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Drug and Multidrug Resistance among *Mycobacterium leprae* Isolates from Brazilian Relapsed Leprosy Patients

Adalgiza da Silva Rocha,[a] Maria das Graças Cunha,[b] Lucia Martins Diniz,[c] Claudio Salgado,[d] Maria Araci P. Aires,[e] José Augusto Nery,[f] Eugênia Novisck Gallo,[f] Alice Miranda,[f] Monica M. F. Magnanini,[g] Masanori Matsuoka,[h] Euzenir Nunes Sarno,[f] Philip Noel Suffys,[a] and Maria Leide W. de Oliveira[g]

Laboratory of Molecular Biology Applied to Mycobacteria, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, RJ, Brazil[a]; Tropical Dermatology Foundation Alfredo da Matta (FUAM), Manaus, Amazonas, Brazil[b]; Dermatology Service, Santa Casa de Misericordia (EMESCAM), Vitória, Espirito Santo, Brazil[c]; Dermato-Immunology Laboratory Universidade Federal do Pará and Dr. Marcello Candia Reference Unit, Marituba, Pará, Brazil[d]; Reference Center in Sanitary Dermatology D. Lebanon (CDERM), Fortaleza, SES, Ceara, Brazil[e]; Leprosy Laboratory, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, RJ, Brazil[f]; Training Center on Dermatology, University Hospital HUCFF and Public Health Institute, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil[g]; and Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan[h]

Skin biopsy samples from 145 relapse leprosy cases and from five different regions in Brazil were submitted for sequence analysis of part of the genes associated with *Mycobacterium leprae* drug resistance. Single nucleotide polymorphisms (SNPs) in these genes were observed in *M. leprae* from 4 out of 92 cases with positive amplification (4.3%) and included a case with a mutation in *rpoB* only, another sample with SNPs in both *folP1* and *rpoB*, and two cases showing mutations in *folP1*, *rpoB*, and *gyrA*, suggesting the existence of multidrug resistance (MDR). The nature of the mutations was as reported in earlier studies, being CCC to CGC in codon 55 in *folP* (Pro to Arg), while in the case of *rpoB*, all mutations occurred at codon 531, with two being a transition of TCG to ATG (Ser to Met), one TCG to TTC (Ser to Phe), and one TCG to TTG (Ser to Leu). The two cases with mutations in *gyrA* changed from GCA to GTA (Ala to Val) in codon 91. The median time from cure to relapse diagnosis was 9.45 years but was significantly shorter in patients with mutations (3.26 years; $P = 0.0038$). More than 70% of the relapses were multibacillary, including three of the mutation-carrying cases; one MDR relapse patient was paucibacillary.

There is no doubt about the efficiency of the currently used multidrug therapy (MDT) scheme for treatment of leprosy, as demonstrated by the strong decrease in disease prevalence since its implementation and the low number of reported relapse cases (18). However, there has been a scarcity of in-depth studies of relapse occurrences in recent decades (27). As is known, differentiating diagnosis of relapse and reactional states poses some difficulties in the field, being responsible for under- or overdiagnosis of both disease stages. This is important because undiagnosed relapse cases could contribute to continuing disease transmission. In addition, hardly any data on the contribution of emergence of drug-resistant strains of *Mycobacterium leprae* to leprosy relapses exist.

Diaminodiphenylsulfone (DDS), also called dapsone, was the first drug to be effective against leprosy worldwide, and the first cases of resistance to dapsone were detected in 1964 and involved two single nucleotide polymorphisms (SNPs) in the gene *folP1*, located in codons 53 and 55 (8, 9, 14, 29). Rifampin is the key component of the standard multidrug regimen used for treatment of leprosy, and it has been shown that PCR-based DNA sequence analysis of the *rpoB* gene of *M. leprae* was in full concordance with rifampin susceptibility testing in the mouse footpad system (17, 30). In addition to dapsone and rifampin, ofloxacin is also used for leprosy treatment and is a quinolone with an action mechanism based on interaction with DNA gyrase (2); SNPs in *gyrA* and *gyrB* confer resistance or hypersensitivity to quinolones (15). Although there is not yet an official definition of multidrug resistance (MDR) in leprosy, in parallel with tuberculosis, we adopt this terminology when we encounter resistance to rifampin and one other drug of the standard MDT regimen.

Emerging drug resistance has been observed among many diseases caused by bacteria, and this could pose a challenge for the treatment of leprosy, a neglected disease with a minimal therapeutic arsenal (22). Brazilian studies show relapse rates below 1% (12, 26), and drug resistance does not seem to be an important problem in the country (10, 21). Nonetheless, a pilot project for optimal detection of relapse and the contribution of drug resistance among leprosy patients of five states in Brazil was started in 2006 (26), in parallel with the initiative of the World Health Organization (WHO) to perform global surveillance of drug resistance in leprosy in 2008 (36).

MATERIALS AND METHODS

Study design and patients. A prospective study for detection of relapse in leprosy patients was designed for more accurate determination of the frequency of relapse by drug resistance among Brazilian leprosy patients, based on evaluation of DNA sequencing in samples from 145 leprosy patients, collected during 2006 to 2008, in five states to which leprosy is highly endemic, including Rio de Janeiro, Espírito Santo, Amazonas, Pará, and Ceará (26). All patients were examined by experienced dermatologists in six state reference units in order to guarantee the quality and uniformity of these procedures. Leprosy relapse detection was based on standardized and optimized diagnostic procedures and criteria for definition of relapse (4) and with inclusion criteria being suffering from active clinical lesions of leprosy, as confirmed by smears and histopathological exams, being considered cured from the first disease course after having undergone the

Received 30 November 2011 Returned for modification 29 December 2011
Accepted 29 March 2012
Published ahead of print 11 April 2012
Address correspondence to Maria Leide W. de Oliveira, mleide@hucff.ufrj.br.

doi:10.1128/JCM.06561-11

Brazilian Leprosy Program treatment regimens. Regarding the official treatment regimens from the National Leprosy Program, it is necessary to clarify that Brazil, before adopting the WHO MDT treatment schemes in 1986 (24 doses), used a scheme called DNDS that consisted of 90 daily doses of 600 mg of rifampin, followed by daily doses of 100-mg dapsone monotherapy, up to 5 years and until slit skin smears became acid-fast bacillus (AFB) negative. For each relapse case, a control case, being a new leprosy case of the same sex, clinical form, and municipality of residence and belonging to the same treatment cohort, was selected from the National Information System for Notification of Diseases (SINAN) and enrolled for clinical and laboratory examinations.

This study was approved by the Ethical Committee of Research of the Federal University of Rio de Janeiro (HUCFF/UFRJ) (no. 019/06). Written consent was obtained from individual subjects by having them sign a standard Brazilian form before being admitted in the study. The epidemiologic, clinical, and demographic data collected from each participant center were stored and analyzed at the UFRJ, using the software program Strata 9.0.

Slit skin smear and histopathology of skin biopsy specimens. As part of the diagnostic procedure, slit skin smear samples were collected from four different body sites at the time of diagnosis of disease relapse, and a skin biopsy was done according to standard recommendations (4). After being cut in half, one part of the skin biopsy specimen was prepared for histopathology exam, and the other half was immersed in 70% ethanol for genetic analysis. In order to standardize the histopathology procedure and reporting of results, a consensus meeting was held with the histopathologists from the participating reference centers and a standard protocol was elaborated.

Extraction of nucleic acids. For extraction of nucleic acids, the ethanol was removed from the biopsy specimen, and the latter was rehydrated, cut into small pieces, and subjected to DNA extraction and purification using the Qiagen DNeasy Blood & Tissue kit (Invitrogen do Brasil). In brief, 180 μl of ATL buffer and 20 μl of proteinase K from the kit were added to the biopsy specimen and subjected to vortex mixing, and after overnight incubation at 56°C, DNA was purified using a spin column from the kit as described by the manufacturer.

Amplification and sequencing analysis of part of *rpoB*, *folP1*, *gyrB*, and *gyrA*. Part of the genes *rpoB*, *folP1*, and *gyrA* was analyzed by direct sequencing of PCR products generated using conditions described previously, using the amplification primers M*rpoB*F and M*rpoB*R (31), *folP*1F and *folP*1R (38), and *gyrA*NF and *gyrA*NR (5, 11, 23) and using touchdown amplification conditions described previously (11). Each PCR mixture contained at least one negative control, and after verification of PCR product quantity and quality on a 3% agarose gel, amplicons were purified using the ChargeSwitch PCR clean-up kit (Invitrogen do Brasil) and sequenced using the same primers as those for generating the PCR fragment of each gene, using the ABI BigDye 3.1 Terminator ready reaction kit (Applied Biosystems do Brasil). For characterization of the *gyrA* SNP at position 297, we followed the approaches described previously (11). Sequences were generated on an ABI 3730 genetic analyzer (Applied Biosystems) and compared with the *M. leprae* sequences NC002677 and z14314 (*rpoB*), AL023093 (*folP1*), and NC002677 (*gyrA*), available at GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez/), and for SNP analysis, sequences were introduced into SeqScape. Control DNAs were purified from *M. leprae* NHDP-63 (kindly donated by Patrick Brennan, Colorado State University), and the plasmids *folP*101, -102, and -103 (a gift from Dianna Williams, Louisiana State University). Following the recommendations of the WHO Global Surveillance of Drug Resistance in Leprosy Protocol, samples with mutations suggestive for drug resistance as determined at FIOCRUZ were send for blind sequence evaluation to M. Matsuoka at the Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

In order to verify the presence of inhibitors in the processed biopsy samples, 23 biopsy samples that gave no PCR product in the *gyrA* system were subjected to a reconstitution experiment to verify the presence of eventual PCR inhibitors. For this, these samples were submitted to the PCR using the same conditions as described above, except for the addition of 1.5 ng of NHDP-63 DNA to each PCR mixture. For evaluation of inhibition, the PCR signal for reactions with biopsy sample was compared to that for reconstituted samples without biopsy sample and two positive controls (without reconstitution), as for the earlier PCR experiments. We used three interpretation criteria, with results having either (i) similar or (ii) less signal than the control samples or (iii) no amplification at all.

