Differentiation in a Single-Tube PCR between Leishmania mexicana and Leishmania braziliensis in Clinical Samples

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Authors’ contributions
This work was carried out in collaboration between all authors. Authors VM, EQR and RL designed the study, authors VM, LB, JLR performed the statistical analysis, authors VM, RL, EQR and JLR wrote the protocol, and authors VM and RL wrote the first draft of the manuscript and managed literature searches. Authors VM, EQR, RDA, MV, RL, LB and JLR managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To assess the usefulness of a single-tube PCR end point for differentiating between Leishmania and Viannia subgenera in clinical samples of cutaneous leishmaniasis.

Methodology: Impression smears of 65 patients from Campeche State, Mexico with leishmaniasis-like lesions were analyzed by conventional Giemsa staining and a new simple PCR end point with primers named LU-5A designed by [Harris et al.1998] and LME (designed for this study and based on a mini-exon of L.(L) mexicana,GenBankX64317.1). This PCR generates an amplicon of 210 and

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1. INTRODUCTION

The diagnosis of cutaneous leishmaniasis (CL) is based on epidemiological data, clinical features, and laboratory tests. In most public health laboratories in Mexico and many others in Central and South America the demonstration of the aetiological agent by microscopic means is the most common method of establishing cutaneous leishmaniasis infection, but this approach does not enable the identification of the species involved [1]. The material for parasitological diagnosis obtained by scraping, fine-needle aspiration, biopsy of lesions or impression smears. This material can also be used for culture and molecular diagnostic techniques in those laboratories with available resources. Detection of parasite nucleic acids, essentially by PCR-based methods, can assist in the diagnosis. PCR-based methods are more sensitive than traditional methods such as culture or microscopy, particularly in those cases with few parasites in the stained preparations. The sensitivity of microscopic techniques ranges from 17 to 83%, whereas that of PCR depends on the DNA target and ranges from 53 to 98% [2-5]. However, knowing the identity of Leishmania species in the lesion is crucial for epidemiology and better treatment management. Multilocus enzyme electrophoresis is considered the gold standard method of identification, but it is time consuming and requires prior isolation and mass culturing of the parasites [6]. Therefore, other methods have been developed such as restriction fragment length polymorphism (PCR-RFLP), multiplex PCR, multilocus sequence typing (MST), nested PCR, and Real-Time PCR that targets kinetoplastid DNA (kDNA), minor-exons, or selected genes such as mannose phosphate isomerase (MPI), 6-phosphogluconato dehydrogenase (6PGD), 18S rDNA and cytochrome b [7-13]. Many of the approaches mentioned above require special equipment, qualified personnel and two different techniques such as PCR and restriction enzyme assay, or manipulation of the sample. A simple PCR able to differentiate between species may be useful in strengthening diagnostic capacity.

The sensitivity and specificity of the PCR assay relies on the primers and gene targets [4-5], the instruments used to obtain the sample, the location from which the sample was obtained [3,11], and the DNA extraction procedure. Other less well-studied factors that may also affect the performance of PCR include the presence of contaminant microorganisms in the ulcers, it is known that 60% of ulcerated lesions containing associated pathogenic or opportunistic bacteria [14].

In Mexico, CL may be caused by L. mexicana and L. braziliensis. Mucocutaneous leishmaniasis (MCL) is caused by the L. braziliensis complex, diffuse cutaneous leishmaniasis (DCL) by L. mexicana and visceral leishmaniasis (VL) by L. (L) chagasi [15-17]. In some regions of the country more than one species coexist, and thus diagnostic confirmation and correct identification of the species is essential. In the Yucatan peninsula of Mexico there is an endemic region of cutaneous leishmaniasis. In 1987 it was thought that Leishmania (L) mexicana was the only species involved in this pathology [18], but 11 years later L. (Vianna) braziliensis was recognized in human samples [19]. It is known that patients infected with L. (V) braziliensis have a higher risk of developing the mucosal manifestation and show varying response rates to therapeutic drugs [20,21]. Therefore, identification of the etiological species is epidemiologically important and may help improve patient management.

