MAJOR ARTICLE

Molecular Detection of Rifampin and Ofloxacin Resistance for Patients Who Experience Relapse of Multibacillary Leprosy

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Molecular detection of rifampin resistance (*rpoB* analysis) in *Mycobacterium leprae* was determined for 49 patients who experienced relapse of multibacillary leprosy and for 34 untreated patients. Molecular detection of ofloxacin resistance (*gyrA* analysis) was determined for the 12 patients who experienced relapse and who had received ofloxacin. Results of molecular tests were compared with the reference susceptibility test in the mouse footpad. Overall, the efficiency of molecular detection—that is, positive DNA amplification—was 95%, whereas that of the in vivo test was 55% ($P < .001$). Results of molecular detection and in vivo test were fully concordant when both were available—that is, for 35 rifampin-sensitive cases of leprosy (no *rpoB* mutation), 4 ofloxacin-sensitive cases (no *gyrA* mutation), 11 rifampin-resistant cases (*rpoB* missense mutations), and 1 ofloxacin-resistant case (*gyrA* mutation). *rpoB* and *gyrA* analysis appears to be an effective method for detection of rifampin and ofloxacin resistance in patients with leprosy.

Until 1982, the standard treatment for multibacillary leprosy was lifelong monotherapy with dapsone, which led to the selection of dapsone-resistant *Mycobacterium leprae* and subsequent therapeutic failure [1, 2]. Multidrug therapy was recommended by the World Health Organization (WHO) in 1982 [3] and includes rifampin, which is more bactericidal against *M. leprae* than the other antileprosy drugs [4, 5]. Rifampin-resistant leprosy has been documented in patients treated by rifampin monotherapy [6, 7]. Ofloxacin and some newer fluoroquinolones also displayed bactericidal effect against *M. leprae* and became important components of regimens to treat rifampin-resistant leprosy [3, 8, 9]. As in tuberculosis, the emergence of multidrug-resistant *M. leprae* strains (dapsone, rifampin, and ofloxacin-resistant), which has been described elsewhere [10], represents a severe threat for control of leprosy.

M. leprae is a slow-growing mycobacterium (division time of 11–14 days) that has not yet been cultured in vitro. Forty years ago, Shepard standardized an in vivo method for growing *M. leprae* in the mouse footpad [11] and drug susceptibility testing [12]. For the testing, mice inoculated with *M. leprae* recovered from patients are treated with antibiotics to determine whether the drug prevents bacterial multiplication [12]. This method is time-consuming; 12 months is required to get results, it requires expensive facilities and expertise, and its success is largely dependent on the biopsy containment and the elapsed time until mouse inoculation. For these reasons, few laboratories perform drug susceptibility testing for *M. leprae.*

The progress of molecular biology offers an unprecedented opportunity for diagnosing drug resistance in

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For the antibiotic susceptibility test in the mouse footpad, animal experimentation guidelines were followed.

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M. leprae by in vitro methods. The molecular mechanism of rifampin resistance was first described in *Escherichia coli* [13] and was elucidated in 1993 for *M. leprae* [14] and *Mycobacterium tuberculosis* [15]. Rifampin resistance is associated with mutations in the *rpoB* gene that encodes the β subunit of RNA polymerase. All the mutations associated with resistance in mycobacteria are localized in the 500–540 domain (numbering system used for *E. coli* RpoB) [16]. Resistance to ofloxacin is known to be associated with mutation in *gyrA,* encoding the A subunit of DNA gyrase, of various mycobacteria [17, 18] including *M. leprae* [10]. Nevertheless, until now, the number of *M. leprae* isolates investigated for rifampin and fluoroquinolone susceptibility by both genetic analysis and standard mouse footpad method is rather small [10, 19, 20].

To establish the ability of *rpoB* gene analysis to detect rifampin resistance and of *gyrA* gene analysis to detect fluoroquinolone resistance, we systematically compared the results of genetic analysis to those of the mouse test for the patients with leprosy for whom we received a biopsy during the past decade. Complete concordance between in vivo and genetic tests was observed for both drugs. The results of this study showed that *rpoB* and *gyrA* analyses of *M. leprae* contained in skin biopsies can be used as a rapid and convenient method for drug susceptibility testing.