TABLE 1 Characteristics of relapse patients[a]

Variable	Value for patients
Sex [no. (%) of patients]	
Male	105 (72.4)
Female	40 (27.6)
Clinical form [no. (%) of patients]	
MB	102 (70.3)
PB	43[b] (29.7)
Treatment regimen of first disease course [no. (%) of patients]	
MDT MB 12[b]	22 (15.1)
MDT MB 24	57 (29.3)
MDT PB	31 (21.3)
ROM[c]	2 (1.3)
DNDS + MDT 24	7 (4.8)
DNDS	14 (9.6)
Substitutive regimen[d]	12 (8.2)
Close contact with leprosy case [no. of patients/total (%)]	38/120 (31.6)
Age [median value, yrs (SD)]	47.5 (10.5)
Time from cure to relapse [median value, yrs (SD)]	
All cases	9.45 (4.95)
DR-MDR cases	3.26 (2.62)
Bacillary index [median value (SD)]	2.85 (1.87)

[a] n = 145 relapse cases.
[b] Multidrug therapy with the number of doses between 12 and 24.
[c] ROM, rifampin plus ofloxacin plus minocycline.
[d] Replacement of rifampin by ofloxacin or of dapsone by clofazimine or combined use of rifampin and clofazimine without dapsone. Statistical analysis was performed using Fisher's exact test.

RESULTS

General patient data. Clinical data confirmed that 145 patients suffered from leprosy relapse, and their characteristics are summarized in Table 1. All of these patients presented the inclusion criteria, having been considered cured after completing the official treatment regimen (Brazil/DNDS or WHO/MDT) and having developed a second course of active leprosy disease, as confirmed by bacilloscopic and histopathological examination, also allowing the classification of the clinical form. Most cases (70%) were multibacillary (MB), while the rest were paucibacillary (PB); among the latter, the majority (88%) were borderline tuberculoid. The bacilloscopy index (BI) of the MB cases ranged between 0.25 and 6.0, with an average of 2.85. The average incubation period from cure to relapse diagnosis was 9.45 years, ranging between 1.5 and 25 years, and was significantly shorter in the four resistant cases (3.26 years; $P = 0.0038$), ranging between 1 month and 6.6 years. In addition, two of these cases had been subjected to more than one treatment regimen. Gender analysis showed that males

TABLE 2 Results of DNA sequencing and mutations in the *folP1*, *rpoB*, and *gyrA* genes of *M. leprae*[a]

Gene	No. (%) of samples with conclusive sequencing for IBCAT of[b]: 0	1	2	*P* value[c]	No. (%) of samples with[d]: SNP present	No SNP
folP1	4/60 (6.6)	22/41 (53.7)	31/44 (70.5)	<0.001	3 (5.3)	54 (94.7)
rpoB	5/60 (8.2)	19/41 (46.3)	33/44 (75.0)	<0.001	4 (7)	53 (93)
gyrA	18/60 (29.5)	27/41 (65.9)	32/44 (72.7)	<0.001	2 (2.6)	75 (97.4)

[a] Total no. of relapsed cases: 145.
[b] IBCAT, categorized bacillary index (0, IB = 0; 1, IB > 0 and < 3+; 2, IB > 3+).
[c] Each *P* value is for the three groups as calculated with Fisher's exact test.
[d] SNPs are drug resistance related only.

(72.4%) were more affected than females (26.4%), and the median age of all cases at time of diagnosis of relapse was 47.5, ranging from 13 to 96 years (Table 1).

Upon analyzing treatment regimens, we observed that most of the MB first-disease cases had been treated with the MDT/WHO scheme, having completed either 24 or 12 doses, as adopted by the National Program; some MB cases, however, instead of having received 12 doses, had been subjected to one of the following: (i) a number of doses that varied between 12 and 24, as a consequence of the reduction of MDT treatment from 24 to 12 doses, as recommended by WHO, (ii) the DNDS regimen only, or (iii) the DNDR regimen and 24 doses of the MDT scheme. The last situation occurred in a considerable number of cases (Table 1) and also in three of the cases with drug-associated mutation (see Table 3).

Ampification and sequencing of *rpoB*, *folP1*, and *gyrA*. The results of amplification and DNA sequencing of part of the genes for *folP1*, *rpoB*, and *gyrA* are presented in Table 2. A total of 92 samples (63.4%) yielded sequence results for at least one gene fragment, and informative sequences were obtained for 57 cases (61.9%) for *folP1*, 57 cases (61.9%) for *rpoB*, and 77 cases (83.6%) for *gyrA*. Drug-associated SNPs were detected among 3 of the 57 samples for *folP1* (5.3%), 4 of the 57 samples for *rpoB* (7%), and 2 of the 77 samples for *gyrA* (2.6%). In addition, a statistically significant difference was observed between BI and sequence results (Table 2).

Among the 23 biopsy samples that were tested for the presence of PCR inhibitors, 21 had positive BI and 1 sample had a BI of 0, and for another sample we had no information on the BI. Among these samples, eight (35%) showed a PCR signal similar to that of the positive controls, nine (39%) give weaker signals, and six (26%) gave no PCR product at all (data not shown), meaning that 65% of this sample selection showed some level of PCR inhibition for *gyrA* (data not shown). We did not test PCR inhibition in the PCR systems for *rpoB* and *folP1*.

Regarding the nature of the SNPs, the three changes in *folP1* were always a transition from CCC to CGC in codon 55 (Pro to Arg); in the case of *rpoB*, all occurred at codon 531, with two presenting a change from TCG to ATG (Ser to Met), one from TCG to TTC (Ser to Phe), and one from TCG to TTG (Ser to Leu); the two cases with mutations in *gyrA* presented a transition from GCA to GTA (Ala to Val) in codon 91. On the patient level, mutations suggestive of drug-resistant strains were observed in four cases, including one patient with a mutation in *rpoB* only, suggesting monoresistance to rifampin, one case with SNPs in both *folP1* and *rpoB*, suggestive of multiple drug resistance (MDR) for rifampin and dapsone, and two cases with mutations in *folP1*, *rpoB*, and *gyrA*, strongly suggestive of MDR against the three main antileprosy drugs (Table 2). The sequence results obtained with the four cases that presented SNPs at Fiocruz were confirmed by M. Matsuoka in the Leprosy Research Center, National Institute of Infectious Diseases, Japan.

In addition to the drug resistance-associated SNP in *gyrA*, this gene fragment also presented a synonymous SNP in position 297, and as demonstrated in Table 2, among the 77 samples that were sequenced, 57 (74.03%) presented the C allele, while 20 samples (25.97%) had the T allele. The four cases with drug resistance-associated SNPs presented the C allele.

Characteristics of patients with mutated strains. Table 3 summarizes the data from DNA sequencing and mutations found in the four patients, three being MDR. The first three cases were residents in former colonies for leprosy patients in the Amazon region, and all were subjected to the aforementioned DNDS regimen in their first disease episode. Case one, from the state of Para, presented the most characteristic resistance features, since his treatment failed in a second treatment course (first the DNDS/Brazil regimen and then two courses of MDT/WHO). His last treatment course ended in 2007, while he presented active lepromatous leprosy (LL) disease in the beginning of 2008. The two cases from Amazonas had also undergone two complete treatment schemes before diagnosis of relapse, and their clinical features provoked the suspicion of drug resistance (DR). For the first of these two patients, a mutation on *gyrA* was found, and we discovered that at the end of the

TABLE 3 Summary of drug-resistant relapse cases[a]

Case no. (state[b])	Sex[c]	Age (yrs)	CF	BI	His	Treatment	IP	Result of DNA sequencing[d]: *folP1*	*rpoB*	*gyrA*
1 (PA)	M	49	LL	5	LL	DNDS-MDT 24	1 mo	55, CCC → CGC (Pro → Arg)	531, TCG → ATG (Ser → Met)	No mutation
2 (AM)	M	63	LL	4,5	LL	DNDS-MDT 24	3.2 yrs	55, CCC → CGC (Pro → Arg)	531, TCG → TTC (Ser → Phe)	91, GCA → GTA (Ala → Val)
3 (AM)	M	46	BL	3,5	LL	MDT 24	3.3 yrs	No mutation	531, TCG → TTG (Ser → Leu)	No mutation
4 (ES)	M	38	BT	6,6	BT	MDT 12	6.6 yrs	55, CCC → CGC (Pro → Arg)	531, TCG → TTC (Ser → Phe)	91, GCA → GTA (Ala → Val)

[a] CF, clinical form; BI, bacilloscopic index; His, histopathologic diagnosis; IP, incubation period of relapse; Pro, proline; Arg, arginine; Ser, serine; Phe, phenylalanine; Leu, leucine; Met, methionine; Ala, alanine; Val, valine; MDT/MB 24 (WHO), rifampin (RMP) (600 mg/month) + clofazimine (CLZ) (300 mg/month) (supervised) + DDS (100 mg) + CLZ (50 mg/day), during a period between 12 and 18 months; DNDS (Brazil), rifampin (RMP) (600 mg/day, 90 days) + dapsone (DDS) (100 mg/day up to 5 years until AFB negative). Case one received three treatment courses.
[b] PA, Pará; AM, Amazonas; ES, Espírito Santo.
[c] M, male.
[d] Codon number, mutation.

second scheme (MDT/WHO), this patient also had received ofloxacin but not according to a standard treatment scheme. Finally, the fourth and quite intriguing case from the state of Espírito Santo, southeast Brazil, was diagnosed with borderline lepromatous leprosy (BL) during first disease, presenting positive BI, but was negative in the second disease course, 7 years later, and classified as suffering from the borderline tuberculoid leprosy (BT) form. Although this patient presented a resistance-associated SNP in *gyrA*, we found no history of treatment with ofloxacin, and eventual reinfection by an ofloxacin-resistant strain acquired from his relatives could be possible. Among the 145 patients, 31% informed that they had relatives that were diagnosed for leprosy within 5 years before relapse diagnosis (Tables 1 and 3).