In Mexico and Latin America in general, many public health laboratories have PCR thermocycler facilities, but the diagnosis of cutaneous leishmaniasis usually based on microscopic

| Keywords: Leishmania mexicana; Leishmania braziliensis; diagnosis; PCR. | 270 bp with L. (L) mexicana (MHOM/MX/84/SETGS) and 230 bp with L. (V) braziliensis (MHOM/BR/75/M2903). |
|---|---|
| **Results:** Out of 65 impression smears of clinical samples 50 were positive according to PCR (76.9%) and 29 were positive according to microscopic observation (44.6%). The primers did not hybridized with human, Trypanosoma cruzi, Staphylococcus aureus, Staphylococcus epidermidis or Pseudomona aeruginosa DNA. Of the positive smears, 84% (42/50) corresponded to L. (L) mexicana and 16% (8/50) to L. (V) braziliensis. |
| **Conclusion:** A one-step PCR with primers LU-5A and LME improves the diagnostic capacity to differentiate between Leishmania and Vianna subgenera in parallel with conventional tests. |
examination. The development of a simple PCR able to differentiate between complexes of *L. (V) braziliensis* and *L. (L) mexicana* could strengthen diagnostic capacity. In addition, it may be used to gather useful information for epidemiology and better treatment management.

### 2. MATERIALS AND METHODS

#### 2.1 Human Clinical Samples and Diagnosis

Clinical samples from 65 patients with leishmaniasis-like lesions were obtained from regional health centers spread over Campeche State, Mexico, these centers being part of the network of State Public Health Services. Males represented 96% of the population studied; the most frequent lesions were single and ulcerated (76%); the duration of the lesion varied from 2 weeks to 4 years (90%); the size of lesions varied from 2 cm² to 40 cm² (the average area was 3 cm²); and secondary infection was present in 51% of patients. There was no mucosal involvement or cutaneous metastases. The impression smears were taken and delivered to the Central Public Health Laboratory in Campeche city (LESP). At LESP the impression smears were stained with Giemsa solution and examined under light microscopy by qualified technicians to establish a diagnosis; thereafter samples were coded and blinded and sent to the Laboratorio de Inmunoparasitologia at the Universidad Autonoma de Campeche (UAC), where the samples were prepared for PCR diagnosis.

#### 2.2 Parasite Cultures and DNA Extraction

*Leishmania (L) mexicana* strain MHOM/MX/84/SETGS, *Leishmania (L) amazonensis* strain MHOM/BR/67/PH8 and *Leishmania infantum chagasi* strain MHOM/BR/74/PP75 were maintained in NNN biphasic medium, or cultured in RPMI enriched with fetal calf serum when DNA parasite was required. In brief, the parasites were cultured for 6 days and then the parasite suspension was centrifuged. The pellet was washed three times with PBS and then suspended in 500 µL of lysis buffer solution (Tris 0.05 M pH 7.6, 100 µL SDS 10% and proteinase K at 10 mg/mL), and incubated for 1 h at 37°C. The digestion reaction was stopped by incubating at 72°C for 5 minutes. Finally the mixture was treated with phenol-chloroform-isoamyl alcohol (PCI 25:24:1) to allow DNA extraction. The DNA was precipitated with 3 M sodium acetate and ethanol (Ac-Eth), and the pellet was suspended in water and kept at -70°C until use.

The DNA from *L. (V) braziliensis* strain MHOM/BR/75/M2903 was donated by Omar Triana (Colombia).

#### 2.3 Bacterial Culture and DNA Extraction

Clinical samples of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* previously identified by biochemical means were cultured on blood agar plates and after 24 h the biomass was suspended in digestion buffer containing lysozyme in order to digest the cell wall. After this step the cellular suspension was treated with lysis buffer, FCI and precipitated with Ac-Eth as described above to extract DNA.

