Research Article

Analysis of Kinetoplast DNA from Mexican Isolates of Leishmania (L.) mexicana

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This study analyzed DNA minicircles of Mexican isolates of L. (Leishmania) mexicana to look for genetic differences between strains isolated from patients with diffuse cutaneous (DCL) and localized (LCL) leishmaniasis. The kDNA was analyzed using polymerase chain reaction (PCR), restriction fragment polymorphism analysis of the PCR products (PCR-RFLP) and the PCR products were sequenced. In the PCR with primers specific for the subgenus Leishmania, the Mexican isolates gave higher amplification products than the other L. mexicana complex strains and with specific primers for the L. mexicana complex they were poorly amplified. In the PCR-RFLP analysis with the Eco RV, Hae III, and Mbo I endonucleases, the Mexican isolates displayed similar restriction patterns, but different from the patterns of the other members of the L. mexicana complex. In the phylogenetic tree constructed, the kDNA sequences of the Mexican clones formed two groups including sequences of LCD or LCL clones, apart from the other L. mexicana complex members. These results suggest that the kDNA minicircles of the Mexican isolates are more polymorphic than the kDNA of other members of the L. mexicana complex and have different recognition sites for the restriction enzymes used in this study.

1. Introduction

Cutaneous leishmaniasis (CL) is the most widespread form of leishmaniasis, causing primary localized skin lesions (LCL) from which parasites can disseminate to the nasopharyngeal mucosa and cause the disfiguring lesions typical of mucocutaneous leishmaniasis (MCL) or disseminated to the entire body as nodular lesions (DCL) [1].

American cutaneous leishmaniasis is characterized by a spectrum of clinical presentations. These include LCL caused by L. (Leishmania) mexicana, DCL caused by L. (Leishmania) amazonensis, L. (Leishmania) venezuelensis, and L. (Leishmania) pifanoi, all belonging to the Leishmania mexicana complex, and MCL caused by members of the Leishmania braziliensis complex [2].

Cutaneous leishmaniasis in Mexico is highly endemic with broad geographical distribution of the different clinical manifestations. In endemic areas, LCL or MCL can be caused by members of both L. mexicana and L. braziliensis complexes [3], making more accurate analysis and identification of the Leishmania strains imperative so that opportune and appropriate treatment can be administered.

Polymerase chain reaction (PCR) approaches have been used in parasite DNA analysis. Several PCR molecular targets have been developed, including minicircle kinetoplast DNA (kDNA) [3], the minixeron (spliced leader RNA) gene [4], and the gp63 PCR-RFLP [5], among others.

Kinetoplast DNA contains approximately 10,000 minicircles per cell that are around 800 base pairs (bp) in size, with a roughly 200 bp conserved region and an approximately 600 bp variable region [6, 7]. The sequence classes of minicircles are organized in a uniform scheme and contain one or several highly conserved regions (CR). There are three highly conserved blocks (CSB) within CRs: CSB1...
2. Materials and Methods

2.1. Leishmania Species and Culture Conditions. This study was reviewed and approved by the Ethics Committee of the Escuela Nacional de Ciencias Biologicas, Instituto Politecnico Nacional, Mexico, in agreement with international ethics guidelines for biomedical research involving human subjects (Ley General de Salud, Mexico).

The study was conducted with seven Mexican isolates of *L. (L.) mexicana* from patients with multiple nonulcerative nodular skin lesions (DCL) or with ulcerative skin lesions (LCL) or the di 2 Interdisciplinary Perspectives on Infectious Diseases ff those in clinical manifestation of the disease are related to any particular minicircles classes.

2.2. DNA Extraction. DNA was prepared, as described elsewhere [3], by centrifuging $10^9$ parasites of an exponential phase of growth culture at 1900 g for 10 min. The pellet was resuspended in 1 mL of NET 100 (100 mM Tris-HCl, pH 8.0; 100 mM EDTA; 100 mM NaCl), 1% SDS, and 4 μL of 10 mg/mL proteinase K (Sigma) and incubated at 37°C overnight, followed by two phenol-chloroform extractions and ethanol precipitation. The DNA extracted was washed with 70% ethanol and dissolved in 100 μL distilled water and kept at -70°C until use.