PATIENTS AND METHODS

Patients. Patients included in the study had multibacillary leprosy, either as a relapse or a new case. "Relapse" was defined as active leprosy lesions in a previously treated patient and a new case as active leprosy in an untreated patient [21]. Skin biopsies were sampled in the country of origin and sent for drug susceptibility testing to the laboratory of the National Reference Center for surveillance of mycobacterial diseases and drug resistance in Paris. The study included the cases of leprosy with a positive skin biopsy (see definition below) and a volume of material sufficient to perform both genetic and mouse testing. Relapsed patients and patients newly diagnosed with leprosy as a control group were included in the study on rifampin (*rpoB* analysis and rifampin-susceptibility in the mouse test). Rifampin-resistant *M. leprae* isolates, previously studied in our laboratory and by Honoré et al. [14, 19] for *rpoB* sequencing, were tested as internal controls for *rpoB* analysis but were not included in the study. Those of patients who experienced relapse and who had received a treatment containing ofloxacin were included in the study on ofloxacin (*gyrA* analysis and ofloxacin susceptibility in the mouse test).

Skin biopsies were minced and ground with glass beads to obtain suspensions in Hanks solution (Sanofi Diagnostics Pasteur, Marne la Coquette, France) according to Shepard's method [11]. After Ziehl-Neelsen staining of 2 smears (10 μL each), acid-fast bacilli (AFB) were counted by light microscopy ($\times$1000 magnification) [22]. Biopsies were considered to be positive when the skin biopsy suspension contained at least 10^4 AFB/mL—that is, 1 bacillus per 40 fields. *M. leprae,* C6 isolated previously [8], served as the wild-type and sensitive control isolate.

Drug susceptibility testing in the mouse footpad. A suspension containing $\sim 5 \times 10^3$ bacilli per 0.03 mL was prepared by appropriate dilution of the initial suspension and inoculated into the left hind footpads of Swiss mice [11, 12]. Inoculated mice were assigned to an untreated control group (10 mice) or a treated group (8 mice) receiving 10 mg/kg rifampin once weekly by gavage [7]. For the biopsies from patients who had received ofloxacin, an additional treated group received 150 mg/kg of ofloxacin 5 times a week by gavage [8]. Treatment started 1 week after inoculation, and 7, 9, and 12 months later, mice were killed and the soft tissue under the skin of the footpad was prepared for AFB enumeration [7, 11, 12]. *M. leprae* bacilli were considered to have multiplied when $\geqslant 10^5$ AFB were observed per footpad in control mice. When no multiplication was observed after 12 months, the drug susceptibility test was unsuccessful [12]. Isolates were defined as sensitive when they multiplied in untreated mice but not in any treated mouse and as resistant when they multiplied in untreated mice and in at least one treated mouse [7, 12].

Genetic tests for the detection of rifampin and ofloxacin resistance. Genomic DNA was extracted from 100-μL samples of the initial biopsy suspension by use of the freeze-boiling technique [23] modified as follows: 5 series of heat-cold shocks, alternately boiling (1 min at 100°C) and freezing (1 min at −196°C in liquid nitrogen) were followed by 2 min of sonication (sonicator 1200, Branson Ultrasonic).

Primers E12 5′-GATCAATATCCGTCCGGTG-3′ and E8 5′-GTGCACGTCACGGACCTC-3′ were used to amplify a 178-bp fragment corresponding to the region of the *rpoB* gene involved in rifampin resistance [15]. Primers LEP1 5′-CCGTAGCCACGCTAAGTCA-3′ and LEP2 5′-CTGGCAACCGAAGTTGCC-3′ were used to amplify a 158-bp fragment corresponding to the region of the *gyrA* gene involved in quinolone resistance, as described elsewhere [10, 24]. DNA extract (10 μL) was added to a 90-μL of PCR mixture containing 5 μL of Taq polymerase 10$\times$ buffer (Eurogentec), 5 μL of 25 m*M* $MgCl_2$, 10 μL of a 2.5 m*M* mix of dNTPs (Eurogentec), 10 μL of each 4 μM primer, and 1 unit of Taq polymerase (Eurogentec). After denaturation for 7 min at 94°C, *rpoB* and *gyrA* PCR amplification were performed as follows: 40 cycles consisting of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C, followed by a final extension step for 10 min at 72°C. Amplified DNA fragments were purified with the Prep-A-Gene Kit (Bio-Rad).

