Detection of *Leishmania braziliensis* in human paraffin-embedded tissues from Tucumán, Argentina by polymerase chain reaction

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American cutaneous leishmaniasis (ACL) is an endemic disease in Northern Argentina. We applied the polymerase chain reaction (PCR) followed by a hybridization labelled probe to 21 paraffin embedded human skin biopsies, already analyzed histologically, from leishmaniasis endemic areas in the province of Tucumán, Argentina. We used primers previously designed to detect a Leishmania*-specific 120-base-pair fragment of kinetoplast DNA minicircle, other two primer pairs that amplify kDNA minicircles belonging to the* L. braziliensis *and* L. mexicana *complexes respectively, and specific oligonucleotide primers to detect* L. (V.) braziliensis *which amplify the sequence of the ribosomal protein L-14 of this species. The PCR-hybridization showed a sensitivity of 90.5% when compared to the histopathology test which was 61.9%. Five of the total samples analyzed were positive for the* L. braziliensis *complex whilst none was positive for the* L. mexicana *complex. The specific primers for* L. (V.) braziliensis *detected the parasite in four samples. These results are consistent with those reported for close endemic areas and demonstrate that the causative agent of human leishmaniasis in the analyzed cases was* L. (V.) braziliensis. *PCR should be used as a diagnostic tool for tegumentary leishmaniasis, especially in the mucosal form, and as a valuable technique for the identification of the* Leishmania *species that causes the disease in certain areas.*

Key words: *Leishmania (V.) braziliensis* - diagnosis - polymerase chain reaction - Argentina

American tegumentary leishmaniasis (cutaneous and mucocutaneous) has been endemic in Argentina since 1916 (Mazza 1926). In the Northern provinces of the country, outbreaks were identified in 1985/1987, 1997/1998, and 2002. In Tucumán province, between 1991 and 1996, 232 leishmaniasis cases were reported (Marcolongo et al. 1993, Yadón 1997, Villalonga 1998).

In the Americas the causal agents of this pathology are the mexicana complex species: *Leishmania (L.) mexicana, L. (L.) amazonensis,* and *L. (L.) venezuelensis*; the subgenera *Viannia* braziliensis complex: *L. (V.) braziliensis*, *L. (V.) peruviana,* and *L. (V.) lainsoni*); and the guyanensis complex: *L. (V.) panamensis* and *L. (V.) guyanensis.* The disease is characterized by cutaneous lesions which may develop to metastatic mucosal sores. It is known that *L. (V.) braziliensis* is the causal agent of cutaneous and mucocutaneous leishmaniasis, and in some immune compromised cases it may present visceral manifestations.

The clinical and epidemiological pattern of the human cases observed in Tucumán were related to leishmaniasis due to *L. (V.) braziliensis* as described in other outbreaks in the country (Sosa-Estani et al. 1998). At present, the characterization of parasites from human patients in Tucumán has not been possible. Studies from epidemic outbreaks in nearby provinces within Argentina, indicate that the parasites belong to the *L. braziliensis* complex (Campanini et al. 1993, Cuba et al. 1996, Sosa-Estani et al. 1998). The presence of *L. (L.) amazonensis* (*L. mexicana* complex) by isoenzymatic analysis has also been reported in the Chaco region, Argentina (Frank et al. 2000).

The most common diagnostic method for cutaneous leishmaniasis used in Argentina is the smear – histopathology exam in combination with clinical and epidemiological data. Mucocutaneous leishmaniasis generally requires a complementary test because of the scarcity of parasites in the lesions.

The polymerase chain reaction technique (PCR) has been successfully employed in the detection of specific sequences of pathogenic agents (Erlich 1991). At present this technique is an adequate method for the leishmaniasis diagnosis, showing a high sensitivity when compared to traditional tests (Rodgers et al. 1990, 1994, De Bruijn & Barker 1992, De Bruyin et al. 1993, Lopez et al. 1993a, b, Meredith et al. 1993, Piarroux et al. 1994). The sensitivity of the laboratory diagnosis increases significantly when PCR is combined with Southern blotting (Andresen et al. 1996).