2.3. Polymerase Chain Reaction. The universal primers AJS1 (GGGGTTTGGTGTAAAAATAG) and DeB8 (CCAGTTTCGCAGCAG) specific for kDNA minicircles of the *Leishmania* subgenus [9], and the M1 (CCAGTTTCGAGCCCCGGAG) and M2 (GGTGTTAAAAAGGGCCCCGAGTGCCT) primers, specific for minicircles of the *L. mexicana* complex [10] were used to amplify kDNA from reference strains and from Mexican isolates. PCR amplifications were done as described elsewhere [9, 10], in a solution of 0.2 mM each of deoxyribonucleotide (Invitrogen Life Technologies, Carlsbad, CA, USA), 50 pmol of each specific primer, 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus), 100 ng of DNA template, 1.5 mM of MgCl$_2$ in a final volume of 100 μL. Samples were denatured at 96°C for 6 min. PCR (35 cycles) consisted of annealing at 60°C for AJS1 and DeB8 primers and 67.5°C for M1 and M2 primers, extension at 72°C for 1 min, and final extension at 72°C for 10 min on a Perkin-Elmer Model 2400 thermal cycler (Perkin Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA). PCR products (10 μL) were fractionated by electrophoresis in 1% agarose or 8% acrylamide gels in TBE (90 mMTris-HCl pH 8.3, 90 mM boric acid, and 2 mM EDTA) stained with ethidium bromide (50 g/mL) and were observed under a transilluminator (SIGMA Chemical Co., St. Louis, MO, USA).

2.4. Restriction Fragment Polymorphism Analysis of kDNA. The kDNA amplicons obtained with the AJS1 and DeB8 primers of the *Leishmania* reference strains and the Mexican isolates were digested with the Bam HI, Eco RV, Hae III, Hind III, Kpn I, Mbo I, Mse I, Msp I, and Xba I endonucleases (Gibco BRL). The restrictions were performed following the supplier’s instructions, briefly: 10 μg of PCR product, 2.5 μL of the correspondent buffer, and 10 U of the endonuclease in a final volume of 25 μL were incubated at 37°C for 2 h; the Taq I was incubated at 67°C. The reactions were analyzed by electrophoresis as mentioned above.

2.5. Sequencing of Minicircles of Mexican Isolates. The kDNA amplicons of the Mexican isolates, MC (from patient with LCL), and GS (from patient with DCL) obtained with the AJS1 and DeB8 primers were gel purified using a QIA quick gel extraction Kit (Qiagen, Germany), ligated into a TA Cloning vector (pCR II Vector) (Invitrogen Life Technologies), and transformed into *Escherichia coli* INV α′. Plasmid DNA was extracted using the Wizard Plus Minipreps DNA Purification System (Promega Corporation, Madison, WI, USA) and sequenced. Nucleotide sequences were determined using a dideoxynucleotide chain termination sequence kit
Table 1: Mexican isolates and reference strains of *Leishmania mexicana* complex used in this study.

(a)

| Reference strains | International code\(^1\) | Source |
|-------------------|---------------------------|--------|
| *L. (L.) mexicana* | MHOM/BZ/62/BEL21          | 1      |
| *L. (L.) mexicana* | MHOM/BZ/62/M379           | 1      |
| *L. (L.) amazonensis* | IFLA/BR/67/PH8    | 1      |
| *L. (L.) amazonensis* | MHOM/BR/73/M2269        | 1      |
| *L. (L.) garnhami* | MHOM/VE/75/PHM76         | 1      |
| *L. (L.) pifanoi*  | MHOM/VE/76/JAP78         | 1      |

\(^1\): The WHO *Leishmania* Reference Collection at the London School of Hygiene and Tropical Medicine, London, UK.

(b)

| Mexican isolates\(^2\) | Origin |
|-------------------------|--------|
| From patients with LCL |
| *L. (L.) mexicana*      | MHOM/MX/88/HRC JS (SOB) | Tabasco |
| *L. (L.) mexicana*      | MHOM/MX/88/HRC MC (MC)  | Tabasco |
| *L. (L.) mexicana*      | MHOM/MX/83/UAYV (YUC)   | Campeche |
| From patients with DCL |
| *L. (L.) mexicana*      | MHOM/MX/84/ISET GS (GS) | Tabasco |
| *L. (L.) mexicana*      | MHOM/MX/85/ISET HF (HF) | Veracruz |
| *L. (L.) Mexicana*      | MHOM/MX/92/INDRE AM (AM)| Veracruz |
| *L. (L.) mexicana*      | MHOM/MX/92/INDRE AG (AG)| Tabasco |

\(^2\): Instituto Nacional de Diagnostico y Referencia Epidemiologicos, Secretaria de Salud, Mexico. Tabasco, Veracruz, and Campeche states are located in the Mexican Southeast.