From 1992 through 1997, purified PCR *rpoB* fragments were subjected to nonradioactive single-strand conformation poly-

morphism (SSCP) analysis [25]. For the biopsies tested in 1998 and 1999, the purified PCR *rpoB* fragments were directly sequenced. All *gyrA* fragments were subjected to direct PCR sequencing.

Nucleotide sequences were determined according to the Sanger's method with radioactive dCTP and manual electrophoresis, as described elsewhere [24], or with fluorescein-labeled ddNTPs and 25 ng of DNA subjected to the dRhodamine terminator cycle sequencing reaction kit (ABI Prism 377, Perkin-Elmer). Sequences were analyzed with the Sequencing Analysis software (ABI Prism, Perkin-Elmer).

RESULTS

Forty-nine positive skin biopsies from patients who experienced relapse of multibacillary leprosy, including the 3 rifampin-resistant controls, and 34 positive biopsies from new cases of leprosy were screened for rifampin resistance by susceptibility testing in the mouse footpad and genetic *rpoB* analysis. Seventy-one of the 83 biopsies have been taken from distinct patients and the other 12 from 6 patients, before treatment and after relapse. Patients were from France (immigrants from Cameroon, Portugal, Sri Lanka) or French territories (West Indies, Guyana, New Caledonia) (27 relapsed cases and 34 new cases of leprosy), Mali (14 relapsed cases), China (7 relapsed cases), and the Philippines (1 relapsed case). Forty-one of the 49 cases of relapse (hereafter referred to as "relapses") occurred in patients who received rifampin as monotherapy or an unsupervised combination. For the 8 other relapses, there was no indication that the patient had received rifampin.

Drug susceptibility testing in the mouse. The mouse test was positive (multiplication of *M. leprae* bacilli) for 46 (55%) of 83 biopsies, equally distributed among relapsed (30 [61%] of 49) and new cases of leprosy (16 [47%] of 34) ($P = .2$). For the 37 other biopsies (sampled from 19 relapsed cases and 18 new cases of leprosy), no multiplication was observed in the untreated mice 12 months after inoculation, indicating that the biopsies did not contain enough viable *M. leprae*. Multiplication in the mice correlated with the number of AFB per milliliter in the biopsy suspension (table 1; $P < .02$). The mean time between the biopsy and its reception in the laboratory was 3.1 days for the biopsies yielding multiplication and 4.9 days for the negative biopsies ($P < .03$).

Rifampin in vivo susceptibility tests demonstrated that 35 isolates were sensitive and 11 were resistant (table 2). Ofloxacin susceptibility tests performed for 12 patients with relapse after ofloxacin treatment were successful for only 5 (42%) of them: 4 isolates were sensitive and 1 was resistant.

Genetic tests. *rpoB* gene amplification was successful for 79 (95%) of 83 biopsies. For the 4 PCR-negative biopsies, no multiplication in the mouse was observed. Amplification efficiency correlated with the number of AFB per milliliter in the biopsy suspension (table 1) and was 100% for biopsies containing $\geq 10^6$ AFB/mL.

Table 1. Efficiencies of in vivo susceptibility testing in the mouse footpad (mouse test) and PCR *rpoB* amplification, according to the number of acid-fast bacilli (AFB) per milliliter in the skin biopsy suspension.

AFB/mL	No. of biopsies tested	Mouse test		*rpoB* amplification	
		Positive	Efficiency, %[a]	Positive	Efficiency, %[a]
10^4 to $<10^5$	3	0	0	1	30
10^5 to $<10^6$	19	8	42	17	89
10^6 to $<10^7$	38	20	53	38	100
$>10^7$	23	18	78	23	100
Total	83	46	55	79	95

[a] Efficiency was defined as [(no. of valid tests/no. of tests run) × 100].