In the present study we used PCR and Southern blotting for the detection of *Leishmania* spp. and identification of the parasite from skin biopsies, histologically examined, of human patients from an endemic area of Tucumán province, Argentina.

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MATERIALS AND METHODS

Samples - In this study we used 22 paraffin-embedded skin biopsies from endemic areas of leishmaniasis in the province of Tucumán, kindly donated by the Pathologic Anatomy Service of the Angel C Padilla Hospital (Tucumán, Argentina). Twenty one of the samples had a clinical diagnosis for leishmaniasis and the histopathology test was positive for 13 of them and negative for the rest (Table I). The only sample with a nonleishmanial clinical diagnosis and a negative histopathology result was that used as a negative control.

DNA extraction - For each sample five to seven 5 µm sections of paraffin-embedded tissue were placed in a tube. The samples were washed with xilene, incubated for 30 min at 37ºC, centrifuged at 13,000 rpm for 5 min and the supernatant discarded. The washing procedure was repeated three times. Then two washes with ethanol 100% were performed followed by two final washes with PBS (NaCl 0.14 M, KCl 2.68 mM, Na_2HPO_4 0.01 M, KH_2PO_4 1.76 mM, pH 7.4). The resulting pellet was resuspended in a 100 µl of Proteinase-K lysis buffer (Tris-HCl 10 mM, pH 8.5, EDTA 10 mM and 4 µl of Proteinase-K (20 mg/ml). The samples were incubated at 60ºC for 2 h. Then the enzyme was inactivated for 5 min at 95ºC and the samples rapidly centrifuged. Two µl of the supernatants of each sample were used for the PCR reaction (modified from Wright & Manos 1990).

PCR procedure - The primers used to detect *Leishmania* spp. were previously designed (Fernandes et al. 1994). Briefly, the PCR reaction mixture (50 µl total volume) consisted of PCR buffer 1X (10mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (Perkin-Elmer), 2.5 U/µl of *Taq* DNA polymerase (Perkin-Elmer), 200 µM of each deoxynucleoside triphosphate (dNTPs) (Pharmacia), 15 pmol of each primer, and 2 µl of the DNA sample. In this reaction DNA amplification was performed for a first cycle at 94ºC for 5 min of incubation, 30 cycles at 94ºC for 30 s, 50ºC for 30 s and 72ºC for 30 s with a final extension cycle of 10 min at 72ºC. In the case of specific DNA amplification of the *L. braziliensis* and *L. mexicana* complexes we used the oligonucleotide primers **B1**/**B2** (De Bruijn & Barker 1992) and **M1**/**M2** (Eresh et al. 1994) respectively. The reaction mixture consisted of 5 µl of PCR buffer (1X), 8 µl of dNTPs, 0.5 µl of each primer (20 pmol/µl), 2 µl de DNA (10 ng/µl), 0.5 µl de *Taq* DNA polymerase (2.5 U/µl) and 33.5 µl of ultrapure water. DNA amplification was performed for 35 cycles using previously established conditions (De Bruijn & Barker 1992, Eresh et al. 1994). For the detection of *L. (V.) braziliensis* DNA we used the primers **L14-A** /**L14-C** (Alonso 1999). The PCR reaction mixture (50 µl total volume) consisted of PCR buffer 1X (10mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (Perkin-Elmer), 2.5 U/µl of *Taq* DNA polymerase (Perkin-Elmer), 200 µM of each deoxynucleoside triphosphate (dNTPs) (Pharmacia), 15 pmol of each primer, and 2 µl of the DNA sample. DNA amplification was performed by a first cycle at 94°C for 3 min of incubation, followed by 30 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1:30 min, with a final extension cycle at 72°C for 10 min. Negative (no DNA and *L. (L.) tropica* DNA) and positive [*L. (V.) braziliensis* or *L. (L.) amazonensis* DNA] controls were used.

