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The isolation and molecular characterization of *Leishmania* spp. from patients with American tegumentary leishmaniasis in northwest Argentina

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ABSTRACT

American tegumentary leishmaniasis (ATL) is a group of zoonotic diseases caused by kinetoplastid flagellates of the genus *Leishmania*. A total of 66 patients diagnosed as positive ATL cases from northwest Argentina were included in this study. *Leishmania* stocks were isolated *in vitro* and analyzed over promastigote cultures sown on FTA through nested PCR and sequence of cytochrome *b* (cyt *b*). The molecular analysis resulted in the incrimination of *L.* (*Viannia*) *braziliensis* as the predominant species in the studied area, identifying two genotypes of *L.* (*V.*) *braziliensis*, 24 cases of Ab-1 cyt *b* and 41 cases of Ab-2 cyt *b*. One *L.* (*V.*) *guyanensis* strain was obtained from a traveler from the Brazilian Amazon. The prevalence of different genotypes was in agreement with previous studies, suggesting the necessity for new systems to study the genetic diversity in more detail. Most of the cases typified in this study were registered in the area of Zenta Valley (Orán, Hipólito Yrigoyen, and Pichanal cities), pointing a link between genotype and geographical origin of the sample. Sex and age distribution of the patients indicate that the transmission was predominantly associated with rural areas or rural activities, although the results might not exclude the possibility of peri-urban transmission. This work represents, so far, the largest isolation and molecular characterization of ATL cases in Argentina.

1. Introduction

Leishmaniasis is a group of worldwide zoonotic diseases caused by kinetoplastid flagellates of the genus *Leishmania*. This tropical disease extends over 88 countries, 72 of them in the developing world, while 13 belong to the category of least developed countries (Desjeux, 2001; WHO, 2004). It is considered as one of the most important diseases by the World Health Organization (WHO), and has been included in the special program of research and training of tropical disease research (TDR), identified as a priority for research and control (WHO, 2004). It presents a variety of clinical manifestations which includes: visceral leishmaniasis (VL), which is the most severe form of the disease; muco-cutaneous leishmaniasis (MCL), a mutilating disease; diffuse cutaneous leishmaniasis (DCL), a long-lasting disease, and cutaneous leishmaniasis (CL) (Desjeux, 2004; Dujardin, 2006; WHO, 2004). In America, CL, MCL, and DCL together receive the denomination of American tegumentary leishmaniasis (ATL), with a wide geographical distribution from the southern United States to the northern Argentina.

ATL is endemic in northern Argentina, and both the largest number of reported cases (53.1%) and the highest incidence have been found in the north of Salta Province, which represents only 0.7% of the country's population, being the primary locus of CL and MCL (Ampuero et al., 2005; Gil et al., 2010; Marco et al., 2005; Salomón et al., 2001; Sosa-Estani et al., 2001). Rural labors and outdoor recreational activities seem to be associated with high rates of incidence of this disease in the given areas, although recent publications provide evidence of the potential urban transmission (Gil et al., 2010; Salomón et al., 2001; Sosa-Estani et al., 2001). In the present study region, *Leishmania* (*Viannia*) *braziliensis* is the main

