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Research Article

Localized cutaneous Leishmaniasis in Chiapas. A case report

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Abstract

Leishmaniasis is a parasitic disease affecting skin, mucocutaneous tissue, and/or internal organs and it is endemic in southern Mexico including Chiapas.The disease is sometimes difficult to diagnose with classical laboratory methods and therefore a molecular PCR approach was utilized in the following case report. A male 55 years-old from Villacorzo, Chiapas, presented himself with a suppurating cutaneous ulcer on the left leg. A biopsy was collected, DNA extracted and PCR analysis using genus- or species-specific primers generated 603 and 790 bp amplicons, respectively, allowing the identification of *Leishmania mexicana*.The PCR assay allowed us the rapid and specific identification of *L.mexicana* as the etiologic agent of this cutaneous lesion in Chiapas.

Keywords: *Leishmania mexicana*, Chiapas, Leishmaniasis, PCR

Introduction

Leishmaniasis is a disease caused by a protozoa parasite of the genus *Leishmania*, family Trypanosomatidae, that include over 20 species. Parasitic diseases induced by these species are distributed world-wide but have in common that are transmitted to people by insect bites, specifically by females of genera *Phlebotomus* (Africa, Europe, and Asia) and *Lutzomyia* (the Americas) [1]. Clinical manifestations of Leishmaniasis include skin injuries in the form of ulcers that can be utilized to clasify the disease as localized cutaneous Leishmaniasis (LCL), diffused cutaneous Leishmaniasis (DCL), or mucocutaneous tissue injuries (mucocutaneous Leishmaniasis –MCL-). The other clinical form is a disseminated infection known as Visceral Leishmaniasis (VL) or kala azar [2,3].

Leishmaniasis is considered as one of the neglected diseases and as such, it is endemic in the world´s poorest regions [4]. In Mexico, a developing country with a high prevalence of Leishmaniasis, the southern states including Yucatán, Quintana Roo, Campeche, Tabasco, Veracruz and Chiapas have been identified as highly, recurrent, transmission areas [5,6]. From 2000 to 2002, a study on VL conducted in the central valley of Chiapas, demonstrate high seroprevalence against *L. mexicana* and *L. chagasi* among patients (77%) [7]. During

2010, government authorities from this state reported cases of Leishmaniasis as follows: LCL (N=4), DCL (N=2), MCL (N=3) and VL (N=1). These cases were registered at the central valley of Chiapas [8]. In 2014, Mexico´s Ministry of Health reported 147, 77, 66, 48 and 34 cases of cutaneos Leishmaniasis in Tabasco, Quintana Roo, Nayarit, Campeche, and Chiapas, respectively; a similar trend has been reported during 2015 [9]. For this reason, this parasitary disease still represents a serious public health problem in Chiapas, southern states of Mexico and perhaps other developing countries. Prompt diagnosis will lead to an appropriate manage of the patient wich in turns should help reducing the burden of complicated cases of Leishmaniasis as well as mortality in endemic zones (e.g, southern Mexico).

Materials and Methods

The central valley of Chiapas has presented a high prevalence of VL [7] so that, since mid-2014, we have been looking for cases of cutaneous Leishmaniasis in this region. The clinical case we present hereby corresponds to a male individual (farmer), aged 55, native and resident of Nuevo Vicente Guerrero, Villacorzo locality (Chiapas). The physical examination revealed the presence of a painless injury (approximately 30 x 25 mm of diameter) that patient referred as been first noticed it at least 6 months earlier. The injury was located in the front of the left leg (as an ulcer) presenting a bleeding chronic process and erythematosus zone. The injury presented a defined indurated margin and dry and scabby zones with possible localized infection (Fig. 1A). After obtaining the patient´s informed consent from the Bioethics Commission of the state of Chiapas (approval ID 5003/5857), it was possible to obtain a biopsy of the affected zone, using a 3 mm surgical biopsy punch (Miltex®) (Fig. 1B). The tissue was placed in 500 µl TE buffer solution (Tris HCl 10 mM, EDTA 1 mM, pH 8.0). Then, it was transported to the laboratory in a cooler.