PCR products were analyzed on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. All products were transferred to a nylon membrane using a southern blot procedure according to standard conditions (Sambrook et al. 1989). Two PCR amplified minicircle kDNA fragments from *L. (V.) braziliensis* strain (MHOM/PE/95/LQ2) and *L. (L.) amazonensis* strain (MHOM/77/LTB0016) were used as probes in different reactions. The amplified products were isolated from an agarose gel and purified by the Quiaex II gel extraction Kit (Quiagen). Twenty five ng of each purified fragment were labelled with digoxigenin coupled to dUTP (deoxyuracile triphosphate) using the DIG DNA Labeling Kit (Boehringer Mannhein). The blot was prehibrydized at 68ºC for 2 h in SSC 5X (sodium chloride 0.15 M, trisodium citrate 0.015 M), sodium dodecyl sulphate (SDS) 0.02%, N-lauroylsarcosine, blocking reagent 2% (Roche). Hybridization with the specific labelled probe *L. (V.) braziliensis or L. (L.) amazonensis* (25 ng), depending on the reaction, was performed at 68ºC overnight. Washing conditions were 2×5 min in SSC 2X, SDS 0.1% at room temperature and 2×15 min in SSC 0.1X, SDS 0.1% at 68ºC. The hybridized products were immunodetected with anti-digoxigenin-AP, Fag fragments and then visualized with the colorimetric substrates NBT/BCIP using the DIG Nucleic Acid Detection Kit (Roche).

RESULTS

From all of the samples with a clinical diagnosis of leishmaniasis DNA amplification with genus-specific primers was positive in 19 of them. The amplified *Leishmania*-specific fragment (120 bp) corresponds to the amplification of a minicircle sequence of the kDNA. These results were confirmed by the Southern blot-hybridization of the PCR products with the specific *L. (V.) braziliensis* probe (Fig. 1).

The entire samples positive for cutaneous leishmaniasis by histopathology were also positive with the PCR hybridization combined technique. *Leishmania* DNA was detected in 6 of the 8 samples that showed a negative diagnosis of leishmaniasis when assessed histopathology. It is noteworthy that one of these 6 samples belonged to a clinically cured patient (Table).

The only sample with nonleishmanial clinical features, used as a negative control, showed negative results when analysed by both methods, i.e., histopathology and PCR-hybridization.

Overall PCR-hybridization showed a sensitivity of 90.5 %, whilst for the histopathology test was 61.9%.

The second step was to determine more precisely the parasite present in the 19 positive samples for *Leishmania* spp.

Firstly, we performed a PCR using primers specific for the *L. braziliensis* and *L. mexicana* complexes followed by a hybridization assay. The amplified products obtained showed a 760 bp fragment in the positive samples in both cases. From all the samples analyzed we were able to de-

tect in 5 of them the parasite as belonging to the *L. braziliensis* complex (Fig. 2). We did not detect any *L. mexicana* complex parasite in the samples.

Secondly, the 19 samples positive by the PCR *Leishmania* specific were analysed with a PCR specific for the species *L. (V.) braziliensis.* A 160 bp fragment was amplified in 4 of the samples. This result was confirmed with a Southern blot and hybridization with a specific probe for *L. (V.) braziliensis*.

There was only one positive sample detected by both PCR-hybridization experiments, i.e., the one using B1/B2 specific primers for the *L. braziliensis* complex and the one using L14-A/L14-C specific primers for the species *L. (V.) braziliensis*.