DISCUSSION

The efficiency of the WHO MDT scheme for leprosy treatment is supported by the dramatic decreases in disease prevalence and the low relapse rates in short and medium time frames. Therefore, relapse has not been considered a problem, and organization of studies of this disease characteristic was somewhat neglected, leading to the recent WHO initiative to organize a resistance surveillance project for relapse cases, 26 years after having started MDT. This was possible due to the development and standardization of molecular genotyping procedures of genes associated with drug resistance (5, 16, 23, 24, 38, 39).

After the introduction of relapse surveillance by the WHO, many of the countries of endemicity reported leprosy relapses. In addition, evaluation of the contribution of drug resistance under an international network has been implemented, focusing on MB relapse cases (36). For good-quality data on relapse rates, in addition to laboratory technology, uniformity of clinical criteria relapse diagnosis is important and needs to be standardized within and among countries. Although it not so difficult to diagnose leprosy relapse during the late MB disease form, recognition of relapse is not so easy during early disease, especially in the borderline spectrum cases of disease and under field conditions (19, 20).

In the present study, 29.7% of the relapses were PB cases, 88.3% of these being BT, and this after clinical examination by experienced leprologists and histopathological confirmation by three different pathologists. This was also the case for the BT patient that presented mutations in the *M. leprae* genes *rpoB*, *folP*, and *gyrA*, and possibly this patient, although being MDR, presented this disease form because he was diagnosed very soon after developing relapse, had a better immune host defense response, or had a different strain causing relapse, either by reinfection or strain selection, as observed in a considerable number of relapse cases in another study (11). On the other hand, selection of a particular part of the *M. leprae* population that caused first disease as being responsible for relapse is in accordance with the work of Toman in 1981 (35) and Colston et al. in 1987 (7), raising the possibility that "persistent" *M leprae* could cause relapse in a large proportion of patients, the persistent bacilli presenting a metabolic state that resists the drug without the presence of drug-associated mutations, also suggested by Pattyn (28) and Balagon et al. (1).

Suspicion of DR or MDR in leprosy is raised mainly because of maintenance of clinical symptoms, with or without evaluation of the presence of bacilli in skin smears and confirmation by growth in the footpads of mice fed with antibiotics. Bacteriological analysis by smear microscopy is not always reliable, however, and advances in the elucidation of molecular events responsible for drug resistance in mycobacteria have allowed the development of alternative tools for drug resistance screening (6). However, due to the need of technical expertise and specialized equipment, this technique is executed in a limited number of centers in Brazil (38). Nonetheless, SNP detection seems to be more sensitive and is certainly much quicker for detecting DR than the mouse footpad-based technique (23, 33). In two very recent studies, sequence analysis for DR in Latin American leprosy patients was reported, the first report presenting two cases with SNPs in *rpoB* and one case in *gyrA*, suggestive for resistance against rifampin and ofloxacin, respectively, among 38 Mexican cases, suggesting the possible reemergence of DR leprosy in a country where leprosy was considered eliminated (22). The second study included 230 mostly new leprosy cases, two being from Uruguay, 10 from Bolivia, 23 from Brazil,.and 197 from Venezuela. Only two relapse cases presented SNPs in the three genes studied, one from Venezuela in *folP1* and one from Brazil in *folP1* and *rpoB* (34).

The mutations observed presently all have been reported in studies in other countries, including the changes in codon 531 of *rpoB*, causing an amino acid change from Ser to either Met (n = 1), Phe (n = 2), or Leu (n = 1), the SNP observed in *folP1* in codon 55 (n = 3), causing the change of Pro to Arg, and the mutation at codon 91 of *gyrA* (n = 2), leading to a change from Ala to Val. These SNPs had been described earlier in several reports, including those of Honoré and Cole (17), Williams et al. (37), Cambau et al. (6), and Gillis and Williams (14). In addition to the nonsynonymous SNP in *gyrA*, we observed the allele distribution in the relapse cases of a recently observed synonymous SNP at position 297 of *gyrA* (11, 25), showing that 74% of the cases carried *M. leprae* of the SNP type *gyrA* C at position 297. Our own previous data (11) and the recently published data from Singh et al. (34) showed the correlation of the synonymous SNP *gyrA* 297T type with the SNP type 3 and of SNP *gyrA* C with type 1 or 4 defined by Monot et al. (25). Previous data showed the higher frequency of the SNP3 type in southeast Brazil (13) and Latin America (34), and the prevalence of the SNP *gyrA* C could be due to sampling from other regions of Brazil.

We did not obtain PCR products and good-quality sequences from all samples, and this is due partly to the inclusion of samples with low or zero bacterial counts and to the presence of PCR inhibitors, as evidenced by the reconstitution experiment. Indeed the presence of PCR inhibitors in skin biopsy samples has been described before (32).

The significant difference between the period of time between first disease and relapse between resistant and nonresistant cases is in agreement with the work of Pattyn et al. (28), suggesting a difference in the incubation period in these two kinds of relapses. One MDR relapse case, however, showed such a short incubation period (1 month) that we suspect that this patient had not really been cured from his second disease course (Table 3). Our observation that all resistant cases were males is in agreement with findings of other studies (29, 30) and could be associated with the higher prevalence of males in MB leprosy and more frequent irregular self-administered drug intake (including quinolones) in males, causing mainly secondary resistance. This is supported by the recent observation of Singh et al. (34) showing the absence of primary drug resistance as demonstrated by the lack of drug-related mutations in strains from new leprosy patients. Indeed, three

out of four of the DR patients are from leprosy colonies that had received a previous Brazilian treatment regimen before MDT/WHO. Possibly, these cases, despite receiving regular monthly doses of the MDT/WHO scheme, might have been noncompliant regarding the daily self-administered dose of combined dapsone and clofazimine.

Although DR does not seem to be a problem in Brazil, one should note that the three older DR cases had skin lesions typical of leprosy and good access to a health unit and yet suffered from late diagnosis, strongly suggesting the need of inclusion of ex-colony areas as "loci" for epidemiological surveillance for relapse, as per norms defined by the Ministry of Health (4). Also, the observation of two cases of strains of *M. leprae* with MDR against the three most common drugs for treatment is concerning and could become a serious threat for leprosy control. In order to comply with the Global Surveillance of DR in Leprosy, the following had been recommended: (i) to provide a technical guideline from the National Hansen's Disease (Leprosy) Control Programme (4) for establishment of relapse surveillance measures, (ii) to include the study of drug resistance, (iii) to provide recommendations for the management of suspected relapse cases, and (iv) to design a specific investigation form for the cases reported as relapse in the SINAN national information system (3). In addition, we suggest the implementation within the leprosy control program of monitoring of DR and MDR patients and their close contacts and organizing a reference framework.

Our data show that development of DR isolates of *M. leprae* is contributing to leprosy relapse in Brazil but that the following are alternative causes: (i) bacterial persistence, (ii) immunosuppression of the host, (iii) pregnancy, (iv) the presence of advanced leprosy, (v) reinfection, and (vi) factors associated with failures in operational health care, such as late diagnosis, inadequate or irregular treatment of the disease, and misclassification of earlier disease (11, 18, 19, 20). We admit, however, that a limitation of this study is the use of PCR sequencing for SNP detection, with limitations regarding the detection of eventual minor mutant populations. In addition, mutations outside the part of the genes that was sequenced could have been missed.

ACKNOWLEDGMENTS

This study was funded by CNPq/DECIT/MS-Brazil, grant N401296/2005-9.

The work was made possible by infrastructure of the Laboratory of Molecular Biology Applied to Mycobacteria and Leprosy Laboratory of FIOCRUZ, Rio de Janeiro, RJ, Brazil, UFRJ coordination, and the collaboration of many health professionals from all six outpatient reference health units and participants from five states of Brazil.