#### 2.4 DNA Extraction of Human Clinical Impression Smears

Each Giemsa-stained smear from 65 clinical samples was added to 100 µL of lysis buffer solution (Tris 0.05 M pH 7.6, 100 µL SDS 10% and proteinase K at 10 mg/mL) as previously described. This suspension was used to extract DNA as described above.

#### 2.5 Primers

We used a set of primers called LU-5A(5′-TTTATTTGGTATGCGAAACTTC-3′) designed by Harris et al. [13], which recognized a common sequence in the mini-exon gene, and LME (5′-GTACAGAAAACGATAATTATAGCGTTAG-3′) designed for this study, which anneals with a specific region of the mini-exon gene of *L. mexicana* (accession number GenBankX64317.1 and emb|FR799555.1) and will theoretically generate a 287-bp amplification product. However, in preliminary data we observed experimentally that the use of this set of primers (LU-5A and LME) generates amplicons of different sizes, depending on the type of *Leishmania* complex. A doublet band of approximately 210 and 270 bp is produced with DNA of *L. (L) mexicana* or *L. (L) amazonensis*; a single band of 230 bp with DNA of *L. (V) braziliensis*; and three bands of 410, 430 and 750 bp when DNA of *L. (D) chagasi* is used.
In order to confirm the quality and efficiency of the DNA extraction from the clinical samples, we used a set of primers for human β-actin gene: H-Cybt Forward: 5’CRTGAGMCAAATATCHTTYT-3’ and H-Cybt Reverse: 5’-AGGAGAGAAGGAAGAGAAAGT-3’ as previously reported [22], which produce an amplicon of 315 bp.

2.6 PCR

Identification of Leishmania: The amplification reaction was performed in a volume of 50 µL, containing enzyme buffer 5 µL (100 mM Tris–HCl, pH 8.4, and 250 mM KCl), 25 mM of dNTP and 1.5 U Taq DNA polymerase, 1.5 mM MgCl₂, 50 pmol of primers, and 1 ng of DNA (only in the positive control for Leishmania) or 5 µL of clinical samples. Initial denaturing took place at 94°C for 5 min, followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 5 min. Positive controls (Leishmania DNA), negative controls (human or bacteria DNA) and blanks (no DNA sample) were run in all experiments.

In order to evaluate the limit of detection of the PCR assay a dilution series of standard DNA of L. mexicana (0.0005–1 ng) was used. To evaluate the specificity of the PCR, human, Trypanosoma cruzi, mouse, Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa DNA was tested.

PCR for human β-actin gene: The amplification reaction was performed in a volume of 25 µL, containing enzyme buffer 2.5 µL (100 mM Tris–HCl, pH 8.4, and 250 mM KCl), 25 mM of dNTP and 1.5 U Taq DNA polymerase, 1.5 mM MgCl₂, 50 to 100 pmol of primers and 1 µL of DNA sample. The cycling annealing temperature was set at 42.5°C for 30 seconds and extension at 72°C for 30 seconds.

2.7 Cloning and Sequencing PCR Products

The amplification product of L. (V) braziliensis was electrophoresed and the band excised and purified using the extraction kit PureLink™ Quick Gel Invitrogen™. Purified DNA was cloned using the PCR®-4TOPO® kit according to the manufacturer’s instructions. Purified recombinant plasmid was sent for sequencing to CINVESTAV Mexico.

The NCBI GenBank was searched for similar sequences using Blast, and Clustal W was used for multiple alignment.

2.8 Statistical Analysis

The Kappa index was calculated using a 2X2 contingency table for comparison between Giemsa-stained smears of clinical samples and the PCR results.

2.9 Ethical Considerations

The study was considered as low risk according to the “Reglamento de la Ley General de Salud; National Secretariat of Health Mexico.” Clinical samples were taken as part of routine laboratory testing. Written consent was obtained from all participants. The study was approved by the Postgraduate and Research General Division of the Universidad Autonoma Campeche.