DNA was then extracted from the tissue using the DNAeasy Blood and Tissue Kits (Qiagen), following the manufacturer´s instructions. The presence of *Leishmania mexicana* was investigated using as template this genomic tissular material and two polymerase chain reactions (PCR) that targeted: 1) the subunit 18S rRNA and ideintified genus level (i.e., *Leishmania*) and 2) an specific gene for *L. mexicana* as specified below. Reactions contained 13.5 µL of the GoTaq®DNA polymerase mix, Green GoTaq®2X (pH 8.5) reaction buffer, $MgCl_2$ 3 mM and 400 mM of each deoxynucleotide triphosphate (GoTaq® Green Master Mix, Promega), 1 µL of specific primers, 2 µL of DNA sample and 8.5 µL of sterile nuclease-free water. The presence of a 603 bp (base pairs) gene which encodes the subunit 18S rRNA (*Leishmania* genus) was investigated in the first PCR [10], using primers R221/R332 [11] at a concentration of 0.63 µM; reaction conditions were previously described [12]. In the second reaction, a specific rRNA internal transcribed spacer (ITS) region encode by *L. (L.) mexicana* complex (790 bp) was amplified using primers IR1/LM17 in a final 0.81 µM concentration. Reaction conditions utilized had been reported earlier by Berzunza-Cruz et. al. (2009). DNA obtained from *L. (L.) mexicana* axenic cultures, strain MNYC/BZ/62/M379 [13] and *Trypanosoma cruzi* strain Y MHOM/BRA/53/Y [14] were used as a positive or a negative control, respectively. DNA from these parasites was purified as mentioned earlier. PCR products were resolved by electrophoresis in 2.5% agarose gels [15] in 1X TAE (Tris acetate 400 mM, EDTA 10 mM, Promega) buffer at 100 Volts during 40 min, gels were stained with GelRed (Biotium) and analyzed in a documentation system (Enduro™ GDS, USA). A 100 bp ladder was utilized (TrackIt™, Invitrogen) as a molecular weight marker.

In order to confirm the identity of both PCR products (603 and 790 bp) obtained from the patient, those products were gel-purified using the QIAquick ® Gel Extraction Kit (Qiagen) and then sequenced at Eurofins Genomics (Huntsville, AL). Sequences were loaded in the GenBank with numbers KU177477 and KU177478, respectively.

Results and Discussion

Molecular results demonstrate the presence of specific PCR amplicons of 603 and 790 bp, corresponding to *Leishmania* genus and L. *(L.) mexicana* species, respectively, using as template DNA purified from the tissue of the presented clinical case. These PCR amplicons were similar to those bands obtained using as a template DNA from L. *(L.) mexicana* strain MNYC/BZ/62/M379 whereas no reaction was observed when DNA from the negative control *Trypanosoma cruzi* strain Y MHOM/BRA/53/Y was utilized as template for the PCRs (Fig. 1C). Sequencing of the 603 bp PCR products demonstrate a 100% homology with the nuclear small subunit ribosomal RNA gene (GenBank accession number X53912) of *Leishmania genus*. As expected, the sequences of the 790 bp PCR product showed the gene includes the 18S ribosomal RNA gene (GenBank accession number FJ948433) of *L. mexicana* strain. Therefore, in this study we rapidly identified a case of *Leishmania mexicana* using DNA purified from the biopsy obtained from a patient presenting an ulcer very suggestive of cutaneous leshmaniasis which was further confirmed by sequencing of the PCR products.

Among target genes used to detect parasitic disease cases caused by *Leishmania* through PCR we can mention: ribosomal genes, kinetoplast DNA, mini-exon derived RNA, a region of the β-tubulin gene [16] and a preserved region of the minicircle present in the genus Leishmania [17]. A comparative study of molecular techniques for the diagnosis of cutaneous *Leishmaniasis* showed that the PCR for amplifying kinetoplast DNA was more sensitive (98.7%), compared with the one amplifying the rRNA ITS gene (91%) or the mini-exon spliced leader (53.8%) [19].

