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A drug resistant leprosy case detected by DNA sequence analysis from a relapsed Mexican leprosy patient

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ABSTRACT. A skin biopsy sample was obtained from a relapsed lepromatous leprosy patient **from the central area of Mexico.** Genes associated with resistance to anti-leprosy drugs were analyzed by DNA sequence assay. A single nucleotide substitution was found at codon 53 (ACC→GCC) in the *folP* gene, which is known to confer dapsone resistance. No mutations in the *rpoB* and *gyrA*, which indicate resistance to rifampicin and fluoroquinoles, were detected. This is the first reported case of dapsone resistant leprosy in Mexico in which the cause of the resistance is shown at genomic level. Evaluation of drug resistance by identifying known mutations in these genes by PCR is simple and reliable. Testing for resistance to anti-leprosy drugs should be performed in relapses or intractable cases for a better outcome.

Key words: Leprosy, *Mycobacterium leprae,* drug resistance, genomic mutation, dapsone.

RESUMEN. A partir de una biopsia de paciente diagnosticado con lepra lepromatosa, del centro de México, se analizaron mediante secuenciación de DNA los genes relacionados con resistencia a los fármacos utilizados en su tratamiento. Se detectó la sustitución de un nucleótido en el codón 53 (ACC→GCC) del gen *folP*, lo que confirma la resistencia a dapsona. No se detectaron mutaciones en los genes *rpoB* ni *gyrA* que confieren resistencia a rifampicina y quinolonas. Este es el primer caso de lepra en México con resistencia a dapsona confirmado genómicamente. El examen de susceptibilidad a la terapia anti-lepra, mediante la detección de mutaciones, es un método simple y confiable que debe ser aplicado en casos de recaída o en aquellos casos que no muestren mejoría con el tratamiento convencional.

Palabras clave: Lepra, *Mycobacterium leprae*, farmaco-resistencia, mutación genética, dapsona.

INTRODUCTION

Leprosy is an infectious disease caused by the obligate intracellular pathogen *Mycobacterium leprae (M. leprae)*. The disease has many features in common with neurodegenerative disorders, and lack of treatment can result in severe neurologic impairment, long term disability, and can lead to stigmatization and social isolation (Lockwood 2002). Since the introduction of multi-drug therapy (MDT), which consists of a combination of dapsone, rifampin and ofloxacin as recommended by the World Health Organization (WHO, 1982), leprosy prevalence has diminished dramatically worldwide. During 2004, the number of cases registered in Mexico was 865, with 287 newly diagnosed cases reported, similar to the previous year. Currently the national prevalence and incidence are 0.12 per 10,000 and 0.38 per 100,000 respectively. According to WHO's definition, the national elimination goal was achieved in Mexico in 1994, since leprosy elimination is defined when a prevalence of 1 case per 10,000 is attained (Lopez Roa and Fafutis Morris 2006).

The current strategy for leprosy control depends mainly on early case detection and providing the recommended anti-leprosy chemotherapy drugs, such as dapsone, rifampin, clofazimine and ofloxacin. Understanding the molecular mechanisms of drug resistance to each of these drugs is essential to providing effective treatment and toward preventing the spread of resistant strains. Drug resistance has been reported beginning in 1964 for dapsone (Pettit and Rees 1964), 1976 for rifampicin (Jocobson and Hastings 1976) and in 1996 for ofloxacin (Ji, Perani et al. 1996). The frequency of cases of drug resistance for dapsone increased primarily due to its use in monotherapy , which was eventually discontinued in favor of MDT. As yet, there have not been any confirmed cases of resistance to clofazimine, although the targeted gene has not been identified. In addition, there have been occasional reports of multi-drug resistant cases (Grosset, Guelpa-Lauras et al. 1989; de la Cruz, Cellona et al. 1996; Cambau, Perani et al. 1997; Matsuoka, Kashiwabara et al. 2000; Matsuoka, Kashiwabara et al. 2003). Since *M. leprae* cannot be cultured *in vitro*, drug susceptibility tests for clinical specimens rely on the mouse footpad assay, which is cumbersome, performed only in a few laboratories worldwide, and

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requires at least a year to obtain results. Recent advances in molecular biology to study the correlations between drug resistance and mutations in relevant genes, had enabled the detection of drug resistance by alternative, simple methods, such as PCR DNA sequencing. In this report, we applied in a case of leprosy to examine the presence of any mutation related with drug resistance to *M. leprae*.

