ARE CYTOCHROME *B* GENE SEQUENCING AND POLYMORPHISM-SPECIFIC POLYMERASE CHAIN REACTION AS RELIABLE AS MULTILOCUS ENZYME ELECTROPHORESIS FOR IDENTIFYING *LEISHMANIA* SPP. FROM ARGENTINA?

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Abstract. Recently, two techniques, polymerase chain reaction (PCR) amplification and sequencing of cytochrome b gene (*cyt* b gene sequencing) and polymorphism-specific PCR (PS-PCR) were recommended for *Leishmania* species identification. Before this study, however, the accuracy of these methods had not been tested against the multilocus enzyme electrophoresis, the current gold standard technique on this task. Therefore, a trial was done for the first time to compare the results obtained by these techniques, using 17 Argentinean *Leishmania* stocks in independent assays. For all the stocks examined, the same results at species level were obtained by the three techniques. Among them, 14 were assigned to *L. (Viannia) braziliensis*, and three to *L. (V.) guyanensis*. The two techniques, *cyt* b gene sequencing and PS-PCR, were able to distinguish between all the proven species responsible for leishmaniases in Argentina. Thus, both techniques were validated and could be used independently for the species designation of *Leishmania* parasites in the country.

INTRODUCTION

The leishmaniases are parasitic diseases that threaten 350 million people in 88 countries on 4 continents, with an annual incidence of new cases estimated between 1.5 and 2 million. They are caused by protozoan flagellates that belong to *Leishmania* genus, which includes around 30 taxa.[1] There is no direct correlation between these *Leishmania* species and the specific clinical patterns induced except several cases,[2] and they have shown different responses to the chemotherapy.[3] In addition, it is common for different species to coexist in the same endemic areas, as seen in Argentina, where *Leishmania (Viannia) braziliensis, L. (V.) guyanensis,* and *L. (Leishmania) amazonensis* have been incriminated as the causative agents of human leishmaniases by molecular methods to date.[4–9] In particular, substantial evidence exists that the diseases caused by *L. (V.) braziliensis* and *L. (V.) guyanensis* differ in their clinical presentation, diagnostic approach required, and therapeutic response to antimonials.[10,11]

For these reasons, the *Leishmania* spp. discrimination is important not only from an epidemiologic perspective but also from the clinical ones to improve the patients' prognosis, diagnostic methods, and to monitor clinical outcomes.[12,13] Moreover, the accurate identification of these parasites must be based on molecular approaches because parasitological, clinical, or epidemiologic features are by themselves insufficient for this task.[14]

Among the molecular methods applied on the *Leishmania* spp. identification, the DNA-based techniques have been used increasingly. One of them, the polymerase chain reaction (PCR) amplification and sequencing of cytochrome *b* gene method (*cyt* b gene sequencing) has recently been established as a useful tool for the identification and phylogenetic studies of the *Leishmania* genus, being able to differentiate among the species and from other trypanosomatids.[15] Another of these techniques, the polymorphism-specific PCR (PS-PCR), has been used for the *Leishmania* spp. identification based on the presence of bands amplified for subgenus or species-specific primers, without requiring other procedures.[16]

Since both *cyt* b gene sequencing and PS-PCR, as with the other DNA-based techniques, have some advantages over the classically used techniques on the *Leishmania* spp. identification, such as isoenzymes, serodemes, or schizodeme analysis,[12] they may have an advantage of the application in field work, particularly in Argentina. However, to be used widely as reliable and effective methods, it is necessary to evaluate their efficacy in restricted endemic areas.[17]

This article reports for the first time a double-blind assay designed to test the accuracy of the *cyt* b gene sequencing and PS-PCR methods on *Leishmania* spp. identification, compared with multilocus enzyme electrophoresis (MLEE), the current gold standard technique,[1] using a panel of Argentinean *Leishmania* isolates, as a required step in their optimization. The results are discussed in terms of the probable applications of these techniques, highlighting their advantages, on the areas of diagnosis, clinico-pathology, and epidemiology of leishmaniases in Argentina.

