziliensis*, KMS 1, was found in both zones. Considering the zoonotic characteristic of ATL, this fact triggers questions such as if the same transmission cycle exists in both areas, is it related to the mobility and the ability of the reservoirs to colonize different environment. Therefore, a better understanding of the disease will be obtained when the transmission cycles have been disclosed in the areas.

Thus, the current data on the zymodemes from humans and dogs will give important information on the causative agents circulating in and around the endemic areas. By performing further analysis using more stocks from humans, it would be possible to disclose the relationships between the clinical features and the zymodeme patterns in the country.

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