3. RESULTS

3.1 PCR for Human β-actin Gene

Six clinical samples out of 65 were negative for β-actin gene (10%), indicating that the extraction of DNA was not as efficient as desired (Fig. 1). However, all 65 clinical samples were analysed by PCR for Leishmania detection.

3.2 Simple PCR with Primers LU-5A and LME

Primers LU-5A and LME were originally designed to identify L. (L) mexicana but produce different amplicons depending on the Leishmania species. For instance, the DNA of Leishmania(L) mexicana and Leishmania (L) amazonensis, both from the L. mexicana complex, produces a doublet band of approximately 210 and 270 bp, but the DNA of L. (V) braziliensis generates a single band of approximately 230 bp (Fig. 2). It is known that the above species of Leishmania are involved in cutaneous or mucocutaneous leishmaniasis. L. (D) chagasi, which causes visceral leishmaniasis, produces bands of approximately 410, 430 and 750 bp. This result supports the potential use of this PCR for differentiating between the two species involved in cutaneous leishmaniasis.

Serial dilution of L. (L) mexicana DNA was carried out to establish the detection limit of the assay, and the results indicate a limit of 100 fg of
DNA when axenic cultures of parasite were used (data not shown).

The set of primers used did not hybridize with human, *T. cruzi*, *S. aureus*, *S. epidermidis* or *P. aeruginosa* DNA (Fig. 3).

### 3.3 Sequencing of PCR Products

In order to determine the sequence of the amplicon generated from DNA of *L. (V) braziliensis*, the fragment was purified, cloned in DH5α and the recombinant plasmid sequenced in both directions. A length of sequence of 229 nt was obtained from a clone named LB2 (Fig. 4). Blast analysis of the sequence in the NCBI GenBank found 100 hits, and those with a higher score and query cover >98% and identity >95% corresponded to a mini-exon of *Leishmania (V) braziliensis*, *L. guayanensis* or *L. panamensis* but not to *L. (L) mexicana*. This finding indicates that the primers LU-5A and LME, which were designed according to the *L. (L) mexicana* mini-exon, are also suitable for amplifying *L. braziliensis*. Given this information, we searched for the possible site at which the primers LU-5A and LME anneal on the mini-exon of *L. (V) braziliensis* and *L. (L) mexicana*. Fig. 5 shows the position and the expected size of the amplicons. The amplicon was 228 bp in *L. (V) braziliensis* and 287 bp in *L. (L) mexicana*.

Thus, the recombinant plasmid of clone LB2 was employed as a positive control instead of genomic DNA of *L. (V) braziliensis* in the PCR assay. In this way the risk involved in manipulating a pathogen such as *L. (V) braziliensis* was avoided.

**Fig. 1. Amplification product of β-actin gene of clinical samples**

The DNA of impression smears were subjected to PCR for β-actin gene amplification. Lanes 1 and 15 correspond to clinical samples; lane 16 molecular marker; β-actin amplicon is 315 bp.
Fig. 2. PCR products using primers LU-5A and LME with DNA of different Leishmania species
Lane 1 blank; lane 2 human DNA; lane 3 Trypanosoma cruzi DNA; lane M molecular markers; lane 4 L (L). mexicana 210 and 270 bp; lane 5 L (L) amazonensis 210 and 270 bp; lane 6 L (V) braziliensis 230 bp; lane 7 L (L) chagasi 410, 430 and 750 bp.

3.4 Human Clinical Samples and Diagnosis
Out of 65 impression smears of clinical samples, 50 were positive according to the PCR (76.9%) and 29 were positive according to microscopic observation (44.6%). Out of the 29 positive samples by microscopy, 27 were correctly identified by PCR, but two were false negative. By microscopy 36 clinical samples were negative but only 15 were negative by PCR. In order to calculate the sensitivity, specificity and index of concordance between the two tests, a 2X2 contingency table was constructed (Table 1). The sensitivity of the PCR was 93% and the specificity was 36%. The concordance between the tests calculated via the Kappa index was 0.27. This value indicates a poor concordance between direct microscopic observation and PCR.