Integrating the SSCP and direct sequencing results, *rpoB* mutations were detected in 12 isolates, and no *rpoB* mutation was found in 67 isolates (figure 1). The *rpoB* mutations and consequent substitutions in the β subunit of RNA polymerase observed in these 12 isolates are detailed in table 2 and figure 2. Mutations affecting the codon at position 531 (numbering system used for *E. coli*) were the most frequent (10 of 12 isolates), 9 of them being TCG → TTG, resulting in Ser531Leu substitution. Other codons, at positions 507, 513, 526, or 533, were substituted either alone (Gln513Val) or in combination (Ser531Met + Leu533Val, Gly507Ser + His526Asp). The wild-type sequence of the *rpoB* gene from 66 *M. leprae* isolates was identical to that registered with GenBank (accession number MLB17906) [26]. For 1 isolate, the *rpoB* sequence showed a silent mutation (no change of the amino acid sequence) at codon 522 (TCG → TCT) and was considered to be wild-type.

gyrA genetic testing was successful for 11 (92%) of 12 biopsies. PCR was negative for 1 case of leprosy for which the biopsy suspension contained $<10^5$ AFB/mL and the mouse test was unsuccessful. A missense mutation in *gyrA* leading to the substitution of an alanine at position 83 (numbering system used for *E. coli*) for a valine was observed in one isolate reported elsewhere [11], whereas the other 10 isolates had the same sequence as the wild-type *gyrA* allele of *M. leprae* C6 [24].

Agreement between in vivo susceptibility and genetic tests. Results of *rpoB* genetic testing fully agreed with those of rifampin in vivo susceptibility tests for all the 46 cases of leprosy (30 from relapses and 16 from new cases, 35 sensitive and 11 resistant) for which both tests were successful (table 2). For 4 cases of leprosy, there was neither *rpoB* amplification nor multiplication in the mouse.

For the 33 remaining cases of leprosy (16 relapses and 17 new cases), there was *rpoB* amplification but no multiplication in the mouse, impeding comparison between both tests. Wild-

type *rpoB* sequence was found for 32 patients, and a Ser531Leu *rpoB* mutation was detected for 1 patient. For the latter patient (described in "rifampin- or ofloxacin-resistant leprosy cases" below), a biopsy taken 3 years before had been shown to contain rifampin-resistant bacilli.

Results of *gyrA* analysis agreed with those of in vivo ofloxacin susceptibility testing for the 5 patients who experienced relapse for which both tests were successful: 4 sensitive and 1 resistant. Genetic analysis showed no *gyrA* mutation for 6 cases of leprosy for which the mouse test was unsuccessful.

Rifampin- or ofloxacin-resistant leprosy cases. Features concerning the 11 patients with multibacillary leprosy caused by rifampin-resistant isolates of *M. leprae* are presented in table 3. All were relapses. All but 1 reported having received a rifampin-containing regimen, but none received the multidrug therapy recommended by WHO. Only 1 biopsy was included and tested for each of those patients, except for patient 9, for whom 2 biopsies were included. The first biopsy, taken in 1995 after treatment by rifampin, dapsone, and prothionamide for 30 months, demonstrated rifampin resistance in the mouse footpad and a Ser531Leu mutation in *rpoB*. The second biopsy, taken in 1998 after treatment with ofloxacin and clofazimine, did not multiply in the mouse, but Ser531Leu *rpoB* mutation and a wild-type *gyrA* were detected.

Patient 5 has been described elsewhere [8] as the first documented case of ofloxacin- and multidrug-resistant (dapsone, rifampin, and ofloxacin) leprosy.

