Short review and detection of Leishmania parasites in skin biopsies from patients coming from Echeandía, Bolívar Province, Ecuador

Breve reseña y detección de parásitos Leishmania en biopsias de piel de pacientes procedentes de Echeandía, provincia de Bolívar, Ecuador

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Abstract

Leishmaniasis is an important neglected tropical disease and the cause of significant morbidity in endemic countries such as Ecuador. It is transmitted by the bite of infected sand flies (Diptera Psychodidae) and has been reported from at least 22/24 provinces in the country including the foothills of the Andean mountains. Herein, we report the detection of Leishmania parasites, using PCR assays, in skin biopsies taken from ulcers of leishmaniasis patients from Echeandía in the province of Bolívar, Ecuador. These results suggest that most of the patients were infected with parasites from the braziliensis complex that among other species, includes Leishmania (Viannia) panamensis which is the most common species that causes cutaneous leishmaniasis in the Pacific coast, and Leishmania (V.) braziliensis, the cause of mucocutaneous lesions and significant patient disfiguration. More studies are needed to further identify the parasite species present in this area and address aspects of the parasite’s life cycle and transmission dynamics. Our preliminary results showed that the R174 and R798 18 S ribosomal DNA primers used in this study are effective to amplify Leishmania DNA and would be a useful tool to molecularly detect New World parasites causing tegumentary leishmaniasis.

Keywords. PCR, Echeandia, leishmaniasis, skin biopsies.

Resumen

La leishmaniasis es una importante enfermedad tropical desatendida y es la causa de significativa morbidad en países endémicos como el Ecuador. La enfermedad es transmitida por la picadura de flebótomos (Diptera:Psychodidae) infectados y ha sido reportada en al menos 22/24 provincias del Ecuador incluyendo las estribaciones de la cordillera de los Andes. En esta publicación reportamos la detección de parásitos de Leishmania mediante ensayos de PCR en biopsias de piel de pacientes con diagnóstico de leishmaniasis provenientes de la zona de Echeandía en la Provincia de Bolivar, Ecuador. Los resultados sugieren que la mayoría de los pacientes fueron infectados por parásitos pertenecientes al complejo braziliensis que incluye entre otras especies a Leishmania (Viannia) panamensis, el parásito más abundante en la costa Pacífica y causa de leishmaniasis cutánea y Leishmania (V) braziliensis, la causa de lesiones mucocutáneas y significativa desfiguración del paciente. Más estudios son necesarios para identificar las especies del parásito que circulan en esta zona y abordar otros aspectos de los ciclos naturales de cada especie del parásito y la dinámica de transmisión. Los resultados preliminares muestran que los cebadores R174 and R798 del ADN 18 S ribosomal usados en este estudio son efectivos para amplificar ADN de Leishmania y pueden ser una herramienta útil en la detección de parásitos del Nuevo Mundo que causan leishmaniasis tegumentaria.

Palabras Clave. PCR, Echeandia, Leishmaniasis, biopsias de piel.

Introduction

Leishmaniasis is a tropical disease caused by obligate intracellular protozoan parasites of the genus Leishmania and transmitted in the New World by the bite of infected sand fly females of the genus Lutzomyia (Diptera: Psychodidae). Cases of leishmaniasis occur mainly in the tropics and subtropics and disease burden is considered greatly underestimated; reports of the disease are only mandatory in 32 of the 88 countries affected and, according to the World Health Organization (WHO) 2010, is one of the most important neglected vector-borne dis-
Leishmania (V.) panamensis is the most common parasitic form which is endemic in the warmer Pacific lowland where at least 22/24 provinces have reported cases of the disease [35]. In Ecuador, at least some patients with Chiclero's ulcer [34], and a description of book publications [24–31]. The most recent reports referred to the knowledge of the parasites [8–11], the clinical forms [17–20], prevention and treatment strategies [21–23] and other aspects of the disease in Ecuador, many studies compiled in a series of book publications [24–31]. The most recent reports have used two PCR assays to detect Leishmania infec-
tion in skin biopsies taken from patients with microscopically confirmed leishmaniasis from the area of Echeandia in the province of Bolivar, Ecuador. To our knowledge this is the first time that this 18sRNA PCR primers have been used to detect Leishmania parasites from Ecuador.

Materials and Methods
Human samples
Tissue samples were taken from 11 patients, with symptoms of leishmaniasis and positive microscopic diagnosis that were admitted to the Echeandía health center in the town of Echeandía, Bolivar province (Figure 1) between August 2003 and February 2004. Patient’s name will not be cited in this report, but is kept as part of the medical records in the health center at Echeandía. Tissue samples were taken from the ulcer border using local anesthetic and the smear from biopsy material placed in a glass slide was stained using Giemsa. (Table 1). The rest of the tissue was stored in L6 buffer in a 1.5 ml eppendorf tube and sent to the USFQ laboratory for further analysis. Biopsies were taken primarily with a diagnosis purpose only in the cases where diagnosis based on clinical manifestations or microscopic examination of ulcer material taken routinely from scraping the ulcer border using a scalpel was not conclusive. This explains the limited number of samples analyzed using molecular analysis. Procedures were carried out by a physician at the health center of Echeandía under the supervision of Dr. Carlos Reck strictly following safety procedures followed by the Echeandía health center.