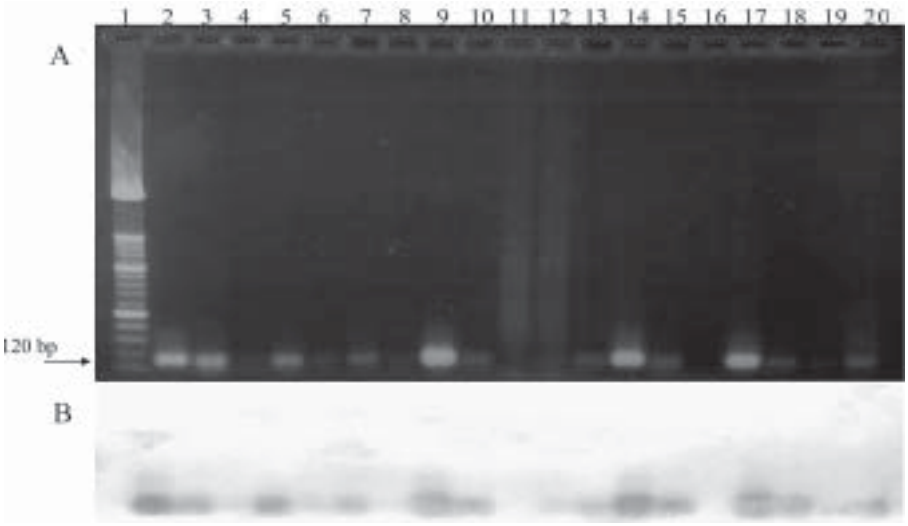

Fig. 1A: polymerase chain reaction amplification of paraffin-embedded biopsies using *Leishmania* genus-specific primers; B: hybridization with a *L. (V.) braziliensis* specific probe. 1: 100 bp molecular weight marker; 2: positive control; 3: L66239; 4: L69252; 5: L1085; 6: L1299; 7: L1418; 8: L1532; 9: L1823; 10: L0413; 11: L1524; 12: L0528; 13: L0032; 14: L0554; 15: L0592; 16: L1957; 17: L1584; 18: L1857; 19: L0349; 20: L0679

TABLE I

Results of the histopathology and polymerase chain reaction (PCR)-hybridization analysis of human paraffin-embedded biopsies from Tucumán-Argentina

	Samples	Lesion	Histopathology	PCR- hybrid *Leishmania* spp.	PCR- hybrid *L. braziliensis* complex	PCR- hybrid *L. (V.) braziliensis*
1	L-66239	*	+	+	-	+
2	L-69252	*	+	+	-	-
3	L-1085	Cutaneous	+	+	+	-
4	L-1299	*	+	+	-	-
5	L-1418	Cutaneous	+	+	-	-
6	L-1532	Cutaneous	+	+	-	-
7	L-1823	Cutaneous	+	+	+	-
8	L-0413 [a]	Cutaneous	-	+	-	-
9	L-0528	*	-	+	-	-
10	L-0032	Mucocutaneous	+	+	-	-
11	L-0554	Cutaneous	-	+	-	+
12	L-0592	Cutaneous	+	+	+	+
13	L-1524	Cutaneous	-	-	ND	ND
14	L-1584	Cutaneous	+	+	-	-
15	L-1857	Mucocutaneous	-	+	+	-
16	L-0349	Cutaneous	-	+	-	+
17	L-0679	Cutaneous	+	+	-	-
18	L-0805	Mucocutaneous	+	+	+	-
19	L-1957	Cutaneous	-	-	ND	ND
20	L-2195a	Cutaneous	-	+	-	-
21	L-2195b	*	+	+	-	-

***** The clinical data supplied by the Anatomy Pathologic Service (Hospital Angel C Padilla, Tucumán, Argentina) of these patients did not show whether the lesion was cutaneous or mucocutaneous; *a*: sample of a patient after complete treatment of the disease; ND: not done.