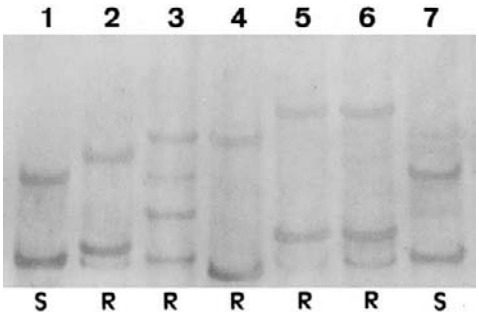

Figure 1. Single-strand conformation polymorphism (SSCP) analysis of PCR-amplified *rpoB* fragments from rifampin-sensitive (S) and -resistant (R) isolates of *Mycobacterium leprae* contained in skin biopsies from patients with multibacillary leprosy. *Lane 1,* wild-type sequence; *lane 2,* Ser531Met + Leu533Val mutations; *lane 3,* Gly507Ser + His526Asp mutations; *lane 4,* Ser531Phe mutation; *lane 5,* Ser531Leu mutation; *lane 6,* Ser531Leu mutation; *lane 7,* wild-type sequence.

DISCUSSION

In leprosy, drug susceptibility testing is indicated for patients with relapse or whose illness fails to respond to treatment to choose the most appropriate antibiotic regimen. In addition, it is the only way to measure the epidemiological trend of primary or secondary resistance to antileprosy drugs. Because drug susceptibility testing by the mouse footpad method is time-consuming, expensive, and technically demanding, leprosy control programs can benefit from a rapid, genetic-based method.

Table 2. Results of the in vivo rifampin susceptibility test in the mouse footpad and *rpoB* analysis for relapses and new cases of multibacillary leprosy.

Cases of leprosy, *rpoB* sequence[a]	No. of patients	In vivo rifampin susceptibility test[b]		
		Sensitive	Resistant	Unsuccessful
Relapsed (n = 49)				
Wild-type	34	19	0	15
Ser531Leu (tcg → ttg)	9	0	8	1[c]
Gln513Val (cag → gtg)	1	0	1	0
Ser531Met + Leu533Val (tcg → atg) + ctg → gtg)	1	0	1	0
Gly507Ser + His526Asp (ggc → agc + cac → gac)	1	0	1	0
No PCR amplification	3	0	0	3
New cases (n = 34)				
Wild-type	33	16	0	17
No PCR amplification	1	0	0	1
Total	83	35	11	37

[a] Sequence of *rpoB* from codon 499 to codon 545 (numbering system used for *Escherichia coli*).
[b] Mice received 10 mg/kg rifampin by gavage once a week.
[c] The same strain was previously associated with resistance as assessed by the mouse footpad testing.