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causative agent of ATL, but *L.* (*V.*) *guyanensis, L.* (*Leishmania*) *amazonensis*, and recently *L.* (*V.*) *panamensis and L.* (*L.*) *infantum* have also been reported (Barrio et al., 2012; Marco et al., 2005; Marco et al., 2006; Marco et al., 2012; Salomón et al., 2012a,b). Since the efficacy of treatment for leishmaniasis is often influenced by the consequence of different sensitivity of *Leishmania* spp. to the drugs, an accurate method for identification of the different species is crucial for an effective remedy (Romero et al., 2001). Parasitological methods remain as the gold standard in leishmaniasis diagnosis because of its high specificity (Reithinger and Dujardin, 2007), especially in the patients infected with the parasites of the *Leishmania* subgenus prevalent in both the old and new world, despite the variable and relatively low sensitivity. Naturally, however, these parasitological methods including *in vitro* cultures cannot differentiate parasite strains and/or species. Moreover, Multilocus Enzyme Electrophoresis (MLEE) still persists as the gold standard for *Leishmania* spp. assignation, and stocks from patients isolated in this region are grouped in two zymodemes assigned to *L.* (*V.*) *braziliensis* and one to *L.* (*V.*) *guyanensis* (Marco et al., 2005). Currently new tools such as PCR followed by sequencing of kinetoplastid cytochrome *b* (cyt *b*) have been proposed as a *Leishmania* spp. confirmatory second line-approach, providing proper identification of different strains (Asato et al., 2009; Castilho et al., 2003; Kato et al., 2010; Luyo-Acero et al., 2003; Reithinger and Dujardin, 2007). Characterization using cyt *b* sequence is a validated method for the identification of the *Leishmania* spp. described in Argentina (Barrio et al., 2012; Marco et al., 2006). In accordance with the diversity suggested by MLEE, cyt *b* gene sequences have assigned two different genotypes for *L.* (*V.*) *braziliensis* named Ab-1 and Ab-2 (Marco et al., 2005; Marco et al., 2006). Likewise, the cyt *b* sequencing has been successfully applied in phylogenetic studies and evaluation of the genetic variability of *Trypanosoma cruzi* I populations in Colombia (Ramírez et al., 2011; Spotorno et al., 2008) and the identification of different genotypes of *Taenia solium* (Ito et al., 2003).

This report focuses on molecular epidemiological studies in northwest Argentina through cyt *b* sequencing combined with *in vitro* cultivation of the parasites, and the characteristics of the disease in this endemic area were also discussed briefly.

2. Materials and methods

2.1. Patients and diagnosis of American tegumentary leishmaniasis

Sixty-six patients diagnosed with ATL from Salta Province, Argentina, were included in this study; the majority of them were from the northern Departments of Orán and San Martin, Salta. These patients were referred by the local physicians to attend to the Instituto de Investigaciones en Enfermedades Tropicales, Subsede Orán, Universidad Nacional de Salta, between July 2009 and November 2012 for a laboratory report. After a positive ATL diagnosis has been made, the clinical controls and treatment protocols were conducted by local physicians in each endemic area. All the patients were treated with 10–20 mg/kg/body weight of pentavalent antimony in a cycle of 25–30 days. In the cases where ulcerative and/or nodulous lesions continued active after the end of the therapy, another cycle of antimonial or amphotericin B treatment was performed. The participation in the study was voluntary, following approved procedures of the Bioethics Commission of the Health Ministry of the Salta Province, Argentina.

2.2. Diagnostic criteria to define patients as ATL-cases

For the parasitological diagnosis, dermal scrapings were obtained from the internal border of the lesions and stained by May Grünwald–Giemsa method. Two specimens were taken on glass slides from each patient's lesions. Each slide was observed in a search for *Leishmania* amastigotes using an optic microscope (1000×) for 40 min. per 100 fields. Leishmanin skin test (LST) antigen derived from *L.* (*V.*) *braziliensis* promastigotes was used in the study, containing 40 μg/ml of the total proteins of the killed parasite strain MHOM/AR/03/OLO1, in 100 μl of a 1:10,000 phenolated saline solution (Frank et al., 2003; Marco et al., 2005). Clinical features of the present subjects were decided based on the presence of compatible lesions and also based on the clinico-epidemiological characteristics. In addition, previous diagnoses of ATL were taken into account in the differentiation of suspected MCL cases.

2.3. Parasites cell culture and in vitro isolation

Lesion aspirate was taken from the patients with a positive parasitological diagnosis. Isolation of parasites was performed by aspirating the lesion borders or lymph node swellings of the patients using a syringe with sterile proline balanced salts solution (PBSS) containing 100 U/mL penicillin (PE) and 50 μg/mL streptomycin (PE), and by inoculating the "Difco" blood agar (USMARU) medium containing 20% defibrinated rabbit blood, following the protocol described previously (Marco et al., 2005). PBSS + PE solution was used as liquid phase, with an incubation temperature of 24 °C. Cultures have been checked by using an invert microscope searching for promastigotes for a maximum of two month (Marco et al., 2006). Seven WHO reference strains were also 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*: MNYC/BZ/62/M379; *L.* (*L.*) *chagasi*: MHOM/BR/74/PP75; and *L.* (*L.*) *infantum*: MHOM/TN/80/IPT1 (Marco et al., 2005). Once the promastigote cultures reached the exponential growth, 1 mL were taken from the liquid phase, transferred to a culture tube with RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin and cultured for 4 days (Marco et al., 2005). For further DNA analysis and to facilitate the preservation and transportation of the material, 100 μL of the culture were sown in filter papers "Classic FTA Cards" (Whatman BioScience MA). The *Leishmania* stocks and reference strains were subsequently cryopreserved and stored in liquid Nitrogen until use (Evans, 1989).