MATERIALS AND METHODS

Parasites. Seventeen *Leishmania* stocks, characterized by MLEE analyzing 12 loci, were included in this study. Sixteen of them were isolated in the northern areas of Argentina, one from a canine and 15 from humans, all American tegumentary leishmaniasis (ATL) cases. Their enzymatic profiles, phylogenetic relationships, and clinico-epidemiologic characteristics were reported previously.[4] The remaining isolate, named MHOM/AR/99/DMZ, was obtained from the stock MHOM/AR/99/JDM1 after performing culture-mouse passages 4 times, as follows: JDM1 promastigotes were cultured for 10 ds in 10 mL of sterile liver infusion tryptose medium

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supplemented with 100 U/mL Penicillin and 50 µg/mL Streptomycin (PE), 20% heat inactivated fetal bovine serum (hiFBS).[18] They were harvested and 0.05 mL of a suspension at 20.10^7 promastigotes/mL was inoculated into right footpads of 4-week-old mice. After 3 weeks of inoculation, the footpad tissue was aseptically removed, homogenized, and cultured again in the same medium. This procedure was repeated 4 times.

Five World Health Organization (WHO) reference strains: *L. (V.) braziliensis*, HOM/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; and one *L. (V.) braziliensis* Ecuadorian strain: MHOM/EC/88/INH-03 were used as controls.

Samples preparation for DNA analysis. Promastigotes of each isolate were cultured in RPMI 1640 medium supplemented with PE and 10% of hiFBS, diluting gradually to a final volume of 8 mL for 7 ds. Two tubes for each isolate were done. After washing 4 times (2,500 rpm, 10 minutes) with sterile PBS, 2 mL of TE buffer (10 mM Tris-HCl, pH = 8, 1 mM EDTA) or 99% ethanol were added to the pellet of each tube. These samples were aliquoted and stored at –20°C until use. This procedure was carried out at the same time that the pellets of promastigotes for MLEE were prepared.

Detection of cytochrome *b* gene using polymerase chain reaction. To extract the genomic DNA from each of the samples prepared, a genomic extraction kit (i.e., Genomic Prep Cell and Tissue DNA Isolation Kit (Amersham-Biosciences, USA)) was used, following the protocol and methods described by the company. The PCR was performed with Ex-Taq polymerase (Takara, Japan) under the following conditions: initial denaturation at 94°C for 3 minutes followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. Two hundred ng of parasite DNA as a template, LCBF1 forward primer (5′-GGTGTAGGTTTTAGTYTAGG-3′), and LCBR2 reverse primer (5′-CTACAATAAACAAATCATAATATRCAATT-3′) were used for the *cyt* b gene amplification. The PCR products were visualized on a 1% agarose gel.

Cytochrome *b* gene direct sequencing procedures. The c*yt* b gene sequences for each sample were determined following the procedure described previously.[15,19] Briefly, the amplified fragments, obtained as was described previously, were electrophoresed on 1% Seakam GTG agarose gel (FMC, USA), excised, and then purified by using the QIA quick Gel Extraction kit (Qiagen, USA). The purified DNA was examined by direct sequencing with the Big Dye Terminator Cycle Sequencing kit (PE Biosystems, USA) and ABI PRISM™ 310 Genetic Analyzer. The sequencing primers used were LCBF1, LCBR2, LCYT B F4L (5′-TGTTATTGAATATGAGGTAGTG-3′) and LCYT B R4 (5′-GAACTCATAAAATAATGTAAACAAAA-3′). The obtained sequences, assembled and edited by Genetyx Mac 11.0.0 (Software Development Co. Ltd, Japan) were compared with the reference strain ones, available from DDBJ/EMBL/ GeneBank nucleotide sequence databases with accession numbers from AB095957 to AB095970.