REFERENCES

1. Balagon MF, et al. 2009. Long-term relapse risk of multibacillary leprosy after completion of 2 years of multiple drug therapy (WHO-MDT) in Cebu, Philippines. Am. J. Trop. Med. Hyg. 81:895–899.
2. Barnard FM, Maxwell A. 2001. Interaction between DNA gyrase and quinolones: effects of alanine mutations at GyrA subunit residues Ser(83) and Asp(87). Antimicrob. Agents Chemother. 45:1994–2000.
3. Brazilian Ministry of Health (Brasil Ministério da Saúde)/SVS. 2009. Monitoramento das recidivas e da resistência medicamentosa em hanseníase no Brasil, nota técnica no. 05/2009PNCH/SVS/MS. Brazilian Ministry of Health, Federal District, Brazil.
4. Brazilian Ministry of Health (Brasil Ministério da Saúde)/SVS. 2010. Instruções Normativas das ações de controle da hanseníase no SUS. Portaria no. 3.125/SVS-SAS. Brazilian Ministry of Health, Federal District, Brazil.
5. Cambau E, et al. 2002. Molecular detection of rifampin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. Clin. Infect. Dis. 34:39–45.
6. Cambau E, Perani E, Guillemin I, Jamet P, Ji B. 1997. Multidrug-resistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. Lancet 349(9045):103–104.
7. Colston MJ. 1987. Application of the thymectomized-irradiated mouse to the detection of persisting *Mycobacterium leprae*. Int. J. Lepr. Other Mycobact. Dis. 55(4 Suppl):859–863.
8. Dallas WA, Gowen JE, Ray PH, Cox MJ, Dev IK. 1992. Cloning, sequencing and enhanced expression of the dihydropteroate synthase gene of Escherichia coli MC4100. J. Bacteriol. 174:5961–5970.
9. Dallas WS, Dev IK, Ray PH. 1993. The dihydropteroate synthase gene, folP, is near the leucine tRNA gene, leuU, on the Escherichia coli chromosome. J. Bacteriol. 175:7743–7745.
10. Damasco MHS, et al. 1986. Secondary dapsone resistant leprosy in Brazil: a preliminary report. Lepr. Rev. 57:5–8.
11. da Silva Rocha A, et al. 2011. Genotyping of *Mycobacterium leprae* from Brazilian leprosy patients suggest the occurrence of re-infection or of bacterial population shift during disease relapse. J. Med. Microbiol. 60:1441–1446.
12. Diniz LM, Moreira MV, Puppin MA, de Oliveira MLWDR. 2009. Estudo retrospectivo de recidiva da hanseníase no Estado do Espírito Santo. Rev. Soc. Bras. Med. Trop. 42:420–424.
13. Fontes AN, et al. 2009. Genetic diversity of *Mycobacterium leprae* isolates from Brazilian leprosy patients. Lepr. Rev. 80:302–315.
14. Gillis TP, Williams DL. 2000. Dapsone resistance in *Mycobacterium leprae*. Lepr. Rev. 71(Suppl):S91–S95.
15. Gootz TD, Barrett JF, Sutcliffe JA. 1990. Inhibitory effects of quinolone antibacterial agents on eucaryotic topoisomerases and related test systems. Antimicrob. Agents Chemother. 34:8–12.
16. Hernández E, et al. 2008. Estudio de resistencia a la rifampicina y la dapsona en tres pacientes con recurrencia de lepra. Rev. Panam. Salud Publica 23:73–77.
17. Honore N, Cole ST. 1993. Molecular basis of rifampicin resistance in *Mycobacterium leprae*. Antimicrob. Agents Chemother. 37:414–418.
18. Jing Z, Zhang R, Zhou D, Chen J. 2009. Twenty five years follow up of MB leprosy patients retreated with a modified MDT regimen after a full course of dapsone mono-therapy. Lepr. Rev. 80:170–176.
19. Kaimal S, Thappa DM. 2009. Relapse in leprosy. Indian J. Dermatol. Venereol. Leprol. 75:126–135.
20. Linder K, Zia M, Kern WV, Pfau RK, Wagner D. 2008. Relapses vs. reactions in multibacillary leprosy: proposal of new relapse criteria. Trop. Med. Int. Health 13:295–309.
21. Madeira-Diório S, Manini MIP, Trino LM, Sartori BGC, Opromolla DVA. 2005. Resistance to dapsone and rifampicin in *Mycobacterium leprae* isolated from leprosy patients from São Paulo State. Hansenol. Int. 30:9–14.
22. Matsuoka M, et al. 2010. Possible mode of emergence for drug-resistant leprosy is revealed by an analysis of samples from Mexico. Jpn. J. Infect. Dis. 63:412–416.
23. Matsuoka M, Kashiwabara Y, Namisato M. 2000. A *Mycobacterium leprae* isolate resistant to dapsone, rifampin, ofloxacin and sparfloxacin. Int. J. Lepr. Other Mycobact. Dis. 68:425–455.
24. Matsuoka M, et al. 2003. A second case of multidrug resistant *Mycobacterium leprae* isolate from a Japanese patient with relapsed lepromatous leprosy. Int. J. Lepr. Other Mycobact. Dis. 71:240–243.
25. Monot M, et al. 2008. Are variable-number tandem repeats appropriate for genotyping *Mycobacterium leprae*? J. Clin. Microbiol. 46:2291–2297.
26. Oliveira MLW, et al. 2006–2007. Research project: magnitude and characterization of leprosy relapse cases in patients that had been submitted to WHO multi-drug therapy and identification of resistant *M. leprae* strains in five Brazilian states; MCT-CNPq/MS-SCTIE-DECIT, no. 35/2005. National Program for Control of Leprosy, Federal District, Brazil.
27. Opomolla DVA. 2001. As reações tipo I. Editorial. Hansenol. Int. 26:89–90.
28. Pattyn SR. 1987. Persisting *Mycobacterium leprae* among THELEP trial patients in Bamako and Chingleput. Subcommittee on Clinical Trials of the Chemotherapy of Leprosy (THELEP) Scientific Working Group of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Lepr. Rev. 58:325–337.
29. Pettit JH, Rees RJ. 1964. Sulphone resistance in leprosy. An experimental and clinical study. Lancet ii(7361):673–674.

30. Prasad PV, Kaviarasan PK. 2010. Leprosy therapy, past and present: can we hope to eliminate it? Indian J. Dermatol. 55:316–324.
31. Ramasoota P, et al. 2000. Multiple mutations in the *rpo*B gene of *Mycobacterium leprae* strains from leprosy patients in Thailand. Southeast Asian J. Trop. Med. Public Health 31:493–497.
32. Santos AR, De Miranda AB, Sarno EN, Suffys PN, Degrave WM. 1993. Use of PCR-mediated amplification of *Mycobacterium leprae* DNA in different types of clinical samples for the diagnosis of leprosy. J. Med. Microbiol. 39:298–304.
33. Sapkota BR, Ranjit C, Macdonald M. 2006. Reverse line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium leprae*. Nepal Med. Coll. J. 8:122–127.
34. Singh P, et al. 2011. Molecular drug susceptibility testing and genotyping of *Mycobacterium leprae* strains from South America. Antimicrob. Agents Chemother. 55:2971–2973.
35. Toman K. 1981. Bacterial persistence in leprosy. Int. J. Lepr. Other Mycobact. Dis. 49:205–217.
36. WHO. 2009. Drug resistance in leprosy: reports from selected endemic countries. Wkly. Epidemiol. Rec. 84:261–268.
37. Williams D, et al. 1994. Characterization of rifampin resistance in pathogenic mycobacteria. Antimicrob. Agents Chemother. 38:2380–2386.
38. Williams DL, Gillis TP. 2004. Molecular detection of drug resistance in *Mycobacterium leprae*. Leprosy Rev. 75:118–130.
39. You EY, Kang TJ, Kim SK, Lu SB, Chae GT. 2005. Mutations in genes related to drug resistance in *Mycobacterium leprae* isolates from leprosy patients in Korea. J. Infect. 50:6–11.

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PLoS NEGLECTED TROPICAL DISEASES

Detection of Antibiotic Resistance in Leprosy Using GenoType LepraeDR, a Novel Ready-To-Use Molecular Test

Emmanuelle Cambau[1,2,3]*, Aurélie Chauffour-Nevejans[2,4], Liana Tejmar-Kolar[5], Masanori Matsuoka[6], Vincent Jarlier[2,4,7]

1 Université Paris Diderot, EA3964, Paris, France, **2** Centre National de Référence des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux, Paris, France, **3** AP-HP, groupe Hospitalier Saint Louis-Lariboisière, Bactériologie, France, **4** Université Pierre et Marie Curie-Paris6, EA1541, ER5, Paris, France, **5** HAIN Lifescience, Nehren, Germany, **6** Leprosy Research Center, National Institute of Infectious Diseases, Higashimurayama-shi, Tokyo, Japan, **7** AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Bactériologie-Hygiène, Paris, France

Abstract

Background: Although leprosy is efficiently treated by multidrug therapy, resistance to first-line (dapsone, rifampin) and to second-line drugs (fluoroquinolones) was described worldwide. Since *Mycobacterium leprae* is not growing *in vitro*, phenotypic susceptibility testing requires a one year experiment in the mouse model and this is rarely performed. Genetics on antibiotic resistance provide the basis for molecular tests able to detect for antibiotic resistance in leprosy.

Methodology/Principal Findings: A reverse hybridization DNA strip test was developed as the GenoType LepraeDR test. It includes DNA probes for the wild-type sequence of regions of *rpoB*, *gyrA* and *folP* genes and probes for the prevalent mutations involved in acquired resistance to rifampin, fluoroquinolones and dapsone, respectively. The performances of the GenoType LepraeDR test were evaluated by comparing its results on 120 *M. leprae* strains, previously studied for resistance by the reference drug *in vivo* susceptibility method in the mouse footpad and for mutations in the gene regions described above by PCR-sequencing. The results of the test were 100% concordant with those of PCR sequencing and the mouse footpad test for the resistant strains: 16 strains resistant to rifampin, 22 to dapsone and 4 to ofloxacin with mutations (numbering system of the *M. leprae* genome) in *rpoB* (10 S456L, 1 S456F, 1 S456M + L458V, 1 H451Y, 1 G432S + H451D, 1 T433I + D441Y and 1 Q438V), in *folP1* (8 P55L, 3 P55R, 7 T53I, 3 T53A, 1 T53V) and *gyrA* (4 A91V), respectively. Concordance was 98.3% for the susceptible strains, two strains showing a mutation at the codon 447 that in fact was not conferring resistance as shown by the *in vivo* method.

Conclusions/Significance: The GenoType LepraeDR test is a commercially available test that accurately detects for antibiotic resistance in leprosy cases. The test is easy to perform and could be implemented in endemic countries.

Citation: Cambau E, Chauffour-Nevejans A, Tejmar-Kolar L, Matsuoka M, Jarlier V (2012) Detection of Antibiotic Resistance in Leprosy Using GenoType LepraeDR, a Novel Ready-To-Use Molecular Test. PLoS Negl Trop Dis 6(7): e1739. doi:10.1371/journal.pntd.0001739

Editor: Christian Johnson, Fondation Raoul Follereau, France

Received March 7, 2012; **Accepted** June 5, 2012; **Published** July 31, 2012

Funding: Funding support for the study on mouse footpad susceptibility testing (animal facilities, animal keeper and technician) was provided by repeated annual grants from the Association Française Raoul Follereau. Development of the DNA strip test work was supported by a specific grant (Magralepre 2007) from Ordre de Malte-France. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Author Liana Tejmar-Kolar works for the Hain Lifescience company. The other authors have declared that no competing interests exist.