2.4. PCR and sequencing of the cytochrome b gene

Classic FTA Cards filter paper fragments of 2 mm diameter containing culture samples were washed three times for 5 min with FTA purification reagent (Whatman BioScience MA) and twice in TE 1/10X, and then used as a template for the first PCR. *Leishmania* spp. were identified by a nested PCR performed with the primers for cyt *b* gene, L.cyt-AS (5′-GCGGAGAGRARGAAAAGGC-3′), and L.cyt-AR(5′-CCACTCATAAATATACTATA-3′), with an initial denaturation at 94 °C for 15 min, and subsequently 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1.5 min and extension at 72 °C for 1 min, and a final extension phase at 72 °C for 7 min. From the first PCR solution 1 μl was used as a template for a second PCR by using the primers L.cyt-S (5′-GGTGTAGGTTTTAGTYTAGG-3′) and L.cyt-R (5′-CTACAATAAACAAATCATAATATRCAATT-3′), performed 10 min at 94 °C for initial denaturation, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and a final extension at 72 °C for 1 min, concluding with a polymerization step at 72 °C for 7 min (Kato et al., 2010). The TaKaRa Ex Taq DNA polymerase hot start version (Takara-Bio, Shiga, Japan) was used in both PCR reactions. The PCR products were visualized in 2% agarose gels, then purified by phenol–chloroform and quantified

using a Spectrophotometer NanoDrop-1000 (Nanodrop Technologies). The amplicons were sequenced using a BigDye terminator cycle sequencing kit, version 1.1 (Applied Biosystems, Foster City, CA), purified by Sephadex G-50 spin columns (Amersham Biosciences, NJ) and analyzed on a Applied Biosystems Hitachi 3130 Genetic Analyzer automated sequencer, employing the primers L.cyt-S and L.cyt-R. The sequences obtained were assembled and edited by Genetyx Mac 11.0.0 (Software Development Co. Ltd, Japan). Data were analyzed performing a NJ tree with 1000 bootstrap replications using the MLSTest v1.0 software (Tomasini et al., 2013) and compared with the Argentinean and WHO reference strains of the genus *Leishmania* reported previously (Marco et al., 2006).

2.5. Statistics

Analysis was performed by using the statistical package GraphPad Prism version 4 (GraphPad Software Inc., San Diego, USA), by using the Goodman and Kruskall, Pearson's Chi-Square test and the probability of finding significant differences was calculated with the Fisher's exact test. A difference was considered to be significant when $p < 0.05$. The data used for statistical analysis were obtained from the patients interview and the statistical analysis was performed to identify any correlation between geographical areas, clinical form, sex or age groups with the ATL diagnosed patients, and the genotypes.

3. Results

In this study, a yield of *Leishmania* spp. isolation of 86.8% was reached by culture technique, meaning that the methodology employed allowed the isolation of 66 stocks over 76 ATL cases caused by the *Viannia* subgenus parasites. Forty-nine (78.1%) patients showed one lesion on the body surface; thirteen (14.6%), two lesions; three (2.9%) showed three lesions and one patient (1.6%), four lesions. Sixty-two (92.4%) patients were diagnosed as having CL, while four (7.6%) corresponded to MCL form. The male–female ratio was 5: 1 showing a higher incidence of ATL in male individuals. The disease is distributed across all age groups (mean age ±SD: 38.2 ± 16.8), although not uniformly, with 10.6% of the people being over 60-years-old while children under 15 years of age represent 9.1% of the total cases, (Fig. 1). Comparison of the incidence of ATL among age groups by Chi-Square test ($p < 0.05$), verified that male in age groups between 16 and 60-years-old were more susceptible to acquiring the disease.