Polymorphism-specific polymerase chain reaction procedures. The DNA samples obtained from the *Leishmania* stocks as was described previously were centrifuged at 8000 rpm for 5 minutes and the pellets were treated with Insta-

Gene Matrix (Bio Rad, Hercules, CA). A dilution of 1/100 in MilliQ water of the resulting materials termed "InstaGene DNA preparations", was used as template and subjected to PCR procedures in a GeneAmp PCR System 2400 (Perkin–Elmer, Wellesley, MA) using Roche GeneAmp XL PCR Kit (Applied Biosystems, Foster City, CA), at a final volume of 15 µL. For *Leishmania* subgenus identification, the following conditions were applied: after initial denaturation at 95°C for 5 minutes, 35 cycles (30 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C) in the first step. This procedure was carried out using the V1 (5′-GCTTCTCGTTTCGCTTTGAAC-3′)– V2 (5′-CAAGACAAGAAAAAAGGCGGC-3′) for the detection of *L. (Viannia)*, and L1 (5′-GGTCACTCGGCATTTTTGC-3′)–L2 (5′-GTGCCCTGACTTGCATGTCTA-3′) for *L. (Leishmania)* subgenus.

In the second step, PS-PCR was performed under the following condition: 37 cycles (30 seconds at 95°C, annealing for 90 seconds at 70°C for the *L. (Viannia)* group or 60°C for the *L. (Leishmania)* group, and extension for 90 seconds at 72°C), using the primers as follows: b1 (5′-GTGGGCGTATCTGCTGATGAC-3′)–b2 (5′-CAAAAAGCGAGGGACTGCGGA-3′) for *L. (V.) braziliensis*, p1 (5′-GGTCGGATCTGCATGCATCAC-3′)–p2 (5′-CAAAAAGCGAGGGACTGCGGG-3′) for *L. (V.) panamensis*, g1 (5′-GGTCGGATCTGCATGCATCAT3′)–g2 (5′-CAAAAAGCGAGGGACTGCGGG-3′) for *L. (V.) guyanensis*, m1 (5′-TGCGAGGATAAAGGGAAAGAG-3′)–m2 (5′-GTGCCCTGACTTGCATGTCTA-3′) for *L. (L.) mexicana*, and a1 (5′-TGCGAGGATAAAGGGAAAGAA-3′)–a2 (5′-GTGCCCTGACTTGCATGTCTA-3′) for *L. (L.) amazonensis*.

The PCR products were separated on 2% agarose gels containing ethidium bromide.

Statistical analysis. To statistically analyze the association between the zymodemes and *cyt* b sequences, the Fisher's exact test was used.

RESULTS

The species assignation of a panel of 17 Argentinean *Leishmania* stocks and 5 WHO reference strains was performed by *cyt* b gene sequencing and PS-PCR in independent determinations, as is shown in Table 1.

All these parasites were characterized by MLEE analyzing 12 enzymatic loci. Their enzymatic profiles, phylogenetic relationships, and clinico-epidemiologic characteristics have been reported previously.[4] Briefly, 14 of the Argentinean *Leishmania* stocks expressed 3 zymodemes, and termed KMS 1 to KMS 3, were assigned to *L. (V.) braziliensis*. These zymodemes differed from the ones expressed for the WHO reference strains. Two other stocks, grouped in the zymodeme KMS 4, were identified as *L. (V.) guyanensis*.[4] The remaining isolate, MHOM/AR/99/DMZ, also expressed this last zymodeme (data not shown).

Cytochrome *b* gene sequencing analysis. On the panel of Argentinean *Leishmania* isolates, the *cyt* b gene amplification was performed applying PCR with LCBF1 and LCBR2 primers (Figure 1).[15] The 866 bp DNA fragments obtained for each one of the samples were sequenced and compared with the *cyt* b gene sequences of WHO *Leishmania* reference strains available from DDBJ/EMBL/GeneBank nucleotide sequence databases. Fourteen of these isolates were assigned

Species differentiation by three techniques: multilocus enzyme electrophoresis (MLEE) analysis, polymerase chain reaction (PCR) amplification and sequencing of cytochrome *b* gene (*cyt* b gene sequencing), and polymorphism-specific PCR (PS-PCR) of 17 Argentinean *Leishmania* stocks