* E-mail: emmanuelle.cambau@lrb.aphp.fr

Introduction

Leprosy, the second communicable disease due to mycobacteria after tuberculosis, is still a preoccupying disease as 230 000 new cases have been reported in 2010 (www.who.int/lep/). This disease remains difficult to diagnose and treat in low- and mid-developed countries, especially in rural areas. Global child rate has remained consistently at around 10% of cases for the last years, showing that transmission is still active [1]. Leprosy can be cured if multidrug therapy (MDT) is properly implemented following WHO recommendations: a 6 month regimen for paucibacillary cases and a 12 month regimen (formerly 24-months) for multibacillary (MB) cases both combining rifampin and dapsone, plus clofazimine for MB cases [2]. The relapse rate ranges between 2% and 5% in leprosy depending of the country, and we learned from tuberculosis that relapse cases are at risk of drug resistance [3]. However, in contrast to what we know for tuberculosis, the prevalence of primary and secondary resistance is unknown for leprosy. Consequently, the risk of resistance cannot be assessed and re-treatment regimen cannot be appropriately design.

Mycobacterium leprae is one of the few bacteria that are not growing *in vitro*. It multiplies only in the mouse footpad [4] and in the nine-band armadillo [5]. The in vivo susceptibility testing model, based on footpad inoculation of mice treated with antibiotics, is available in only an handful of highly specialized laboratories and cannot be spread because it requires one year lasting experiment (*M. leprae* doubling time is about 10 to 14 days) and expensive facilities [4,6]. Resistance to anti-leprosy drugs, such as dapsone, rifampin and fluoroquinolones, has been

Author Summary

Although leprosy is a curable disease using a combination of antibiotics for one year, the transmission is still active with 230,000 new cases in 2010. Drug resistance has been described and may prevent eradication of the disease. The infectious agent causing leprosy, *Mycobacterium leprae*, is not growing *in vitro* and antibiotic susceptibility testing is possible only in the mouse footpad model that requires a one year experiment. Consequently this testing is rarely done and antibiotic resistance rates in leprosy are unknown. This is the reason why we endeavored to set a new diagnosis test that detects for antibiotic resistance in *M. leprae*. The test is based on the method of a DNA strip test with a multiplex PCR followed by reverse hybridization. It was developed as an easy-to-use test and it will be available in endemic countries, where these kinds of strip tests are already used for detection of drug resistance in tuberculosis. The results of the new test, Genotype LepraeDR, performed on 120 *M. leprae* strains were concordant with those of the standard PCR sequencing and mouse footpad susceptibility testing.

described since 1967 using this in vivo model [6]. Multi-drug resistance, i.e. resistance to at least two of these drugs, has been described in Africa [7], Asia [8] and South America (unpublished data).

In the late 1990's, thanks to PCR and determination of the *M. leprae* genome [9], molecular methods detecting antibiotic resistance have been set. Rifampin resistance was associated to mutations in the *rpoB* gene encoding the β subunit of RNA polymerase [10], dapsone resistance to mutations in the *folP1* gene encoding the dihydropteroate synthase [11,12] and fluoroquinolone resistance to mutations in the *gyrA* gene encoding the subunit A of DNA gyrase [7]. Various methods have been described to detect the mutations listed above such as PCR- sequencing, heteroduplexes formation, and DNA array [13,14,15,16,17,18]. However, all these methods require specialized laboratories and are not commercially available. No easy-to-use methods are available in the endemic areas.

The DNA strip assay is a methodology widely used for molecular detection of resistance in tuberculosis [19]. The test is based on a classic PCR and reverse hybridization. Because this methodology has been demonstrated to be simple and robust in developing countries, we aimed to develop a new test based on this technology that easily detect for drug resistance in leprosy.

Materials and Methods

M. leprae strains

Hundred and twelve skin biopsies containing *M. leprae* were studied for the evaluation of the test. They have been sent for leprosy diagnosis to the National Reference Center for mycobacteria (NRC-Myc, Paris, France) from 1989 to 2010 and were all smear-positive for acid fast bacilli (AFB) with a minimum amount of 5×10^4 AFB/ml. The samples were anonymized and the collection was used under the IRB approval for diagnosis specimens received at Assistance publique Hôpitaux de Paris, Biology laboratories of Pitie-Salpetriere Hospital. The selected biopsies (54% of the collection) were consecutive biopsies for which mouse culture was performed and for which enough quantity of specimen was available for performing the molecular studies. Skin biopsies were prepared as described previously for mouse inoculation and molecular experiments [17,20].

Eight *M. leprae* strains, which were previously described and propagated in the nude mouse footpad, were taken as reference strains [8,21].

DNA from several mycobacterial strains other than *M. leprae* were tested for analytical specificity: 3 *M. ulcerans*, 5 *M. marinum*, 5 *M. chelonae*, 1 *M. scrofulaceum*, 1 *M. kansasii*, 1 *M. intermedium*, 1 *M. terrae*, 1 *M. malmoense*, *1 M. fortuitum*. In addition, ten biopsies known to be negative for mycobacteria were also tested for specificity.

GenoType LepraeDR probe description

The design of the mutated (MUT) and wild type (WT) probes were based on the mutations reported in the literature for the resistant strains: in the rifampin resistance determining region (RRDR) in *rpoB* [10,17,22], in the region determining dapsone resistance (DRDR) in *folP1* [11,12,20] and in the quinolone resistance determining region (QRDR) in *gyrA* [7,23]. The probes are listed in Table 1. Wild type probes, one to four according to the gene, were chosen to span the region affected by drug resistance mutations: WT1 to WT4 for *rpoB* (the 430–458 region, numbering system of the *M. leprae* genome TN, GenBank n°NC 002677), WT *folP1* for the 53–55 region and WT *gyrA* for the 89–91 region. Some of the most prevalent mutations in *rpoB* (S456L and H451Y), in *folP1* (P55L) and in *gyrA* (A91V) were included in the strip as specific probes.

GenoType LepraeDR testing

Strips were coated at Hain Lifescience factory (Nehren, Germany) with the different specific oligonucleotides (DNA probes) using the DNA strip technology. Amplification, hybridization and interpretation were performed in a similar procedure as for other GenoType tests [19]. Briefly, 35 µl of 5′-biotinylated primers and nucleotide mix, 5 µl of polymerase buffer, 2 µl of 25 mM $MgCl_2$ stock solution, 3 µl of water and 5 µl of total DNA (20 to 100 ng) were mixed with 1 U of Hot Star Taq polymerase (Qiagen) per reaction. The PCR run was comprised of 35 cycles. After denaturation at 95°C for 15 min, ten cycles at 95°C for 30 sec and at 58°C for 2 min were followed by 25 cycles with a first step at 95°C for 25 sec, a second step at 53°C for 40 sec and a

Table 1. Probes and primers used in the GenoType Leprae DR test for molecular detection of antileprosy resistance.

Antibiotic	Gene	Probe	Targeted codon(s) or mutation*
Rifampin	*rpoB*	WT1	432
		WT2	438–441
		WT3	451
		WT4	456–458
		MUT1	S456L
		MUT2	H451Y
Ofloxacin	*gyrA*	WT	89–91
		MUT	A91V
Dapsone	*folP1*	WT	53–55
		MUT	P55L

na, non applicable.
*numbering system used in the *M. leprae* genome of strain NT (sequence NC 002677 in GenBank).
doi:10.1371/journal.pntd.0001739.t001

third step at 70°C for 40 sec. PCR ended with 8 min at 70°C. Hybridization was performed using the TwinCubator at a temperature of 45°C. The denaturation solution was mixed with 20 µl of the amplified sample and submitted to the usual protocol for hybridization.

In order to assess positive and negative bands, each DNA strip was stuck on an evaluation sheet after the hybridization, and a template was aligned side by side of the respective strip, with at the top the conjugate control band and at the bottom the coloured M marker band. Positive control bands, i.e. that should appear positive to make the test valid, were the conjugate control, the amplification control, the identification control for the *M. leprae* species and amplification controls of the *rpoB*, *folP1* and *gyrA* genes.

Interpretation was as follows for each gene/antibiotic: the strain was predicted to be susceptible when all WT bands were positive and all MUT bands were negative; the strain was predicted to be resistant when at least one MUT band was positive or at least one WT band was negative.

DNA extraction and reference PCR-sequencing

PCR sequencing was performed routinely and prospectively in the frame of NRC-Myc activities, as individual susceptibility to rifampin (*rpoB*) and dapsone (*folP1*) for all the 112 biopsies whereas ofloxacin susceptibility was tested for 52 biopsies. PCR sequencing was performed specifically in the frame of the present study for the 8 reference strains.

Total DNA was extracted from biopsies containing *M. leprae* following the heat-shock procedure [24]. DNA was subjected to three PCRs, one amplifying the RRDR in *rpoB* gene, one the DRDR in *folP1* and one the QRDR in *gyrA*, as previously described [10,25]. Typical reaction mixtures (50 µl) contained 1 × reaction buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 1 µM of each primer (Proligo France SAS), 1.25 U of *Taq* polymerase (Q-Biogene, Illkirch, France) and 5 µl of DNA extract. PCR-amplified fragments were purified by using Montage™ PCR Centrifugal Filter Devices (Millipore, Molsheim, France) and sequenced by the dideoxy-chain termination method with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Courtaboeuf, France). The oligonucleotide primers used for DNA sequencing were those used for PCR. The nucleotide and deduced amino acid sequences were analyzed with the Seqscape v2.0 software (Applied Biosystems).

Antibiotic susceptibility testing in the mouse

Animal experiments were performed in accordance with prevailing regulations regarding the care and use of laboratory animals by the European Commission. The experimental protocol was approved by the Departmental Direction of Veterinary Services in Paris, France.