Table 1
Cytochrome *b* gene sequencing based-genotype assignation and geographical origin.

Geographical origin	Genotype	
	L. (*V.*) *braziliensis* AB-1	*L.* (*V.*) *braziliensis* AB-2
Hipólito Yrigoyen	4	9
Orán	12	27
Pichanal	4	4
Orán (rural)	0	1
Rivadavia Banda Sur	2	0
Embarcación	1	0
Jujuy Province	1	0

In order to identify *Leishmania* species, 66 samples from *in vitro* culture derived from ATL-cases were analyzed by a nested PCR and sequencing cyt *b* gene. This method successfully identified two genotypes of *L.* (*V.*) *braziliensis*, Ab-1 cyt *b* (24), and Ab-2 *cyt* b (41), previously reported in the same endemic area (Marco et al., 2006), and one *L.* (*V.*) *guyanensis* strain obtained from a traveler who had visited an Amazonian leishmaniasis-endemic area in Brazil. The two cyt *b* gene sequences, Ab-1 and Ab-2 (Marco et al., 2006), differed by only one nucleotide base over 817 compared, and showed 99.9 and 99.8% similarity to the *L.* (*V.*) *braziliensis* MHOM/EC/88/INH-03 strain sequence (GeneBank accession No. AB095967) (Marco et al., 2006). The present Brazilian strain showed the same sequence as *L.* (*V.*) *guyanensis* reference strain MHOM/BR/75/M4147 (GeneBank accession No. AB095969) (Marco et al., 2006) (Fig. 2).

Most of the cases typified in this study were registered in the areas of Orán (23°8′0″S 64°20′0″W), Hipólito Yrigoyen (23°16′00″S 64°15′00″W), and Pichanal (23°19′7″S 64°13′32″W), cities located in the Zenta Valley, with 60, 20 and 12% of the cases respectively, being the only area in which the Ab-2 genotype was found (Table 1) with a correlation of $p = 0.04$ between genotype and geographical origin of the sample when Fisher test was applied. When examining possible associations of the identified genotypes with clinical forms (Fig. 3), number of lesions and patient's sex, no links could be observed.

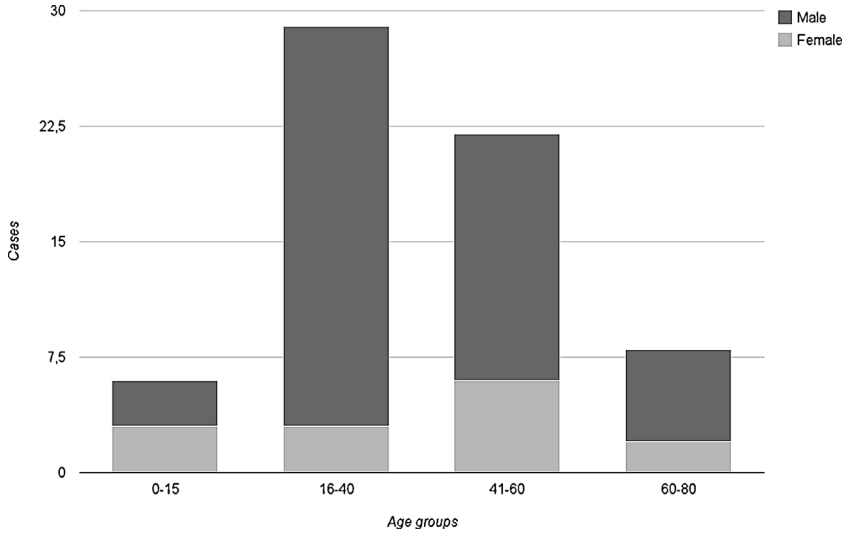

Fig. 1. Leishmaniasis cases distribution across age groups and gender in the study area, northwest Argentina. The male–female ratio shows a higher incidence of ATL in male individuals. Age groups distribution shows the people who had over 60-years-old and children under 15-years-old represent the minority of cases. Female ( ), Male ( ).