2.6. Sequence Alignments And Phylogenetic Analysis. The sequences were edited with the DNAMAN, Chromas version 2.0, and Seaview software \[11\]. Multiple sequences were aligned using Clustal-X Ver. 1.83 \[12\]. An unrooted phylogenetic tree was constructed using the neighbor-joining method \[13\] with the Clustal-X program. Evolutionary distances were calculated using Kimura's two-parameter method \[14\], with MEGA (Molecular Evolutionary Genetics Analysis), Version 3.1. \[15\]. A total of 1000 bootstrapping replicates were used for the neighbor-joining analysis to obtain relative support for internal nodes. The kDNA sequence data were compared with the sequences of the other members of the *L. mexicana* complex previously published in GenBank.

3. Results

PCR specific for the *Leishmania* subgenus performed with the AJ51 and DeB8 primers resulted in the amplification of the kDNA of *L. (L.) mexicana* BEL21 and *L. (L.) mexicana* M379, the reference strains, and the Mexican isolates (lanes 6–12) giving 700 to 870 bp amplification bands; *L. (L.) amazonensis* M2269 and PH8 as well as *L. (L.) garnhami* HM76 and JAP78 gave bands less than 700 bp (Figure 2).

PCR specific for the *L. mexicana* complex, with the primers M1 and M2, resulted in the amplification of the *L. (L.) amazonensis* reference strain, giving 700 bp amplification bands; the other references strains gave 800 bp bands. The Mexican isolates were poorly amplified, giving 680 bp bands (data not shown).

Restriction length polymorphism analysis. In endonuclease digestion of kDNA amplicons with the Eco RV endonuclease, the Mexican isolates displayed a six band pattern (lanes 6–12), the reference strain *L. (L.) mexicana* Bel21 showed eight bands (lane 5), the other *Leishmania* strains showed two bands and some were not restricted (Figure 3). With Hae III the Mexican isolates all displayed the same pattern, which was several bands in length, the *L. mexicana* complex reference strains also displayed a restriction pattern of several bands, but a different size (data not shown). With Mbo I, all the Mexican isolates displayed a similar two-band restriction pattern (lanes 6–12) and the *Leishmania* reference strains showed two bands and some were not restricted (Figure 3). With Xba I only, the Mexican isolates were restricted to displaying patterns several bands long (data not shown); the Hind III, MspI, and Spe I endonucleases restricted only certain strains, but not the Mexican isolates (data not shown). The Bam HI and Kpn I endonucleases did not digest any *Leishmania* strains (data not shown).

In the cloning and sequencing of kDNA minicircles of the Mexican isolates, seven minicircle clone sequences were obtained, five from the LCL isolated and two from the DCL (data not shown). All seven clones showed high...
Homology in their conserved part, presenting the highly conserved block CSB3 (GGGGTTGGTGTA) known as the universal minicircle sequence (UMS) (Shlomai, 2004), but after position 19 two groups of minicircle sequences have been found, one 757–759 bp (LCL6, LCL14, and LCL15), which presents the ACTCCTGGATTATT motif, and the 790 to 791 bp group (LCL7, LCL17, LCL5, and LCL4), which presents the TATCCCTCATCCT motif. The comparison of the sequences of these two groups revealed differences in deletions and substitutions of bp along the kDNA minicircle (Figure 5).

Based on sequence alignments of kDNA minicircles of Mexican isolates and the other members of the L. mexicana complex, phylogenetic trees were constructed. The consensus tree showed nine groupings. The first and second groups were formed with LCL and DCL Mexican clones. A further two groups were formed with clones of L. (L.) mexicana from international strains. Another two groups were formed with L. (L.) amazonensis and L. (L.) mexicana clones. Another node was formed with two groups including only L. (L.) amazonensis clones. The last group was formed with clones of L. (L.) mexicana (Figure 6).

4. Discussion

In the amplification of kDNA with the primers specific for the Leishmania subgenus, the Mexican isolates showed PCR products that had a slightly higher molecular weight than the L. (L.) Mexicana M379, L. (L.) mexicana Bel21, L. (L.) amazonensis, and L. (L.) pifanoi reference strain PCR products (Figure 2). On the other hand, with the specific primers for the L. mexicana complex none of the Mexican isolates were properly amplified as the other members of the L. mexicana complex had been (data not shown). These results could be due to the fact that the specific primers for Leishmania subgenus have the UMS sequence as do the Mexican isolates, and the sequences of the M1 and M2 primers have less homology with the Mexican isolates than with the other members of the L. mexicana complex, indicating that the kDNA minicircles of the Mexican isolates are different from the other members of the L. mexicana complex.