Stocks designation	Species assignment*	Zymodeme	*Cyt* b gene sequence	PS-PCR‡							Clinical form
				V	L	p	b	g	a	m	
Argentinean isolates											
MHOM/AR/99/AZ3	*L. (V.) br*	KMS 1	Ab-1	+	−	−	+	−	−	−	CL
MHOM/AR/02/RLS6	*L. (V.) br*	KMS 1	Ab-2	+	−	−	+	−	−	−	CL
MHOM/AR/02/NSS3	*L. (V.) br*	KMS 1	Ab-1	+	−	−	+	−	−	−	MCL
MHOM/AR/02/MAS5a	*L. (V.) br*	KMS 1	Ab-2	+	−	−	+	−	−	−	CL
MHOM/AR/02/MAS5b	*L. (V.) br*	KMS 1	Ab-2	+	−	−	+	−	−	−	CL
MHOM/AR/03/AAS4	*L. (V.) br*	KMS 1	Ab-1	+	−	−	+	−	−	−	MCL
MHOM/AR/03/OLO1	*L. (V.) br*	KMS 1	Ab-1	+	−	−	+	−	−	−	CL
MHOM/AR/03/MRO2a	*L. (V.) br*	KMS 1	Ab-2	+	−	−	+	−	−	−	CL
MHOM/AR/03/MRO2b	*L. (V.) br*	KMS 1	Ab-2	+	−	−	+	−	−	−	CL
MHOM/AR/03/HNO3a	*L. (V.) br*	KMS 2	Ab-2	+	−	−	+	−	−	−	CL
MHOM/AR/03/HNO3b	*L. (V.) br*	KMS 2	Ab-2	+	−	−	+	−	−	−	CL
MHOM/AR/03/FDO4	*L. (V.) br*	KMS 2	Ab-1	+	−	−	+	−	−	−	CL
MHOM/AR/03/CFO5	*L. (V.) br*	KMS 2	Ab-2	+	−	−	+	−	−	−	RCL
MCAN/AR/02/LPO1	*L. (V.) br*	KMS 3	Ab-2	+	−	−	+	−	−	−	CL
MHOM/AR/98/LBC1	*L. (V.) gu*	KMS 4	AB095969†	+	−	−	−	+	−	−	CL
MHOM/AR/99/JDM1	*L. (V.) gu*	KMS 4	AB095969	+	−	−	−	+	−	−	CL
MHOM/AR/99/DMZ	*L. (V.) gu*	KMS 4	AB095969	+	−	−	−	+	−	−	CL
Reference strains											
MHOM/BR/75/M4147	*L. (V.) gu*	KMS 4	AB095969	+	−	−	−	+	−	−	CL
MNYC/BZ/62/M379	*L. (L.) me*	KMS 5	AB095963	−	+	−	−	−	−	+	CL
MHOM/BR/73/M2269	*L. (L.) am*	KMS 6	AB095964	−	+	−	−	−	+	−	CL, DCL
MHOM/BR/75/M2904	*L. (V.) br*	KMS 7	AB095966	+	−	−	+	−	−	−	CL
MHOM/PA/71/LS94	*L. (V.) pa*	KMS 8	AB095968	+	−	+	−	−	−	−	CL

L. (V.) br, Leishmania (Viannia) braziliensis; *L. (V.) gu*, *L. (V.) guyanensis*; *L. (V.) pa*, L. (V.) panamensis; *L. (L.) am*, L. (Leishmania) amazonensis; *L. (L.) me*, L. (L.) mexicana; KMS, Kochi Medical School; CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; RCL, recurrent cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis.
* Done separately by MLEE, *cyt* b gene sequencing, and PS-PCR; †GeneBank accession numbers of the *cyt* b sequences; ‡The pair of primers V and L were used for *Viannia* and *Leishmania* subgenus differentiation, respectively. The pairs of primers p, b, g, a, and m were used for detection of panamensis, *braziliensis, guyanensis, amazonensis,* and *mexicana* species, respectively.

to *L. (L.) braziliensis*, since the two *cyt* b gene sequences found, named Ab-1 (5) and Ab-2 (9), showed 99.87% and 99.75% similarity to the MHOM/EC/88/INH-03 *L. (V.) braziliensis* strain sequence (GeneBank accession No. AB095967). The sequences Ab-1 and Ab-2 differed by only one nucleotide base (Figure 2). Statistically, no association between the zymodemes reported and the present sequences was found ($P > 1.00$). A third *cyt* b gene sequence found, and shown for the remaining 3 stocks of the panel, was indistinguishable from the *L. (V.) guyanensis* reference strain MHOM/BR/75/M4147 sequence (GeneBank accession No. AB095969, Table 1).

