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Identification of Primary Drug Resistance to Rifampin in *Mycobacterium leprae* Strains from Leprosy Patients in Amazonas State, Brazil

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The aim of this study was to identify polymorphisms in the *folp1*, *gyrA*, and *rpoB* genes in leprosy patients treated in Amazonas State, Brazil. Among 197 slit-skin smear samples from untreated or relapsed patients, we found three cases of primary resistance to rifampin and one confirmed case of multidrug resistance.

Efforts to reduce leprosy led the World Health Organization (WHO) in 1982 to initiate a program introducing multidrug therapy (MDT); a combination of rifampin, clofazimine, and dapsone successfully reduced the number of patients under treatment (1). However, the introduction of MDT did not alter the number of new cases. Yearly, 35,000 leprosy patients have been diagnosed in Brazil in the past decade (2), and drug resistance to MDT components has been observed (3, 4).

Mycobacterium leprae, the causative agent of leprosy, is an obligate intracellular slow-growing bacterium, which has a highly conserved genome (5). Here, we used DNA sequencing in *folp1*, *rpoB* (encoding targets of the first-line drugs dapsone and rifampin, respectively), and *gyrA* (encoding targets of second-line drugs) to assess drug resistance in slit-skin smear samples obtained from leprosy patients treated at the Alfredo da Matta Tropical Dermatology Foundation (FUAM), Manaus, Amazonas, Brazil, from May 2009 to January 2011. The study was approved by the ethics committee of research of this institution (no. 05/2009). Written consent was obtained from each subject before admission in the study.

The samples were collected as done in the routine procedure for the determination of the bacterial index (BI) and transferred into 70% ethanol Eppendorf tubes or FTA elute cards (Whatman, Inc., Florham Park, NJ, USA). The BI of the slit-skin specimens varied between zero (0) and 6+. For DNA extraction, we used either the Qiagen DNeasy blood and tissue kit (Life Technologies, Brazil) for the samples stored in ethanol or the manufacturer's instructions for FTA cards.

Fragments of the three genes were separately amplified using previously described PCR conditions and primers (4). The amplified DNA fragments were confirmed using gel electrophoresis in 2.0% Metaphor agarose (Ludwig Biotec) diluted in Tris-borate-EDTA (TBE) buffer. The PCR products were then purified using the PCR cleanup system kit (Promega). For sequencing, the same primers used to generate the PCR fragment of each gene (4) were used with the ABI BigDye 3.1 Terminator ready reaction kit (Applied Biosystems do Brasil). The target regions of the *folP1* (GenBank accession no. AL583917, gene ML583917), *rpoB* (GenBank accession no. AL583923, gene ML1891), and *gyrA* (GenBank accession no. AL583917, gene ML0006) genes were used as standard wild-type strains, and the reported mutations were labeled in these fragments.

From 197 samples, there were 56 lepromatous leprosy (LL), 54 borderline lepromatous, 27 borderline borderline, 51 borderline tuberculoid, 6 tuberculoid, and 3 indeterminate cases, according to the criteria of Ridley and Jopling (6). All patients were treated according to WHO recommendations for paucibacillary and multibacillary leprosy. Among those, there were new ($n = 126$) and relapse cases ($n = 39$), reentry cases ($n = 3$), and patients with leprosy reactions ($n = 29$). There were 153 males and 44 females. A total of 76, 102, and 79 samples were PCR positive for *rpoB*, *folp1*, and *gyrA*, respectively.

Among the samples screened, we found a total of 4 of 76 PCR-positive (5.2%) specimens carrying previously described *rpoB* polymorphisms. One of the four cases was a multidrug-resistant strain in a relapse case, where polymorphisms at *folp1* and *gyrA* were also detected.

Analyzing the *rpoB* region, three samples showed a GAT→TAT (Asn→Tyr) single nucleotide polymorphism (SNP) at codon 410, which was previously reported in leprosy (7, 8). All of these were new cases clearly demonstrating primary drug resistance to rifampin in Amazonas State. Two of these patients were male and one was female, and all of them had a BI of 4+ with LL as the clinical form. The relapse case showed at codon 425 a TCG→TTC (Ser→Phe) polymorphism in *rpoB*, commonly associated with drug resistance in leprosy (3, 7, 9) and also presented a SNP at codon 91 in the *gyrA* gene altering GCA to GTA (Ala→Val). Another polymorphism in the *folp1* gene was detected at codon 55 (CCC→CGC), changing proline to leucine, which is commonly associated with dapsone resistance (7, 10–13), demonstrating multidrug resistance (MDR) (12). This was a male

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patient who lived in a leprosarium and received dapsone monotherapy treatment for >10 years, later received 24 doses of MDT, and finally received another 24 doses of MDT together with ofloxacin after MDR detection.

We also detected single nucleotide variations in *rpoB*, *folp1*, and *gyrA* in the vicinities of known mutations, but those have never been described with resistance in leprosy. It is likely that these SNPs are not associated with drug resistance, but they might be used for molecular epidemiology and strain identification. In three new cases, we detected polymorphisms in the *folp1* gene at codons 50 (GGC→GGT [Gly]), 44 (GCG→GTC [Ala→Val]), and 64 (GTT→GTC [Val]). Also, a new case showed one polymorphism at codon 89 in the *gyrA* gene of CCG→CTG (Pro→Leu). None of these SNPs are known to be related to drug resistance, neither in *M. leprae* nor *Mycobacterium tuberculosis* complex.

Among the 39 relapse cases, we detected polymorphisms in the *rpoB* gene at codons 437 (GCC→GAC [Ala→Asx]) and 444 (GTG→GCG [Val→Ala]). The second sample showed two different populations, one with the amino acid alanine and the other one with a valine in the *rpoB* gene, suggesting a double infection. This sample was obtained from a potential relapse case, and this SNP might be used to distinguish strains in order to define reinfection. A common SNP that was not associated with resistance was identified in the *gyrA* gene, and it was a synonymous SNP at position 99 (CGC→CGT [Arg]) found in four new cases (5% of the total polymorphisms). Curiously, all of these cases were from multibacillary patients (LL, 3; BL, 1) with high bacillary loads (BI ≥ 3).

The detection of drug-resistant *M. leprae* strains has been reported in Brazil (14–16). Nevertheless, circulating genotypes and resistant strains are not routinely surveyed worldwide (17). Here, we confirm the occurrence of multidrug-resistant and also primary-resistant cases in this geographical area. It is likely that the resistant strains are actively circulating in the north of Brazil, suggesting an urgent need for a drug resistance monitoring policy and a careful posttreatment follow-up of cured patients in order to detect relapse earlier and rapidly identify resistant strains.

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