The PCR can also identify the species of Leishmania involved in the ulcer lesion and distinguish between L. (V) braziliensis and L. (L) mexicana. Our data indicate that 84% (42/50) of clinical samples corresponded to L. (L) mexicana and only 16% (8/50) to L. (V) braziliensis (Fig. 5).

4. DISCUSSION
In most public health laboratories in Mexico and many others in Central and South America microscopy is the most prevalent method for establishing cutaneous leishmaniasis infection [1]. In this work we used the primer LU-5A, which was initially used for a multiplex PCR assay designed by Harris et al. [13], and recognizes a common sequence in the mini-exon gene of the genus Leishmania. The second primer LME was designed for this study and was directed to a specific region of the mini-exon gene of the L. (L) mexicana sequence (accession number GenBankX64317.1 and embl|FR799555.1). In preliminary data we observed experimentally that the use of this set of primers (LU-5A and LME) generates amplicons of different sizes, depending on the species of Leishmania. A doublet band of approximately 210 and 270 bp is produced with DNA of L. (L) mexicana or L. (L) amazonensis; a single band of 230 bp with
DNA of L. (V) braziliensis; and three bands of 410, 430 and 750 bp when DNA of L(D) chagasi was used. This finding allowed us to continue searching for a simple PCR to particularly L. mexicana and L. braziliensis, both of which are involved in cutaneous leishmaniasis in Mexican patients in the region of Yucatan, Mexico.

Establishing which Leishmania species are involved in the lesion has epidemiological and prognostic consequences. For example, it is known that patients infected with L. (V) braziliensis have a higher risk of developing the mucosal manifestation and varying response rates to therapeutic drugs [20,21].

**Fig. 3. Specificity of primers LU-5A and LME in the PCR assay**

Lane 1 Blank; lane 2 Genomic L.(L) mexicana DNA 210 and 270 bp; lane 3 Recombinant plasmid contained mini exon L (V) braziliensis 230 bp; lane 4 Human DNA; lane 5 S. aureus DNA; lane 6 S. epidermidis DNA; lane 7 P. aeruginosa DNA; lane 8 Trypanosoma cruzi DNA; lanes 9-13 negative human DNA; lane 14 molecular markers

**Clone LB2**

GTACAGAAACTGATACCTTATATAGCTTAGGGAAGCCCAGGGTCAGTGTGAGG
GGGGTGCGTTGTTGGGCGTCCCGAACCCTGTCATGCGCGCGCGCGCCTTGGCGCCACCTC
CAGGACACACCCGGCCACCAACACACAAAACAAAACACAGCGCAGATGGCGACCCAAAATGTGG
CCGGAAGAAGGTCCCGGAAAGTTTCGATACCAATAAAA

**Fig. 4. Nucleotide sequence of amplification PCR product of L(V) braziliensis**
Multilocus enzyme electrophoresis is considered the gold standard technique for differentiating species of Leishmania, but it is time consuming and requires prior isolation and mass culturing of the parasites [6]. However, other approaches have been developed such as restriction fragment length polymorphism (PCR-RFLP), multiplex PCR, Real-Time PCR and optimization of single-tube nested PCR [7-9,12,13,23]. The sensitivity and specificity of the PCR assay rely on the primers and gene targets, and it has been demonstrated that the highest sensitivity is achieved using primers targeting kinetoplastid DNA (kDNA), followed by rRNA gene internal transcribed spacer, heat shock 70, spliced leader mini-exon and glucose-6-phosphate dehydrogenase, with specificity ranging from 98% to 42% [4,5].