***Leishmania* species identification by polymorphism-specific polymerase chain reaction.** This analysis is based on the occurrence of either *Leishmania* subgenus or species-specific PCR products corresponding to DNA fragments, which had originally been amplified by arbitrarily primed
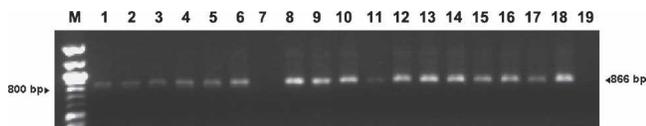

Figure 1. Polymerase chain reaction (PCR) products obtained with the primers LCBF1 and LCBR2 for the 17 Argentinean *Leishmania* isolates. Lane M: size marker (100 bp ladder, BioLabs inc, New England); lane 1, LP01; lane 2, NSS3; lane 3, LBC1; lane 4, DMZ; lane 5, JDM1; lane 6, AZ3; lane 8, OLO1; lane 9, MR02a; lane 10, MR02b; lane 11, HN03; lane 12, HN03; lane 13, FD04; lane 14, CF05; lane 15, MAS5, lane 16, AAS4; lane 17, MAS5; lane 18, RLS6; lane 7 and 19, negative controls. Arrow shows 886 bp (expected amplicon size). For isolates identification see Table 1.

PCR.[16] First, the samples were simultaneously tested in separate reactions, with *Leishmania* subgenus-specific primers. All the isolates of the Argentinean panel were identified as *L. (Viannia)* subgenus, since a 168-bp PCR-product with V1-V2 primers, but none with L1-L2 primers were detected. A 78-bp band with the L1-L2 primers was only observed for the WHO reference strains belonging to the *L. (Leishmania)* subgenus used in this study. In the second step, the species identification was performed using primers b1-b2, p1-p2, and g1-g2 for the samples identified as *L. (Viannia)* subgenus or a1-a2 and m1-m2 for the strains revealed as *L. (Leishmania)* subgenus. Thus, a 103-bp PCR-product with the primers b1-b2, but none with the other two pairs of primers was seen in 14 of the Argentinean samples; therefore, they were identified as *L. (V.) braziliensis*. The 3 remaining stocks were assigned *to L. (V.) guyanensis*, since only a 79-bp band with the primers g1-g2 was seen. (Table 1, Figure 3).

Both, *cyt* b gene sequencing and PS-PCR have agreed with MLEE on the *Leishmania* species identification for each one of the stocks and strains analyzed (Table 1).

DISCUSSION

The clinico-epidemiologic relevancy of the *Leishmania* species identification has induced the development and improvement of new molecular tools such as *cyt* b gene sequencing and PS-PCR, which, as another DNA-based tool, may have many advantages over the classically used methods.[12,15,16] However, it becomes necessary to test their accuracy on this task comparing with the gold standard technique, a validation assay, as an important previous step in their optimization for

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INH-03/M2904        121 GATAAGAAGTACACATATTTGTTTTACATCATTACTATTTTTTCTTCTTTATGTTCATAT 180
Ab-1                121 T........................................................... 180
Ab-2                121 T........................................................... 180
M4147/LBC1/JDM1/DMZ 121 ...............................................G............ 180


INH-03/M2904        361 TGGTGTTTGATTATGTTATTGAATTTGAGGAAGTGAATATATAAATGATTTTACTTTACT 420
Ab-1                361 ........................................................... 420
Ab-2                361 ..................................G........................ 420
M4147/LBC1/JDM1/DMZ 361 .........C................................................. 420
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FIGURE 2. Cytochrome *b* gene alignment. The substitution observed for the Argentinean stocks, Ab-1 and Ab-2 compared with MHOM/EC/88/INH-03 or HOM/BR/75/M2904 strain fragments sequence (GeneBank no AB095967 and AB095966, respectively) of *Leishmania (Viannia) braziliensis* are shown. For comparison, the align fragment of the sequence AB095969, corresponding to the *L. (V.) guyanensis* strains (M4147, LBC1, JDM1, and DMZ) was also included.