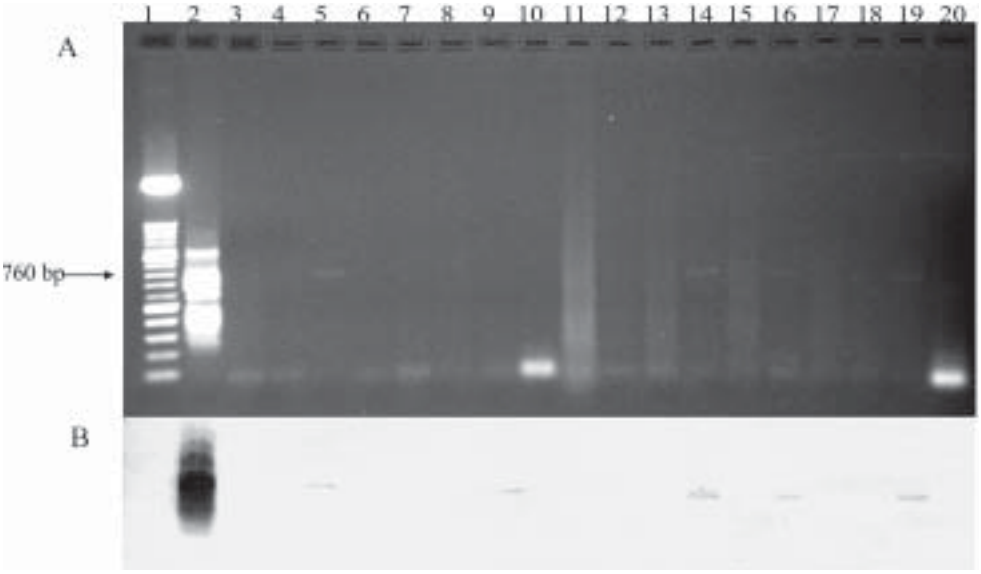

Fig. 2A: polymerase chain reaction amplification of paraffin-embedded biopsies using *Leishmania braziliensis* complex-specific primers; B: hybridization with a *L. (V.) braziliensis* specific probe. 1: 100 bp molecular weight marker; 2: positive control; 3: L66239; 4: L69252; 5: L1085; 6: L1299; 7: L1418; 8: L1532; 9: L1823; 10: L1957; 11: L0528; 12: L0032; 13: L0554; 14: L0592; 15: L1584; 16: L1857; 17: L0349; 18: L0679; 19: L0805; 20: L2195

DISCUSSION

This study reveals that *L. (V.) braziliensis* was the causal agent of leishmaniasis in the human samples analyzed using a PCR-hybridization assay.

The results presented here demonstrate that the leishmanial etiology was established in 61.9% by the conventional method and in 90.5% by PCR-hybridization. No false positive results were obtained. Laskay et al. (1995), using oligonucleotide primers 13A and 13B were able to detect *L. aethiopica* in paraffin-embedded tissue, in 7 out of the 22 cases where the conventional methods failed. They proposed the PCR as a valid diagnostic method for leishmaniasis, especially for chronic cases of the pathology. Furthermore, Medeiros et al. (2002) using the same primers found positivity in 81.5% of the examined samples by PCR, with a specificity of 100%, while the histological test was positive in 50% of the cases. Safei et al. (2002) found a sensitivity of 92% in the diagnosis of cutaneous leishmaniasis in paraffin-embedded biopsies using PCR, and a specificity of 100%.

The PCR combined with Southern blotting can increment the sensitivity of the method. Andresen et al. (1996) reached a 93% of positives using this combination when compared to the results obtained by PCR when used alone (86%) in contrast with the sensitivity detected by histopathology (76%).

Five of the total samples analysed by the complex specific primers B1/B2 (De Bruijn & Barker 1992) were positive for the *L. braziliensis* complex.

Furthermore, the PCR using the primers L14-A and L14-C (Alonso 1999) specific for *L. (V.) braziliensis*, which amplify the sequence of the ribosomal protein L14 of this species (Gonzalez et al. 2004), detected the parasite in 4 samples, only one of them was also amplified by the PCR specific for the *L. braziliensis* complex.