The *M. leprae* strains were subjected to the mouse footpad susceptibility testing that included 10 untreated Swiss mice as a control group, a rifampin-treated group of 8 mice and a dapsone-treated group of 30 mice as described previously [17],[20]. Dapsone susceptibility testing was stopped in 2001 because of new governmental regulation for antibiotic-free animal feeding. An additional group of 8 ofloxacin-treated mice was inoculated, as described in [7], for the biopsies sampled in patients who have been treated by fluoroquinolones.

Evaluation of the diagnosis performances

The results of the GenoType LepraeDR test were compared to those of the PCR sequencing method for all the 120 *M. leprae* strains (60 in the case of ofloxacin and *gyrA*).

The results of the GenoType LepraeDR test were also compared to the results of the mouse footpad model for *M. leprae* strains that yielded interpretable susceptibility results, i.e. 84 strains tested in vivo for rifampin susceptiblity, and among them 56 for dapsone susceptibility and 5 for ofloxacin susceptibility.

Results

Performances of GenoType LepraeDR for detection of *M. leprae*

The DNA strip tests were validated with regard to the *M. leprae* identification band, which was positive with an intensity equal or higher than that obtained with the universal positive control, demonstrating the presence of *M. leprae* DNA. Thus, the overall sensitivity of GenoType LepraeDR for detecting *M. leprae* was 100%.

Analytical specificity tested with either DNA from another mycobacterial species (n = 19) or negative skin biopsies (n = 10) was 100% since no positive signal was obtained for the *M. leprae* identification band. However, hybridization was observed for DNA from *M. intermedium* and *M. malmoense* with two of the wild type *rpoB* bands, due to a high identity between the *rpoB* genes of these mycobacterial species.

Performances of GenoType LepraeDR for detecting mutations in the genes involved in antileprosy drug resistance

The mutations found in the *M. leprae* strains by PCR-sequencing are listed in the Table 2. Representative results of the DNA strip tests are shown in Figure 1 for resistant strains and in the Figure 2 for susceptible strains.

The results of the DNA strip test were concordant with those of PCR sequencing for all the 16 *rpoB* mutations conferring rifampin resistance (Table 3). We observed a positive signal at probes rpoBMUT2 for the 10 strains harboring the mutation S456L and at rpoBMUT1, for the strain harboring the H451Y mutation, since these mutations are present onto the strip as a mutated probe. As expected for these strains, no signals were observed for the wild type probes rpoBWT4 and rpoBWT3, respectively. For the others mutations, the test detected the *rpoB* mutation through the lack of hybridization with the wild type probes that include the mutated codon (Table 1), e.g. with rpoBWT4 for the two strains harboring the mutation S456M or S456F, with rpoBWT2 for the strain with the mutation Q438V, rpoBWT1 and rpoBWT3 for the strain harboring the two mutations G432S + H451D and

Table 2. List of mutations present in the *M. leprae* resistant strains.

Mutations in the region determining resistance in the following genes (N strains)		
rpoB	***folP1***	***gyrA***
S456L (10)	P55L (8)	A91V (4)
S456F (1)	P55R (3)	
S456M + L458V (1)	T53I (7)	
H451Y (1)	T53A (3)	
G432S + H451D (1)	T53V (1)	
T433I + D441Y (1)		
Q438V (1)		

doi:10.1371/journal.pntd.0001739.t002

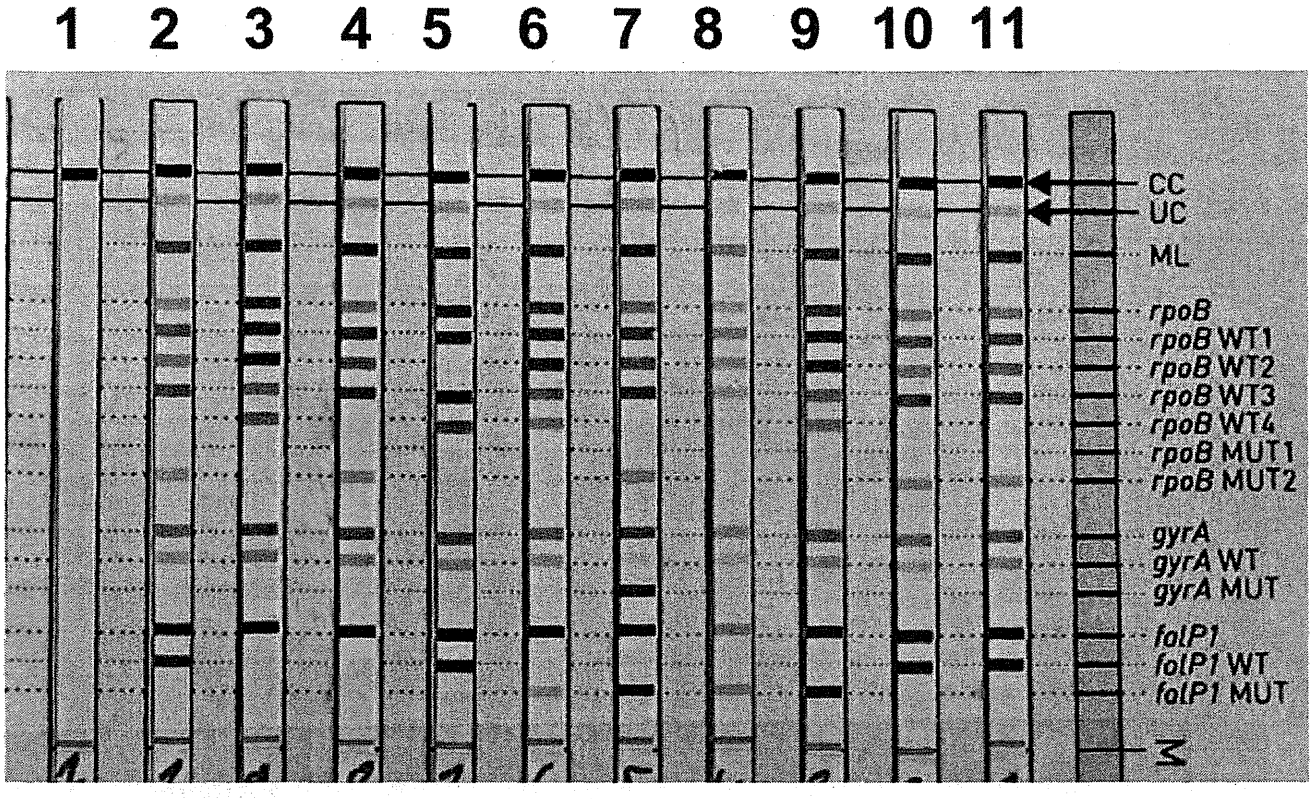

Figure 1. Mutations conferring resistance in *Mycobacterium leprae* are detected by the GenoType LepraeDR DNA strip test. Lane 1 is a negative control (only the CC band). Lanes 2 to 11 showed various profiles for resistant strains: lane 2, *rpoB* mutation S456L with wild type *gyrA* and *folP1* alleles; lane 3, wild type *rpoB* and *gyrA* alleles with a *folP1* mutation to be defined; lane 4, *rpoB* mutation S456L with a wild type *gyrA* allele but a mutation in *folP1*; lane 5, *rpoB* mutation (Q438V) with wild type *gyrA* and *folP1* alleles; lane 6, wild type *rpoB* and *gyrA* alleles with a P55L mutation in *folP1*; lane 7, *rpoB* mutation S456L with a A91V *gyrA* mutation and a P55L mutation in *folP*; lane 8 and lane 9, wild type *rpoB* and *gyrA* alleles with a P55L mutation in *folP1* ; lane 10 and lane 11, *rpoB* mutation S456L with wild type *gyrA* and *folP1* alleles. The numbering system used is that of the *Mycobacterium leprae* genome strain NT (n°NC 002677).
doi:10.1371/journal.pntd.0001739.g001

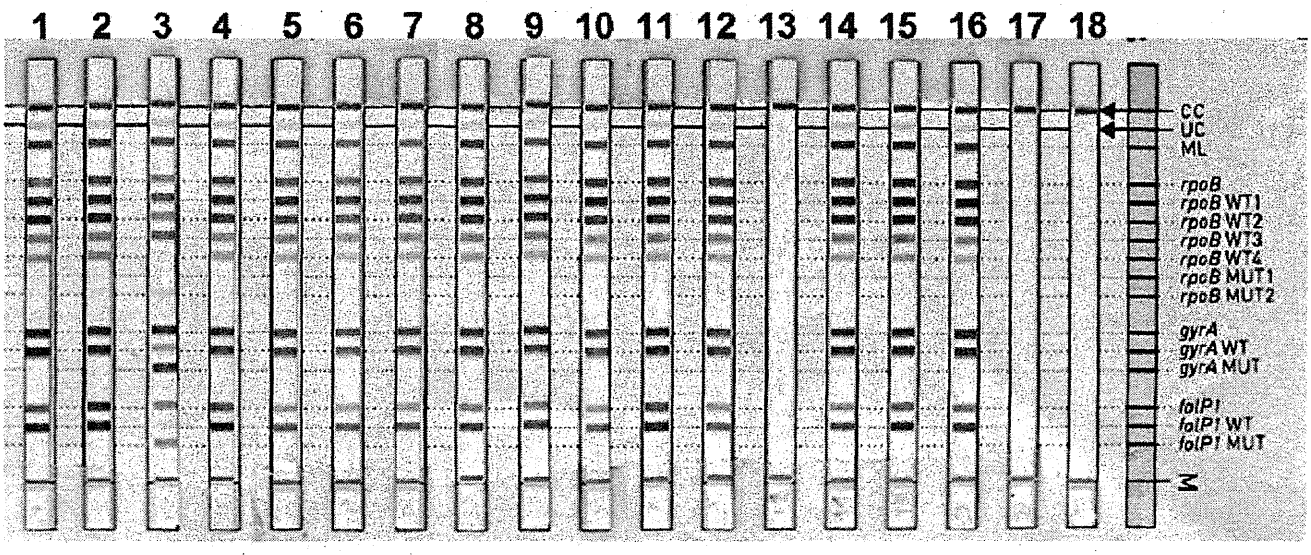

Figure 2. *Mycobacterium leprae* susceptible strains showed a wild type profile in the GenoType LepraeDR test. Lane 1 to 16 (except lane 8) showed wild type profiles for susceptible *M. leprae* strains. Lane 8 showed a multiresistant profile with mutations in *rpoB*, *gyrA* and *folP1* genes. Lanes 17 and 18 showed result of negative controls.
doi:10.1371/journal.pntd.0001739.g002

Table 3. Concordance of results for the DNA strip test (GenoType LepraeDR) and the susceptibility phenotypic and genotypic pattern of antibiotic resistance for the *M. leprae* strains studied.