applying them in field studies. Besides, the tremendous diversity that exists in the *Leishmania* genus necessitates the comparison, using local *Leishmania* isolates from the geographic areas in which the novel techniques are going to be applied.[20]

Under this context, in the current study it was proven that the *cyt* b gene sequencing and PS-PCR methods precisely identified the *Leishmania* spp. for each one of the local stocks well characterized by MLEE, the gold standard technique, and that they were able to differentiate among the 3 proven species responsible for ATL in Argentina: *L. (V.) braziliensis*, *L. (V.) guyanensis*, and *L. (L.) amazonensis*.[4–9] In addition, the *cyt* b gene sequences have shown clear interspecific differences,[15] but high homogeneity among the zymodemes of the two local species analyzed, allowing an accurate species differentiation.

Therefore, these two techniques were validated and could have an application on the species identification of ATL causal agents in this country.

Among the properties of these two molecular methods supporting their applicability in field studies, it should be highlighted that they may not require the isolation and mass cultivation of the parasites, since they can be performed on samples taken directly from the patient (host) lesions and tissues, or from vectors,[19,21] in contrast with the huge amount of parasites required by MLEE method; the two methods need relatively shorter times than MLEE for processing; and in the case of PS-PCR, the technique could be applied in laboratories of relatively few resources to analyze great numbers of samples. Nevertheless, the application of the PS-PCR method is only restricted to the differentiation of the 5 major *Leishmania* spp.- *braziliensis, guyanensis, panamensis, amazonensis*, and *mexicana,* responsible for ATL. In the case of the *cyt* b gene sequencing, it could be used for molecular phylogenetic relationship analysis, as a second-line technique in the identification of samples in which the PS-PCR technique may not be conclusive, as in the possible appearance of a *Leishmania spp.* out of its range, or for the confirmation of very relevant cases.[15,19]

In conclusion, both the *cyt* b gene sequencing and the PS-PCR techniques showed a total agreement with MLEE, the current gold standard method on the identification of *Leishmania* spp. on a panel of Argentinean isolates. Hence, this is evidence that their results concerning the present task are very reliable, representing an advantage in the study of the leishmaniases in Argentina, where simple, field-applicable, and reliable diagnostic techniques are extremely necessary.

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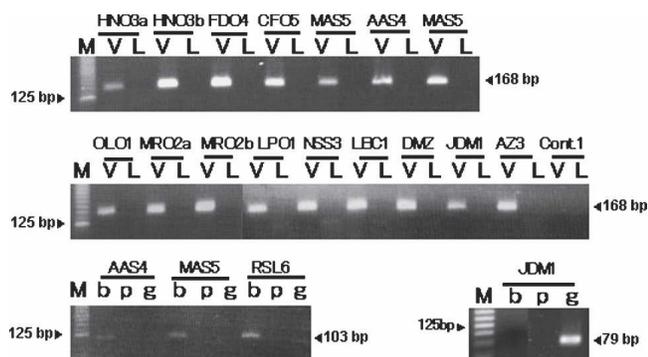

FIGURE 3. Argentinean *Leishmania* subgenus and species assignation by polymorphism specific (PS)-PCR. Products amplified using V1-V2 (V, expected amplicon size: 168 bp) and L1-L2 (L, 78 bp, no band seen) for *Viannia* and *Leishmania* subgenus respectively, b1-b2 (b, 103 bp), p1-p2 (p, 79 bp, no band seen) and g1-g2 (g, 79 bp) for *L. (V.) braziliensis, L. (V.) panamensis* and *L. (V.) guyanensis* species, respectively. For isolates identification see Table 1. Lane M: 25 bp DNA Ladder (Gibco BRL). The products were separated on 2% agarose gels containing ethidium bromide.

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