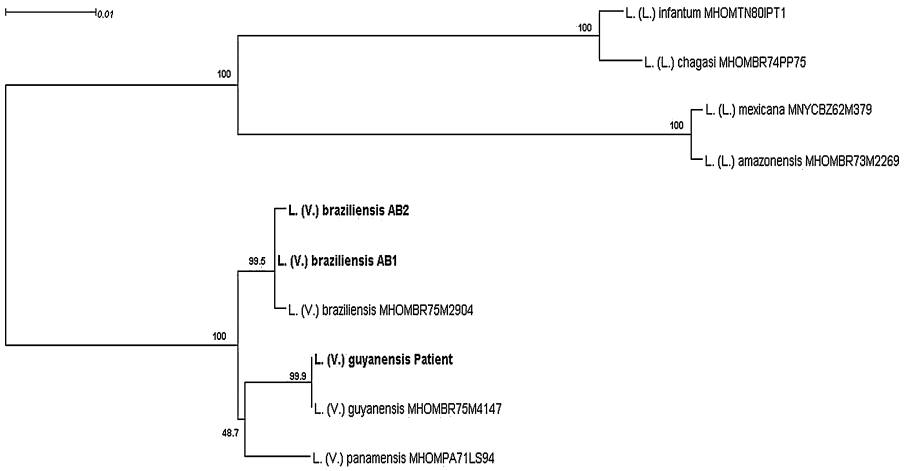

Fig. 2. Relationship among Argentinian *Leishmania* stocks (*L.* (*V.*) *braziliensis* (genotypes Ab-1 and Ab-2) and *L.* (*V.*) *guyanensis*); and WHO reference strains (M2904, LS94, M4147, M2682, M379, M2269, PP75, and IPT1). The dendrogram was built by the NJ method with 1000 bootstrap repetitions using MLSTest 1.0 software.

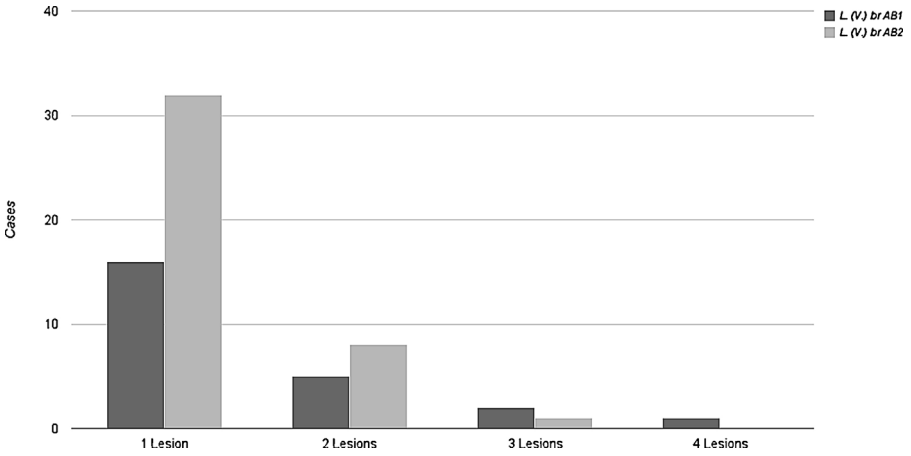

Fig. 3. Cytochrome *b* gene sequencing based-species assignation and number of lesions for patients, most of the patients showed one lesion on the body surface; no correlation was found between the number of lesions and the identified genotype. *Leishmania* (*V.*) *brazilensis*: Genotype Ab-1 (■), Genotype Ab-2 (■).

4. Discussion

The present work represents the largest example of isolation and typing of ATL cases in Argentina to date. The cyt *b* gene sequencing was completely successful when applied on *in vitro* culture samples sown on FTA cards.

The successful isolation from patients proved the viability of *Leishmania* parasites and allowed the application of molecular methods using the purified DNA of *in vitro* parasites. DNA extracted directly from clinical samples mainly consists of the host genetic materials. Therefore it makes the application of certain molecular techniques such as random amplified polymorphic DNA (RAPDs), microsatellites and Multilocus sequence typing (MLST), difficult. Consequently, the use of *in vitro* cultures opens a new range of research possibilities for the molecular epidemiological study of leishmaniasis in Argentina. Furthermore, the parasite DNA concentration could be adjusted, which will increase the efficacy of PCR-based techniques. In this case, the nested PCR of cyt *b* gene, applied over direct clinical sample templates, only achieved a 50–60% success rate (data not shown) compared with 100% from the culture, even when the gene located on the maxicircle, presented 50–100 copies per cell.