M. leprae strains	N diagnosis tests with interpretable results			Concordance GenoType LepraeDR N strains (%)	
	In vivo susceptibility testing*	PCR sequencing	DNA strip test	versus in vivo Susceptibility testing	versus PCR sequencing
Total tested for at least one antibiotic	84	120	120	84 (100%)	120 (98%)
Rifampin resistant	13	16	16	13 (100%)	16 (100%)
Rifampin susceptible	71	104	104	71 (100%)	102** (97%)
Dapsone resistant	8	22	22	8 (100%)	22 (100%)
Dapsone susceptible	48	98	98	48 (100%)	98 (100%)
Ofloxacin resistant	1	4	4	1	4
Ofloxacin susceptible	4	56	56	4	56 (100%)

*For strains growing in vivo and yielding interpretable susceptibility results. Tests were stopped for dapsone due to new regulation for antibiotic animal feeding. Tests for ofloxacin were restricted to patient with previous treatment by fluoroquinolones.
**including two strains with a mutation at codon 447: Ser447Cys for one strain and a silent mutation for the second strain (see text for details).
doi:10.1371/journal.pntd.0001739.t003

rpoBWT1 and rpoBWT2 for the strain harboring the two mutations T433I + D441Y. For two strains carrying a mutation at the codon 447, they were not detected by the DNA strip test since no probe spanning this codon is included in the strip because this mutation was not known to confer resistance. The first of these strains showed a silent mutation and the second showed a mutation leading to the substitution S447C. Although the latter strain appeared susceptible to rifampin in the routine mouse footpad testing, we repeated this test using decreasing dosages of rifampin in order to be sure that the S447C mutation does not confer resistance in *M. leprae* as a similar mutation does in *M. tuberculosis* [26], even at a low level. For this purpose, three groups of mice (10 mice per group) were treated by 10 mg/kg (normal dosage), 5 mg/kg or 2.5 mg/kg rifampin. Growth was not observed in any of these groups but occurred in the control untreated group, demonstrating that the strain was really susceptible to rifampin and that the S447C mutation was not conferring resistance. Moreover, the patient, who was an immigrant from Senegal, was cured after being treated by the standard MDT, i.e. the combination of rifampin, dapsone and clofazimine. For the other 102 other strains, no mutations were detected by the RRDR sequencing in *rpoB* and the DNA strip test.

Concordance was observed between the DRDR sequence in *folP1* and the DNA strip test: 22 strains with a *folP1* mutation involved in dapsone resistance and 98 strains with a wild-type *folP1* sequence (Table 3). Hybridization was observed with the *folP*1 MUT probe for the 8 strains with the *folP1* P55L mutation. For the 14 strains harboring other mutations at codon 55 (P55R) or at codon 53 (T53I, T53A, T53V), there was no signal with the wild type probe, showing that there was a mutation.

Finally, we observed a concordance between the QRDR sequence in *gyrA* and the DNA strip test results: 56 strains with a wild type sequence showed a *gyrA* WT band and the four strains with the mutation A91V showed the *gyrA* MUT band (Table 3).

Concordance between susceptibility phenotype and genotype determined by the DNA strip test

Concordance was observed between the phenotypic susceptibility results assessed by the mouse footpad model and the genotype detected by the GenoType LepraeDR test. Results are detailed in Table 3 with regard to the antibiotic tested.

Concordance between rifampin phenotypic susceptibility in vivo and the results of GenoType LepraeDR was obtained for all the 84 strains tested. Thirteen rifampin-resistant strains showed either the rpoBMUT1 band (S456L) for 9 strains, or the absence of at least one rpoB WT band for the remaining 4 strains, which indicated a mutation in the RRDR. The exact nature of the *rpoB* mutation was further identified by PCR-sequencing. All the 71 susceptible strains were founded susceptible by the DNA strip test since all the rpoB WT bands were positive and all of the MUT bands were negative.

Concordance between dapsone phenotypic susceptibility and detection of *folP1* mutation by the DNA strip test was obtained for the 48 susceptible and the 8 resistant strains. For all the resistant strains, the folP WT band was negative, indicating a mutation in the DRDR. The folP MUT band was positive for two of these strains, indicating a mutation P55L. In the 6 remaining strains, the exact nature of the *folP* mutation was identified by PCR-sequencing. For the 48 dapsone-susceptible strains, the folP1 WT band was positive and the MUT band was negative

Finally, results of ofloxacin phenotypic susceptibility were concordant with the results of *gyrA* obtained by the DNA strip test for the five strains tested in the mouse footpad: one was resistant and showed a positive gyrA MUT band (mutation A91V) with a negative WT band, and the four susceptible strains showed a positive gyrA WT band and a negative MUT band.

Discussion

Leprosy, after centuries of endemicity when the disease lasted the whole patient life due to a lack of efficient treatment, became a curable disease by combining rifampin and dapsone into a multidrug therapy regimen [2]. Consequently, a dramatic decrease in the prevalent active cases occurred during the two last decades. However, the incidence rate did not decrease showing that leprosy is still an actively transmitted disease [1]. Acquired resistance has been observed for each of the antileprosy drugs following their successive introduction as antileprosy agent [27,28]. Multidrug resistant strains resulting from the accumulation of distinct resistant traits have been described in several endemic regions [7,22]. Proportions up to 80% of secondary resistance (patients previously treated) and 40% of primary resistance (patients never treated) to dapsone and up to 40%

secondary resistance to rifampin, have been reported through local and limited studies [28,29,30]. Since *M. leprae* is not growing in vitro, it is not possible to measure resistance rates at large scale in endemic countries. Even in highly specialized leprosy centers where the animal model has been set up, it is nowadays very difficult to sustain animal facilities because of ethic rules and safety measures. Molecular detection of resistance to antileprosy drugs has been introduced since genetic bases of resistance were deciphered by expert scientific labs in France, US and Japan Cambau 1997 [10,11,12,31]. We previously showed that mutations in the target genes in clinical *M. leprae* strains were associated with acquired resistance demonstrated by in vivo drug susceptibility testing: in *rpoB* for rifampin resistance, in *folP1* for high and medium level dapsone resistance, and in *gyrA* for ofloxacin resistance [7,17,20]. These studies demonstrated concordance between genotypic and in vivo phenotypic results. Therefore, in-house molecular detection is being used for individual diagnosis of leprosy cases in countries where PCR sequencing is affordable [15,31,32,33,34,35,36].

Following years of using various in house molecular methods to rapidly detect for drug resistance in *M. tuberculosis*, particularly to detect for multi-drug resistant cases, i.e. cases resistant to isoniazid and rifampin that cannot be cured by the standard regimen, standardized and commercially available kits, such as the line probe assays, InnoLiPA Rif.Tb and GenoType MTBDR, and more recently GeneXpert RifTB, have been introduced and are recommended in low-income but highly epidemic countries (www.who.int/tb/strategy/en/).

WHO launched in 2008 a programme of surveillance of drug resistance in leprosy using molecular methods relying on a handful of national and supranational reference laboratories. First results obtained for cases reported in 2008, 2009 and 2010, showed that rifampin, dapsone and fluoroquinolone resistance were described but the resistance rates varied from 0 to 10% [37]. This needs confirmation at a larger scale and for an extended time. However this showed that the rates of resistance to antileprosy drugs can be measured by using molecular methods.

The DNA strip technology has been developed as GenoType kits and applied to the molecular detection of antibiotic resistance in various infections such as tuberculosis and *Helicobacter pylori* diseases [19,38]. This approach has been shown to be easy to use, requiring only a classic thermocycler and a hybridization chamber at a constant temperature of 45°C. This is the reason we choose to develop a standardized test based on the DNA strip technology able to detect for molecular detection of resistance in leprosy.

The new test, GenoType LepraeDR, was evaluated by systematically testing 120 *M. leprae* strains studied for genotypic and phenotypic characters of resistance [17,20,22]. The results yielded by the test were shown to be 100% concordant with those of the in vivo susceptibility testing whereas the results of PCR sequencing was 98.3% for rifampin, 100% for dapsone, and 100% for fluoroquinolones. Moreover, the two *rpoB* mutations not detected by the test, located at the codon 447, a codon not included in the test, were in fact not conferring rifampin resistance.

We focused deliberately the present evaluation on AFB-positive specimen from multibacillary leprosy cases for two reasons: (i) first the AFB positivity represents a major clue in leprosy diagnosis that allows concentrating subsequent tests on mot probable cases, an important point in low income countries and (ii) second, the risk of developing acquired resistance by selection of resistant mutants are highest in multibacillary cases. We did not evaluate the performances of the test on either AFB-negative specimen nor on specimen other than skin biopsies (e.g. nasal wabs). The specificity of the test with regard to other mycobacterial species involved in skin infections was assessed for Buruli ulcer and infections due to *M. marinum*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. terrae* and other less common mycobacteria. Because of the high identity of the *rpoB* gene between some mycobacterial species, the results of resistance mutation in *rpoB*, *gyrA and folP* genes by the test can be interpreted only when the test identifies the species as *M. leprae* (positive ML band).