Interestingly, the Mexican isolates digested with the Eco RV (Figure 3), Hae III (data not shown), and Mbo I endonucleases (Figure 4) displayed similar restriction patterns between them, totally different from the digestion patterns displayed for the other members of the L. mexicana complex. Furthermore, with the Xba I endonuclease only the Mexican isolates were restricted (data not shown). These results clearly suggest that the kDNA minicircles of the Mexican isolates are more polymorphic than the kDNA of the other members of the L. mexicana complex and have different recognition sites for the several restriction enzymes
Figure 5: Alignment of the kDNA minicircles sequences classes (from 1 to 200 bp). Clones of the Mexican isolates: LCL4, DCL7, LCL17, DCL15, LCL6, and LCL14. Sequences of other members of the *Leishmania mexicana* complex published in the GenBank: *L. (L.) mexicana* Y07807, AF54187, Y145437; *L. (L.) amazonensis* M 94090; *L. (L.) mexicana* Z11551, Z11552, Z11554, Z11556, and Z11549; *L. (L.) amazonensis* M21325, M 21327; *L. (L.) mexicana* Z11553, Z11555.

Figure 6: Phylogenetic tree constructed with sequences of kDNA minicircles. *Leishmania mexicana* complex members published in the GenBank: *L. (L.) mexicana* Y07807, AF54187, Y145437; *L. (L.) amazonensis* M 94090; *L. (L.) mexicana* Z11551, Z11552, Z11554, Z11556, and Z11549; *L. (L.) amazonensis* M21325, M 21327; *L. (L.) mexicana* Z11553, Z11555; clones of *L. (L.) mexicana*. Mexican isolates clones: LCL4, DCL7, LCL17, LCL5, DCL15, LCL6, and LCL14.
used in this study. Furthermore, in studies of PRC-RFLP of kDNA [16] found six basic digestion patterns in Mexican isolates of *L. (L.) mexicana* that were different from the digestion patterns displayed for the other members of the *L. mexicana* complex reference strains.

The Mexican isolate clone sequence classes of minicircles formed two groups including DCL and LCL clones in each group, indicating that a particular minicircle class is not exclusive of one strain of *Leishmania*, furthermore the different clinical manifestation of the disease (LCL or DCL) is not related to any particular minicircle classes (Figure 5). Comparing these sequences with the sequences of other members of the *L. mexicana* complex, it was observed that they did not have extensive sequence homology. These results agree with Barker’s studies [17] showing that the minicircles of representative strains of the main complexes of *Leishmania* do not share extensive sequence homology.

In the phylogenetic tree constructed with these sequences, the other members of the *L. mexicana complex* formed groups with *L. (L.) mexicana* and *L. (L.) amazonensis* clones apart from the groups formed exclusively with the Mexican clones. These may indicate that the Mexican isolates have sequence classes of minicircles that are different from the other members of the *L. mexicana* complex isolated in other countries (Figure 6).

On the other hand, Gutiérrez-Solar et al. [18] found greater heterogeneity between minicircles of geographically separated isolates than minicircles of the same stock. Within human species of *Leishmania*, Rogers and Wirth [19] described highly homogeneous sequences only in minicircles of geographically related isolates.

On the other hand, in studies with *L. (L.) amazonensis* resistant to tunicamycin, Lee et al. [20] found that certain minor minicircle sequence were selected to replicate and to replace the dominant type of minicircles and become predominant.

Although *Leishmania* kDNA is very polymorphic, the PCR of kDNA analysis is a very useful and sensitive enough for detecting *L. donovani* genes in skin biopsy specimens from patients and is recommended as a confirmatory diagnostic tool for PKDL [21], and for characterization of cutaneous isolates of *Leishmania* by isoenzyme typing combined with kDNA restriction analysis [22].

In conclusion it seems that the minicircle classes could change in the kDNA network [8]. We cannot say that one or another minicircle sequence class within the kDNA network is permanent or typical of a virulent or less virulent *Leishmania* strain, and the clinical manifestation of the disease (LCL or DCL) is not related to any particular minicircle classes. Furthermore there is no relation with their geographical distribution.

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