The oligonucleotides B1/B2 used in this assay are capable of detecting 1 fg of purified DNA, equivalent to about 1000 kDNA minicircles of 760 bp. This method was evaluated using a diversity of crude DNA samples: human biopsies and lymph aspirates; sand flies samples and biopsies from wild animals (De Bruijn & Barker 1992). De Bruijn et al. (1993) in a study performed in Cali, Colombia, using this method obtained 84.6% sensitivity for PCR compared to traditional methods.

We consider it possible that the oligonucleotide primers B1/B2 may not detect DNA of all the species belonging to the complex or its variants. These primers were design based on a fragment of 208 bp of the minicircle of kDNA common to the species *L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) peruviana,* and *L. (V.) panamensis.* These four species were considered integrants of the complex *L. braziliensis*. Moreover the common DNA sequences of *L. (V.) braziliensis, L. (L.) amazonensis* and *L. (L.) major* are also between this conserved region.

At present the *L. braziliensis* complex is formed by the species: *L. (V.) braziliensis, L. (V.) peruviana, L. (V.) colombiensis, L. (V.) naiffi, L. (V.) lainsoni,* and *L. (V.) shawi*, being the species *L. (V.) guyanensis* and *L. (V.) panamensis* included in the complex *L. guyanensis*. Taking this into account the primers B1/B2 can probably not detect the DNA of all the species included in the *L. braziliensis* complex.

The use of primers B1/B2 has not previously been reported in formalin fixed and paraffin embedded tissues samples. However, because of the results obtained by the PCR genus-specific we can exclude the possibility of PCR inhibition. The latter detected *Leishmania* DNA in all the histopathologic positive samples for cutaneous leishmaniasis. Furthermore, the negative control gave a negative

result in both PCR assays.

The results obtained by the PCR specific for *L. (V.) braziliensis* did not completely match those obtained by the specific primers of the *L. braziliensis* complex. This could be attributed to the fact that the primers L14-A/L14-C could amplify DNA fragments of only certain strains of *L. (V.) braziliensis*. PCR inhibition is discarded for the same reason as cited above for the primers B1/B2. Primers L14-A/L14-C were design based on the reference strains *L. (V.) braziliensis* (MHOM/PE/95/LQ-8) from Cuzco, Peru, and LR537, LR538, LR539, LR548, LR557, LR560 which were isolated from areas close to Cuzco. These oligonucleotides were also tested on other species, *L. (L.) infantum*, *L. (L.) mexicana*, *L. (L.) chagasi*, *L. (L.) forattini*, and with *Trypanosoma cruzi*, all showing a negative result (Alonso 1999).

The results of this study are reinforced by the clinical and epidemiological pattern of the human cases observed in Tucumán, which were related to leishmaniasis caused by *L. (V.) braziliensis* as observed in other outbreaks in the country (Sosa-Estani et al. 1998).

Diverse molecular and biological methods have proved that the outbreak that occurred in the north-western province of Salta in 1985 was caused by *L. (V.) braziliensis*, although the zymodeme analysis showed a close relationship with *L. (V.) guyanensis* and *L. (V.) panamensis* (Segura et al 2000). Furthermore, *L. (V.) braziliensis* was detected in patients from Santiago del Estero province (next to Tucumán) by monoclonal antibodies and isoenzyme techniques (Torno-Cafasso et al. 1995, Cuba-Cuba et al. 1996). However, in the Chaco region (north-eastern Argentina) the specie *L. (L.) amazonensis* was detected (Frank et al. 2000).

In summary, the results presented here demonstrate that the causative agent of human leishmaniasis in the analysed cases using PCR-hybridization is *L. (V.) braziliensis*. Finally, this study also suggests the presence of other species of the parasite belonging to the *L. braziliensis* complex, or other strains of *L. (V.) braziliensis* that were impossible to detect.

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