Although we do not have a clear explanation for the two amplicons produced from Leishmania (L) mexicana, our hypothesis is based on empirical results that indicate that 210 bp may be a product of incomplete hybridization of primers LU-5A and LME with the Leishmania (L) mexicana DNA genome; when purified amplicons of 210 and 270 bp were excised directly from the gel and then re-submitted to a second round of PCR amplification, only the 270 bp product was generated. LU-5A and LME hybridized better with
the larger fragment than the smaller one. This finding is expected because theoretically the PCR product should be close to 287 bp, and hence the smaller fragment may be a non-specific PCR product. In addition, in some clinical samples the 210 bp band was lacking. However, sequencing of the PCR products would be necessary to confirm the nature of the two amplicons.

In the case of *L. (V) braziliensis* the PCR product was approximately 230 bp. In order to confirm that this 230 bp product corresponds to *L. (V) braziliensis*, the fragment was purified, cloned and sequenced. The sequenced product was 229 nt in length and corresponds to the mini-exon of *L. (V) braziliensis* (Fig. 4). According to blast analyses this sequence presents high homology with the mini-exon of *Leishmania (V) braziliensis, L.guayanensis* and *L. panamensis* but not to *L. (L) mexicana* (data not shown).

Using the sequencing data to search for similar sequences in the NCBI Gen Bank and multiple alignment with Clustal W it was possible to identify the potential site at which the primers align on the mini-exon genes. In the case of *L. (V) braziliensis* 228 bp and for *L. (L) mexicana* it is 287 bp. This finding explains why a single set of primers can generate two different amplicons depending on the species of *Leishmania* (Fig. 5).

In this study 65 impression smears from patients with ulcers were analysed for *Leishmania* parasite. The Laboratorio Estatal de Salud Publica de Campeche is the Public Health Laboratory in charge of establishing the diagnosis of this tropical disease. The slides were stained and the parasite was searched for microscopically. Out of 65 impression smears 29 (44.6%) were positive, and there were usually two to three slides per patient. One of these slides was sent and processed by PCR at the Centro de Investigaciones Biomedicas, Universidad Autonoma de Campeche; the PCR identified 50 positive samples (76.9%) out of 65. Positive, negative and blank controls were included in all PCR runs. These data confirm that PCR is more sensitive than direct observation, as previously reported [2-5]. The sensitivity of microscopic techniques ranges from 17 to 83%, whereas that of PCR, depending on the DNA target, ranges from 53 to 98%. However two samples gave negative results by PCR despite positive results microscopically. DNA extraction is a critical step in the PCR method, regardless of the sensitivity of the PCR. The detection of a constitutive gene such as β actin in the extracted DNA material is a good way of verifying the quality of the DNA extraction method. In our work 10% of samples were of poor quality, as it was not possible to detect the β actin gene. It is possible that poor extraction may have influenced the false negative result obtained.

Other factors that may affect the performance of the PCR include the presence of contaminants in the lesion; it is known that 60% of ulcerated lesions having associated pathogenic or opportunistic bacteria [14]. The presence of *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa* in ulcerated lesions is common, and it was thus necessary to check whether the DNA of such microorganisms hybridized with primers in the PCR assay. However, our data indicate that the presence of this DNA did not influence the performance of the PCR (Fig. 3).

Our study found that 84% (42/50) of clinical samples corresponded to *L. (L) mexicana* and 16% (8/50) to *L. (V) braziliensis*. The fact that *L. (V) braziliensis* was present in our sample of patients should be of concern to health authorities, because the mucosal manifestation of the disease is more complicated to treat and does not resolve spontaneously. It is thus recommended to include diagnostic tools able to identify the individual species in lesions, particularly *L. (V) braziliensis* and *L. (L) mexicana* in the Yucatan, an endemic zone for CL.

The Kappa index was calculated using a 2X2 contingency table and for comparison with our PCR results. We considered Giemsa-stained technique as the reference laboratory test. The specificity of the PCR was 36%, but its sensitivity was high (93%). The concordance Kappa index was poor, at 0.27 (Table 1). These values were expected because PCR is more sensitive than conventional techniques.

5. CONCLUSION

In conclusion the simple PCR presented here may help strengthen diagnostic capacity in parallel with conventional tests.

COMPETING INTERESTS

Authors have declared that no competing interests exist.
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