MATERIAL AND METHODS

Bacterial material. A slit skin sample was collected from a relapsed patient from Guanajuato state, in the central area of Mexico. This patient was diagnosed with lepromatous leprosy in 1957 and treated by dapsone monotherapy for 5 years. The patient showed a relapse in 1974, and was treated again with dapsone for one year. When the patient again relapsed in 1994, treatment with MDT was prescribed for 2 years. The Bacterial index (BI) was found to be negative following 1995. A slit skin smear sample was collected when the patient attended his periodic follow-up in 2002. Material obtained by this procedure was placed in 70% ethanol and kept at room temperature until DNA extraction.

M. leprae DNA was isolated from the slit skin smear sample by treating the sample with lysis buffer at 60ºC overnight, as described previously (Matsuoka, Zhang et al. 2004). In brief, bacilli were partially purified by centrifugation and resuspended in 50 ul of Tris-HCL with 0.05% Tween 20 and 1 mg/ml proteinase K. The bacillary suspension was treated overnight at 60ºC followed by a freeze and thaw cycle.

PCR amplification and DNA sequencing. The sequences to detect mutations corresponding to drug resistance to dapsone, rifampin and fluoroquinolones were determined by direct sequencing of PCR products. DNA fragments of the *folP, rpoB* and *gyrA* genes containing known mutation sites that confer drug resistance to dapsone, rifampin and ofloxacin were amplified by nested PCR, a G mixture of FailSafe PCR system (EPICENTRE, Madison, Wis USA) was used for amplification. The primers used for the PCR to amplify each gene are as follows. The *folP* gene: first round, FK1 (5' to 3'): CTTGATCCTGACGATGCTGT, FH2 (5' to 3'): GGTGGTCTGTGTAGCAACTG. Second round, FH1 (5' to 3'): ATCCTGACGATGCTGTCCA. FK2 (5' to 3') : TGTAGCAACTGCTAGGCAC; *rpoB* gene: first round, RH1 (5' to 3'): ACGCTGATCAATATCCGTCC, RH2 (5' to 3'): CATAAGCTAGAGCAGCGACT. Second round, RK1 (5' to 3'): CTGATCAATATCCGTCCGGT, RK2 (5' to 3') : GCTGTTACTTGGCTAGTCTG; *gyrA* first round, FN (5' to 3'):CAGGTGACGGTTCTATACAG, RN(5' to 3'): AATGGGCCGCTTGGCTTTAAC. Second round, FN2 (5' to 3'): GATGGTCTAAAACCGGTACATC, RN (5' to 3'): AATGGGCCGCTTGGCTTTAAC.

The DNA fragments amplified by PCR were recovered with a MinElute gel extraction kit (QIAGEN GmbH, Hilden Germany) after confirming the amplification of the PCR product by agarose gel electrophoresis followed by observation under UV illumination. A sequencing reaction was made with a Big Dye terminator cycle sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) and the nucleotide sequence was read by an ABI Prism 310 genetic analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed using DNASIS software (Hitachi Software Engineering, Yokohama Japan).

RESULTS

PCR products of the expected length, (239 bp for the *folP* fragment, 256 bp for the *rpoB* fragment and 227 bp for the *gyrA* fragment), were obtained from the slit skin smear sample (Fig. 1). The sequence analysis of the PCR products revealed a single mutation in the folP gene at codon 53 (ACC→GCC) resulting in the substitution of threonine for alanine (Fig. 2). No mutations in the DNA at previously reported known resistant conferring sites within the *rpoB* gene (at positions 513, 516, 526, 531 and 533) nor in *gyrA* (at positions 89 and 91) were found. Therefore, while the *M. leprae* isolated from this relapse patient is sensitive to rifampin and flouroquinolones, it exhibits the molecular signature of dapsone resistance.