In a previous work, the cyt *b* sequencing technique showed total agreement with MLEE on the identification of *Leishmania* spp. on a panel of Argentinean isolates (Marco et al., 2006). In our study the identification of *Leishmania* spp. using cyt *b* sequencing technique applied over the DNA material from promastigote cultures on FTA cards resulted in the incrimination of *L.* (*V.*) *braziliensis* as the predominant species that produces ATL cases in the studied areas, this being consistent with previous characterizations of *Leishmania* stocks isolated in the northwest of Argentina (Marco et al., 2005; Marco et al., 2006; Marco et al., 2012). In accordance with previous studies (Marco et al., 2006), no new genotypes (Ab-1 and Ab-2) were found. In addition, a correlation was found between genotype Ab-2 and the Zenta Valley area. Although more isolates from the Ab-1 exclusive area need to be analyzed, the present results indicate an unequal pattern in the distribution between Ab-1 and Ab-2, suggesting that the different genotypes might be associated with different transmission cycles. Nonetheless it is worth noting that an isolate from a patient infected in Jujuy (24°20′S, 65°02′W) was assigned to *L.* (*V.*) *braziliensis*, this species being reported and isolated for the first time in this northwestern Argentinean Province.

Since the efficacy of treatment for leishmaniasis is often influenced by the fact that different *Leishmania* species have a different drug sensitivity pattern (Romero et al., 2001), it should be noted that a strain identified as *L.* (*V.*) *guyanensis* was isolated from a North American tourist who noticed that the lesion appeared and evolved while he was traveling through the Brazilian Amazon. The movement and migration of infected subjects between different endemic

areas of South American countries highlights the need for precise *Leishmania* spp. identification.

In general, the patients were working age men, most of whom practiced rural professions or outdoor activities, which was reflected in the high male–female ratio (5:1) and the different incidence of ATL among the age groups, implying that the transmission was predominantly associated with rural areas in this study region. The above ratio is higher than any other previously recorded in Argentina and South America in general (3-2:1) (Salomón et al., 2001; Guerra et al., 2006) and much higher compared with the disease cases predominant in the urban areas in which cases affected males and females equally in all age groups (de Miranda et al., 2011). However, the constant presence of cases in women, children, and non-working aged men makes it necessary to take into account the fact that the families in the endemic areas often accompany husbands in rural activities (Salomón et al., 2001). In any case, these results do not exclude the possibility of domestic or peridomestic transmission suggested by previous studies (Ampuero et al., 2005; Salomón et al., 2006, 2008; Gil et al., 2010).

In conclusion, the cyt *b* sequencing method performed on templates from *in vitro* cultures improve the outcome of the identification of *Leishmania* spp. in northwest Argentina, allowing the largest isolation and typing of ATL cases in the northwest region. Moreover, even though the cyt *b* sequencing allows a precise identification at species level, it does not provide much information about strain diversity of the *Leishmania* spp. prevalent in the area; it will therefore be necessary to devise new systems to study this issue in more detail, using microsatellites or MLST. Since the first ATL case was reported in Argentina in 1916, this disease has remained endemic and widespread in most of the northern Provinces (Salta, Jujuy, Catamarca, Tucumán, Misiones, Corrientes, Chaco, Santiago del Estero, and Formosa) (Salomón et al., 2012a,b) where it is caused principally by *Leishmania* (*V.*) *braziliensis*, although have also been reported *L.* (*V.*) *guyanensis*, *L.* (*L.*) *amazonensis* and recently *L.* (*V.*) *panamensis* as a causal agents (Marco et al., 2005; Marco et al., 2006; Marco et al., 2012; Salomón et al., 2012a,b). Achieving a precise identification of genotypes of *Leishmania* circulating in the area, and associating them with clinical properties, will provide a better understanding and knowledge of the transmission and the genetic diversity of parasites involved. This would offer advantages such as improvements in deciding the type of treatment and/or designing control strategies in the given endemic areas.

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