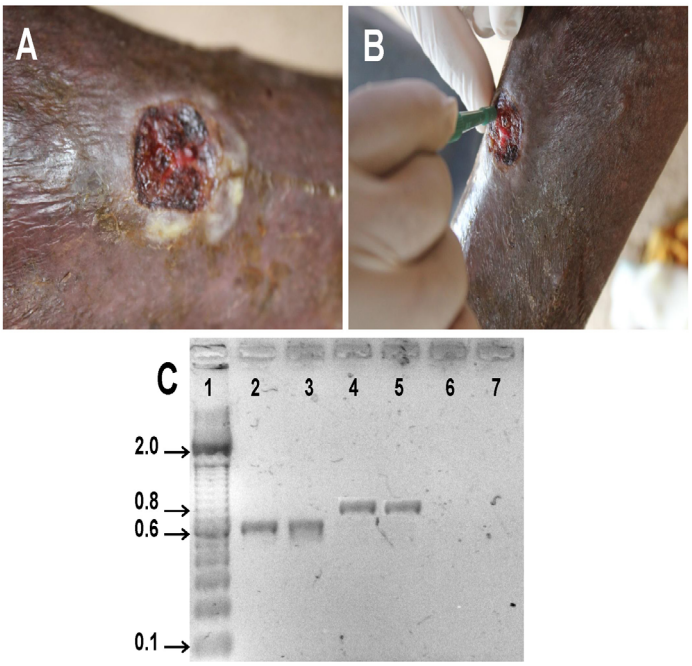

Figure 1. Molecular identification of *Leishmania mexicana* in a case of cutaneous Leishmaniasis. A) Cutaneous wound localized in the front of the left leg of an affected patient. B) Tissue biopsy collection. C) PCR reactions to identify *Leishmania mexicana*. Lane: 1, MWM, fragments at left are shown in Kb. Lanes 2 and 3, PCR amplicons using as a template DNA from L. *(L.) mexicana* strain MNYC/BZ/62/M379 or DNA from the patient´s biopsy, respectively, and genus-specific primers. Lanes 4 and 5, PCR amplicons obtained using DNA from L. *(L.) mexicana* DNA, strain MNYC/BZ/62/M379 or DNA from the patient´s biopsy, respectively, using primers to identify at the specieis level. Lanes 6 and 7, absence of amplicons using DNA from *T. cruzi*, Y strain (MHOM/BRA/53/Y) and using primers specific for *Leishmania genus* and *L. (L.) mexicana*, respectively.

MWM: molecular wight marker; Kb, kilobases.

Lemrani et. al.(2009) utilized a PCR aiming to amplify genes encoding the small ribosomal subunit of *Leishmania* species in biopsies of Moroccan patients which presented cutaneous ulcers; the demonstrate specificity, and sensitivity, was 100, and 84.6%, respectively [19]. In Durango and Sinaloa (Mexico), clinical cases of *L. mexicana* were detected with a PCR that amplifies the ribosomal ITS region, followed by the amplicon digestion with *Hae*III endonuclease, allowing the species identification [20,21]. Monroy-Ostria et. al (2014) determined a high prevalence (94%) of *L. mexicana* in cutaneous lesions of patients from Campeche, Mexico, by means of a ITS1 PCR-RFLP technique [22]. The PCR described by Berzunza-Cruz et. al. (2009) for identifying L. *(L.) mexicana* has high sensitivity using DNA obtained from the tissues of patients that presents LCL and DCL (81.2 and 100%, respectively) [13]. Our case study was the first patient from central valley of Chiapas for whom such a molecular technique has allowed the rapid, sensity and specific diagnosis of cutaneous Leishmaniasis caused by L. *(L.) mexicana*. Recently, these reactions have also been used to demonstrate the presence of *Leishmania* species in bats from Chiapas and Tabasco [23] showing a new potential reservoir of these parasites. In our setting, this disease can be diagnosed through: 1) amastigote microscopic identification (in tissues stained with Giemsa) that could lead to a false negative results, as there are multiple cases reported where tissues of patients affected with LCL or DCL did not show detectable levels of parasites [13] and 2) indirect immune fluorescence which can have a limited used to carry out a cutaneous diagnostic, due to its low sensitivity, variable specificity, high antibodies titers in affected and asymptomatic patients or long– term antibodies presence, difficulting diagnosis or disease recurrent periods [16].

Conclusion

Given the above mentioned, and the fact that there are PCR machines virtually everywhere, including research setting such as public health laboratories in Chiapas, routinely use of PCR for the diagnostic of any form of Leishmaniasis, including cutaneous, should be implemented. Correct and fast identification of the parasites may be beneficial for the prognosis of patients infected with Leishmania species and should help to reduce the burden of severe form of *Leishmaniasis* and perhaps mortality. These molecular approaches, and data supporting its use such as those presented here, should be useful for those living in the developing world, including central and south America, where Leishmaniasis is endemic.

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