DISCUSSION

Elucidation of bactericidal or bacteriostatic activity for some antileprosy drugs has enabled us to perform susceptibility tests by sequence analysis of genes which code for target proteins for each drug (Williams and Gillis 2004). Using this approach, a high prevalence of drug resistance to anti-leprosy drugs has been revealed for relapsed patients or intractable cases in Asia (Maeda 2001). We decided to examine a sample from a relapsed patient from Mexico to determined if similar drug resistance markers could be identified in this country. Mutations at known mutation sites in the *folP*, *rpoB* and *gyrA* genes were analyzed, since it is known that drug resistance to dapsone, rifampin and quinolones is evoked by well characterized amino acid substitutions in dihydroptorate synthase, the β-subunit of mRNA polymerase and the A subunit of DNA gyrase, respectively

The mutation observed at codon 53 (ACC→GCC) in the *folP* gene of the sample DNA has been shown to be associated with dapsone resistance in a report that compared mutations at known sites in the relevant gene and dapsone resistance by the standard susceptibility test using the mouse footpad method (Williams, Pittman et al. 2001).

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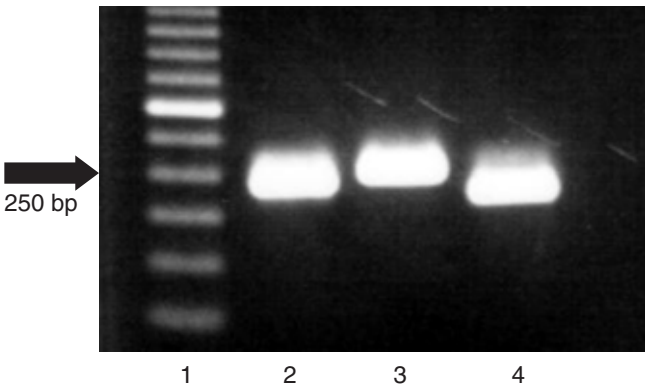

Figure 1. *Electrophoresis of PCR products. Lane 1: 50bp ladder size marker, Lane 2: PCR products of the folP, Lane 3: PCR products of the rpoB, Lane 4: PCR products of the gyrA.*

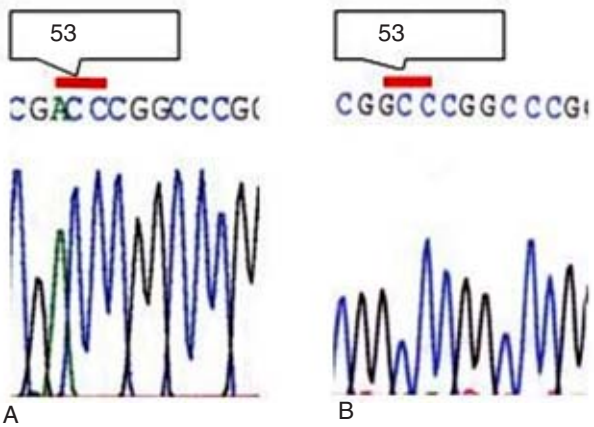

Figure 2. *The mutation at codon 53(ACC→GCC) in the folP gene. A: Wild type* M. leprae. *B:* M. leprae *from the relapsed case.*

To the best of our knowledge, this is the first case of a mutation related with drug resistance to anti-leprosy drug therapy reported in Mexico. However, drug-resistance particularly to dapsone, has been commonly observed in relapsing Asian patients especially in those individuals who have received long-term monotherapy treatment (Matsuoka, Kashiwabara et al. 2000; Maeda, Matsuoka et al. 2001; Matsuoka, Kashiwabara et al. 2003; Ozarmagan, Sutlas et al. 2004). In agreement with this the Mexican patient had received monotherapy for 5 years, followed by an additional year of monotherapy treatment following the first relapsed. During the second relapse, the patient treated with the standard MDT for 2 years, during which time, the BI became negative after one year of treatment. This suggests the effectiveness of MDT, even for patients who develop dapsone resistance.

DNA sequencing analysis is a much simpler method than the mouse footpad assay, and is definitively quicker, two to three days versus 12 months, respectively. Furthermore, material isolated from slit skin smears placed in ethanol are easily handled, transported and can be kept at room temperature until the DNA is extracted. Altogether, these conditions greatly facilitate sampling of clinical materials, especially in remote or rural places, where these patients often reside. The method could be applied for drug resistant testing in patients with relapses, intractable cases as well as in epidemiological studies to assess the prevalence of drug resistance. We would like to suggest this approach to design better therapeutic approaches, as a means to select the drugs with the best potential efficacy, and to improve treatment outcomes for patients with this disease.

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