DNA isolation and PCR Assays
For all the PCR assays, DNA was extracted from the biopsy samples using a DNeasy tissue kit (Quiagen®) following the manufacturer’s instructions. For the PCR assays, two different assays were used to detect Ecuadorian Leishmania parasites: (1) An assay based on a multiplex PCR [45] which uses one forward primer (Lu 5A) common to all Leishmania species and three reverse primers (LB-3C, LM 3A, LC3L) specific to species belonging to the Leishmania braziliensis, Leishmania mexicana and Leishmania donovani parasite complexes which amplify PCR
Preliminary results - Date: April 12/04

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
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<th>Place</th>
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<td>+</td>
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<td>Leg</td>
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Table 1: Information from patients with Microscopic diagnosis (Giemsa) of Leishmaniasis and results from two molecular detection PCRs (RNA mini exon PCR assay and 18S Ribosomal DNA PCR assay.

products as detailed below and (2) an assay specific to the genus *Leishmania* [46] that targets a portion of the 18S ribosomal gene and was evaluated at the Royal Tropical Institute, KIT Medical Research (primers were kindly provided by Dr H. Schallig). In both cases, procedures described in the bibliography were modified and DNA amplification was done using PCR Ready to go Beads (Amersham Pharmacia®), a commercially available product that is easy to use where all the reagents for the PCR reaction come ready to use compacted in a solid bead inside a small eppendorf tube.

**RNA mini exon gene PCR Assay**

Based on a multiplex PCR assay, the RNA mini exon gene repeats were used to detect all three New World complexes of *Leishmania* parasites. The PCR primers used were as follows: the forward primer (Lu - 5A, 5’-ttt att ggt atg cga aac ttc - 3’), reverse primers (LB - 3C, 5’- cgt cgcc gaa ccc gtc gtc - 3’) specific for the braziliensis complex of *Leishmania* species amplifying a fragment of 146 - 149 bp or dimers of 375bp length as reported in the bibliography, reverse primer (LM - 3A, 5’- gca ccc cac cgg a/gcg ac - 3’), specific for the mexicana complex that produced segments of 218 – 240 bp long and reverse primer (LC - 3L, 5’- gcc cgc gc/tg tca ccacca t – 3’) specific for the donovani complex that produced fragments of 351 - 397 bp length. **PCR** reactions for each *Leishmania* complexes were conducted separately using the same forward primer and different reverse primers under the same thermal cycler conditions: 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 sec, 54 °C for 45 sec, 72 °C for 30 sec with a final extension of 72 °C for 5 min.

**PCR assay with 18 S ribosomal DNA primers**

For the PCR reaction, 2.5 µl of DNA were amplified in a final volume of 25 µl with primers (R174, 5’- ggttcc ttctgatttacg-3’) and (R798, 5’- ggcggtaaaggccgaat ag – 3’) to yield a fragment of 600 bp length. The thermal cycling conditions used were as follows: 50 °C for 5 min, 94 °C for 10 min and 40 cycles of 94 °C for 75 sec, 60 °C for 1 min, 72 °C for 2 min with a final extension of 72 °C for 50 min.

For both assays, PCR products were analyzed by gel electrophoresis using 2% Ultra Pure or MetaPhor® agarose for the fine separation of small DNA fragments and visualized under a UV light following standard procedures. Interpretation of the patterns was based on the size and on the presence or absence of amplified DNA bands.

**Results and Discussion**

From the tissue biopsies analyzed for the presence of *Leishmania* parasites using PCR, only 6 (54.5%, n=11) were positive as tested with the two sets of primers used in this study as reported in Table 1. Electrophoresis from the PCR products using Ultra Pure agarose using the RNA mini exon PCR assay showed poorly-defined bands but with clear signs of DNA amplification. MetaPhor® agarose prepared gels showed evidently more defined and clear bands of 149 bp confirming the presence of parasites from the braziliensis complex (Figure 2). Presence of additional bands of 375 bp possibly corresponded to dimers of two linked DNA fragments, that were previously reported in the bibliography [45].

Electrophoresis from samples 5, 7 and 8 revealed an unknown pattern of 4 bands using the RNA mini exon...
PCR assay (gel not shown) suggesting an unspecific amplification; no amplification was detected using the 18 S ribosomal DNA primers (Figure 3). Both PCR assays showed consistent positive/negative results in all the tissue biopsies except for samples 11 and 16. Sample 11 examined with LU5A-LB3C (braziliensis complex) primers showed a strong band of 149 bp and four additional bands with a similar band pattern to the one observed in samples 5, 7 and 8. However, was not recognized by the 18S ribosomal DNA primers. Sample 16 showed no amplification using the LU5A-LB3C set of primers to the braziliensis complex (gel not shown) but showed a weak but distinct band of 600 bp using the 18 S ribosomal DNA primers suggesting the presence of Leishmania parasites (Figure 3).

The results which showed a different band pattern than the expected according to the bibliography using the RNA mini exon PCR assay may suggest that further standardization is needed and the use of beads instead of mixing the reagents individually may have affected the amplification. On the other side, it is also possible that the different band pattern might correspond to different Leishmania species within the braziliensis complex of parasites. Several past studies have reported co-infection of two parasite species [47], the presence of new parasite species (Le. major-like) [48] and hybrid species in Ecuador [49]; recently Le. naiffi was for the first time found in Ecuador [50] and Lutzomyia tuberculata was for the first time found constituting a new sand fly species record for the country [51], thus the immense biodiversity of parasite and vector species still need to be further investigated. According to the information reviewed, Le. (V) panamensis is the only parasite species found in the Northern part of Bolívar province [7]. More studies are needed to further identify the parasite species circulating in Echeandía and to further evaluate the PCR assays used in this study. To our knowledge this is the first time that this 18S ribosomal DNA PCR assay is tested with Leishmania samples from Ecuador. These preliminary results suggest that R174 and R798 primers may be an effective alternative and a useful tool for the detection of Leishmania parasites in Ecuador and in the New World, nevertheless, other species-specific PCR methods would be necessary to identify to species the Leishmania parasites circulating in this region of Ecuador.

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References