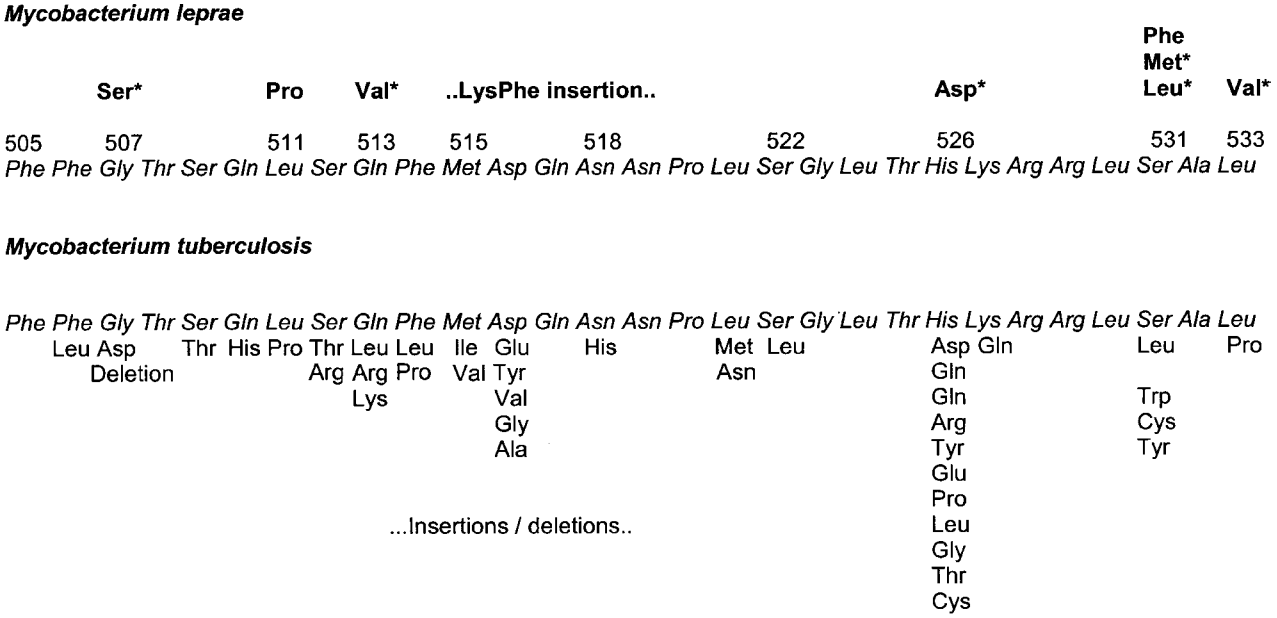

Figure 2. Substitutions in the β subunit of the RNA polymerase associated with rifampin resistance in *Mycobacterium leprae* and comparison with those described elsewhere [16] in *Mycobacterium tuberculosis.* Mutations are in bold type, and an asterisk indicates the mutations identified in this study. Wild-type sequences are in italics, and the numbering system is that used for *Escherichia coli.*

In this study, rifampin and ofloxacin susceptibility of *M. leprae* was determined by both the reference mouse footpad method and sequence analysis of the *rpoB* and *gyrA* genes. Molecular detection of *M. leprae* resistance was fully concordant with the drug susceptibility test in the mouse footpad on the basis of 46 isolates for rifampin and 5 isolates for ofloxacin. All resistant isolates (11 rifampin and 1 ofloxacin resistant) harbored mutations leading to substitutions in the drug target. Conversely, all sensitive isolates harbored the wild-type sequence or silent mutation. Full concordance has been also observed previously for 15 other isolates (10 resistant and 5 sensitive) tested in our laboratory for rifampin susceptibility and analyzed for *rpoB* by Honoré et al. [19].

The significant proportion of cases of leprosy (nearly half in this study) for which the mouse test was unsuccessful is a serious drawback of this test. Because in our study success was equally distributed among untreated patients and patients who experienced relapse, it is unlikely that failure of in vivo multiplication was caused by a previous treatment. Instead, the main causes of multiplication in the mouse appeared to be a low bacterial load in the biopsy and a long interval between collection and inoculation. In contrast, genetic analysis was successfully conducted in >95% of the cases of leprosy and in >85% of the cases of leprosy for which the mouse test was unsuccessful. This indicates that genetic analysis is less dependent on the sample conservation, which is another advantage of the molecular method. The efficiency of the genetic method was 100% when AFB $\geqslant 10^6$/mL, as described for other amplification methods on *M. leprae* [27].

Molecular detection of drug resistance in mycobacteria has been based on the observation of mutations in the genes encoding the target of the drugs or involved in the activation of the drug [16, 28]. Different methods, based on PCR amplification, have been used to detect these mutations, such as SSCP [15, 19], heteroduplexes [20], and automated sequencing. The time required by these methods is similar, ranging 2–5 days under routine conditions. The substitutions observed in rifampin-resistant isolates of *M. leprae* were all located in the 500–540 domain described as the site for rifampin resistance mutations in mycobacteria [16]. Although crystallography analysis of the β subunit has not yet been performed, His526 and Ser531 seem to be key residues for rifampin binding to the β subunit of RNA polymerase [29, 30]. These 2 positions were indeed implicated in 23 (92%) of 25 rifampin-resistant *M. leprae* isolates described so far [19, 20, this study], a proportion similar to that observed in rifampin-resistant *M. tuberculosis* [16]. Therefore, the observation of a 526 or a 531 substitution in *M. leprae* RpoB is most likely predictive of rifampin resistance [30]. More information is needed on substitutions at other positions and on the combination of substitutions, such as the ones we observed in 2 isolates.

The *gyrA* mutation, observed in 1 isolate of *M. leprae* and associated with ofloxacin resistance, is the same as that was observed in in vitro mutants of *M. smegmatis* obtained in 1-

Table 3. Characteristics of the 11 patients with rifampin-resistant (assessed by in vivo test in the mouse) leprosy.

Patient no.	Year of biopsy	Age in years	Country	Case	Year of first diagnosis	Previous treatment and duration	Susceptibility to other drugs
1	1989	41	FWI	Relapse	1956	Dds 1956–1960; Slf + Rif 1980–1982	Dds^R, Pth^S
2	1990	58	FWI	Relapse	1959	Dds 1959–1968; Slf 1968–1980; Slf + Dds + Rif + Clf 1980–1987	Dds^R
3	1991	45	FWI	Relapse	1961	Dds 1961–1974; Dds + Rif 1974–1975	Dds^R, Pth^S
4	1992	55	FWI	Relapse	1956	Dds 1956–1973; Rif 1973–1974; Dds + Slf 1975–1991	Dds^R, Pth^S
5	1992	35	Mali	Relapse	1979	Dds 1979–1991; Rif + Ofx 1 month 1992	Dds^R, Pth^R, Ofx^R
6	1992	50	FWI	Relapse	1958	Dds 1958–1977; Rif 1977–1979; Rif + Pth	Dds^R, Pth^R
7	1993	57	Cameroon	Relapse	1960	Dds 1961–1975; Pth + Rif 1975–1992	Dds^S, Pth^S
8	1995	64	FWI	Relapse	1946	Dds 1946–1978; Slf 1979–1982; Rif 1983–1995	Dds^S, Pth^S
9	1995	30	FWI	Relapse	1980	Dds + Rif + Pth 30 months	Dds^S, Pth^S
10	1997	45	FWI	Relapse	1950	Dds for 11 years	Dds^S, Pth^S
11	1999	69	FWI	Relapse	1947	Dds 1947–1972; Dds + Rif 1973–1976	Dds^R

NOTE. Clf, clofazimine; Dds, dapsone; FWI, French West Indies; Ofx, ofloxacin; Pth, prothionamide; R, resistant; Rif, rifampin; S, sensitive; Slf, sulfonamides.

step selection by ofloxacin [18]. Substitutions at the position 83 in the A subunit of DNA gyrase are commonly associated with quinolone resistance through a decrease in DNA gyrase affinity for quinolones [31].

From the studies on rifampin-resistant cases of leprosy reported by Grosset et al. [7] and in the present study, secondary rifampin resistance rates in leprosy were 56% (22 of 39 patients) in Grosset et al. [7] and 40% (12 of 30) in this study, which were higher than that found (20%) for tuberculosis, but the total number of leprosy cases studied is low still [32]. No secondary resistance to rifampin after multidrug therapy has been reported to date [33]. Primary resistance (rifampin resistance in previously untreated patients with leprosy) has not been described either.

The moderate efficiency (46 [55%] of 83) of the in vivo reference method for susceptibility testing compared with its cost and the organization needed to maintain the mouse-footpad test facilities puts in doubt the future of mouse testing. The genetic approach is much easier to implement. Nevertheless, it is premature, because of the relatively small number of cases of leprosy analyzed worldwide [19, 20, this study], to state, first, that all rifampin-resistant isolates of *M. leprae* will harbor *rpoB* mutations, particularly considering the results obtained on rifampin-resistant *M. tuberculosis* [16]; and second, that any mutation in the 500–540 domain leads to rifampin resistance. As soon as high sensitivity (no false sensitivity) and high specificity (no false resistance) will be established for genetic tests, it will be tempting to replace the mouse footpad testing with them. The few laboratories with expertise in in vivo drug susceptibility testing will serve as reference for external quality control of genetic results, and for the investigation of the susceptibility of isolates harboring undescribed mutations and of isolates with no mutation from patients not cured by rifampin. The mouse footpad will also remain a way to grow *M. leprae* and thus to test the activity of new drugs for the improvement of leprosy therapy [8, 12].

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