Various other methods have been described to detect mutations in *rpoB*, *gyrA* and *folP* such as PCR sequencing, heteroduplexes, and DNA array [13,14,15,16,18]. There were mostly used in large laboratories affiliated to Universities of high income countries and collecting strains from endemic countries [34,39]. Since the reverse hybridization technology is already used in several countries endemic for tuberculosis, the same technology could be also used for the diagnosis of resistance in leprosy in countries where leprosy is still a preoccupying disease, with two objectives: (i) diagnosing resistance at the individual level and (ii) assessing rates of secondary and primary resistance in collaboration with health authorities [1,37]. Although leprosy is now diagnosed in the field using clinical findings only and no laboratory support is available, such a test can be used complementary to the clinical diagnosis of multibacillary leprosy for (i) relapse cases, especially those who have not been treated by MDT, i.e. before 1982, and (ii) survey of resistance in new cases in defined areas or periods for epidemiological surveillance on the behalf of leprosy public health programmes. Therefore the specimen can be send to a regional lab, especially one used to similar molecular test detecting resistance in tuberculosis. In addition, clinical microbiology laboratories in high income countries, which have usually moderate expertise in leprosy diagnosis and resistance detection, would appreciate the robustness of the test, and such a test can help in diagnosing cases from immigrants or national intertropical territories [40,41]. Using this technology routinely at the French National Reference Center for mycobacteria during the last two years, we diagnosed 35 cases of leprosy in patients living in France and detected 4 cases with dapsone resistant strains (*folP1* mutations as P55L in 3 strains and T53A in one strain) and 1 case with an ofloxacin resistant strain (*gyrA* A91V mutation) (data not shown). These results, obtained independently of the present evaluation, support the practical interest of this technology.

Acknowledgments

We thank Romain Roth dit Bettoni pour technical assistance in some of the experiments.

Author Contributions

Conceived and designed the experiments: EC LTK VJ. Performed the experiments: ACN LTK MM. Analyzed the data: EC VJ. Contributed reagents/materials/analysis tools: LTK MM ACN. Wrote the paper: EC VJ.

References

1. Rodrigues LC, Lockwood D (2011) Leprosy now: epidemiology, progress, challenges, and research gaps. Lancet Infect Dis 11: 464–470.
2. WHO (1982) Chemotherapy of leprosy for control programmes. Geneva: World Health Organization.
3. AmericanThoracicSociety (2003) Treatment of tuberculosis. American Journal of Respiratory and Critical Care Medicine 167: 603–662.
4. Shepard C (1960) The experimental disease that follows the injection of human leprosy bacillus into footpads of mice. Journal of Experimental Medicine 112: 445–454.
5. Truman RW, Singh P, Sharma R, Busso P, Rougemont J, et al. (2011) Probable zoonotic leprosy in the southern United States. N Engl J Med 364: 1626–1633.

6. Ji B (1985) Drug susceptibility testing of *Mycobacterium leprae*. International Journal of Leprosy 55: 830–835.
7. Cambau E, Perani E, Guillemin I, Jamet P, Ji B (1997) Multidrug resistance to dapsone, rifampin and ofloxacin in *Mycobacterium leprae*. Lancet 349: 103–104.
8. Maeda S, Matsuoka M, Nakata N, Kai M, Maeda Y, et al. (2001) Multidrug resistant *Mycobacterium leprae* from patients with leprosy. Antimicrob Agents Chemother 45: 3635–3639.
9. Cole S, Eiglmeier K, Parkhill J, James K, Thomson N, et al. (2001) Massive gene decay in the leprosy bacillus. Nature 409: 1007–1011.
10. Honore N, Cole ST (1993) Molecular basis of rifampin resistance in *Mycobacterium leprae*. Antimicrob Agents Chemother 37: 414–418.
11. Kai M, Matsuoka M, Nakata N, Maeda S, Gidoh M, et al. (1999) Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. FEMS Microbiology Letters 177: 231–235.
12. Williams D, Spring L, Harris E, Roche P, Gillis T (2000) Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. Antimicrobial Agents Chemotherapy 44: 1530–1537.
13. Sapkota BR, Ranjit C, Neupane KD, Macdonald M (2008) Development and evaluation of a novel multiple-primer PCR amplification refractory mutation system for the rapid detection of mutations conferring rifampicin resistance in codon 425 of the *rpoB* gene of Mycobacterium leprae. J Med Microbiol 57: 179–184.
14. Matsuoka M, Aye KS, Kyaw K, Tan EV, Balagon MV, et al. (2008) A novel method for simple detection of mutations conferring drug resistance in *Mycobacterium leprae*, based on a DNA microarray, and its applicability in developing countries. J Med Microbiol 57: 1213–1219.
15. Sapkota BR, Ranjit C, Macdonald M (2006) Reverse line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium leprae*. Nepal Med Coll J 8: 122–127.
16. Kim SK, Lee SB, Kang TJ, Chae GT (2003) Detection of gene mutations related with drug resistance in *Mycobacterium leprae* from leprosy patients using Touch-Down (TD) PCR. FEMS Immunol Med Microbiol 36: 27–32.
17. Cambau E, Bonnafous P, Perani E, Sougakoff W, Ji B, et al. (2002) Molecular detection of rifampin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. Clinical Infectious Diseases 34: 39–45.
18. Williams DL, Pittman TL, Gillis TP, Matsuoka M, Kashiwabara Y (2001) Simultaneous detection of *Mycobacterium leprae* and its susceptibility to dapsone using DNA heteroduplex analysis. J Clin Microbiol 39: 2083–2088.
19. Hillemann D, Weizenegger M, Kubica T, Richter E, Niemann S (2005) Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol 43: 3699–3703.
20. Cambau E, Carthagena L, Chauffour A, Ji B, Jarlier V (2006) Dihydropteroate synthase mutations in the *folP1* gene predict dapsone resistance in relapsed cases of leprosy. Clin Infect Dis 42: 238–241.
21. Matsuoka M (2011) History and characteristics of isolates maintained at the leprosy research center. Nihon Hansenbyo Gakkai Zasshi 79: 247–256.
22. Maeda S, Matsuoka M, Nakata N, Kai M, Maeda Y, et al. (2001) Multidrug resistant *Mycobacterium leprae* from patients with leprosy. Antimicrobial Agents Chemotherapy 45: 3635–3639.
23. Matrat S, Cambau E, Jarlier V, Aubry A (2008) Are all the DNA gyrase mutations found in *Mycobacterium leprae* clinical strains involved in resistance to fluoroquinolones? Antimicrob Agents Chemother 52: 745–747.
24. Woods S, Cole S (1989) A rapid method for the detection of potentially viable *Mycobacterium leprae* in human biopsies: a novel application of PCR. FEMS Microbiol Lett 65: 305–310.
25. Williams DL, Spring L, Harris E, Roche P, Gillis TP (2000) Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. Antimicrob Agents Chemother 44: 1530–1537.
26. Cambau E, Perani E, Guillemin I, Jamet P, Ji B (1997) Multidrug-resistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. Lancet 349: 103–104.
27. Jacobson RR, Hastings RC (1976) Rifampin-resistant leprosy. Lancet 2: 1304–1305.
28. Pearson J, Rees R, Waters M (1976) Sulfone resistance in leprosy. A review of 100 proven clinical cases. Lancet 2: 69–72.
29. Guelpa-Lauras C, Cartel J, Constant-Desportes M, Millan J, Bobin P, et al. (1987) Primary and secondary dapsone resistance of *M. leprae* in Martinique, Guadeloupe, New Caledonia, tahiti, Senegal, and Paris between 1980 and 1985. International Journal of Leprosy 55: 672–679.
30. Grosset J, Guelpa-Lauras C, Bobin P (1989) Study of 39 documented relapses of multibacillary leprosy after treatment with rifampin. International Journal of Leprosy 57: 607–614.
31. Kai M, Nguyen Phuc NH, Nguyen HA, Pham TH, Nguyen KH, et al. (2011) Analysis of drug-resistant strains of *Mycobacterium leprae* in an endemic area of Vietnam. Clin Infect Dis 52: e127–132.
32. Matsuoka M, Budiawan T, Aye KS, Kyaw K, Tan EV, et al. (2007) The frequency of drug resistance mutations in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from Myanmar, Indonesia and the Philippines. Lepr Rev 78: 343–352.
33. Parashar D, Chauhan DS, Sharma VD, Katoch VM (2006) Applications of real-time PCR technology to mycobacterial research. Indian J Med Res 124: 385–398.
34. You EY, Kang TJ, Kim SK, Lee SB, Chae GT (2005) Mutations in genes related to drug resistance in *Mycobacterium leprae* isolates from leprosy patients in Korea. J Infect 50: 6–11.
35. Shetty V, Wakade A, Ghate S, Pai V, Ganapati R, et al. (2003) Viability and drug susceptibility testing of *M. leprae* using mouse footpad in 37 relapse cases of leprosy. International Journal of Leprosy 71: 210–217.
36. Matsuoka M, Kashiwabara Y, Liangfen Z, Goto M, Kitajima S (2003) A second case of multidrug-resistant *Mycobacterium leprae* isolated from a Japanese patient with relapsed lepromatous leprosy. Int J Lepr Other Mycobact Dis 71: 240–243.
37. Anon (2010) Surveillance of drug resistance in leprosy: 2009. Weekly epidemiological record 85 : 281–284.
38. Cambau E, Allerheiligen V, Coulon C, Corbel C, Lascols C, et al. (2009) Evaluation of a new test, genotype HelicoDR, for molecular detection of antibiotic resistance in *Helicobacter pylori*. J Clin Microbiol 47: 3600–3607.
39. Ramasoota P, Wongwit W, Sampunachot P, Unnarat K, Ngamying M, et al. (2000) Multiple mutations in the *rpoB* gene of *Mycobacterium leprae* strains from leprosy patients in Thailand. Southeast Asian J Trop Med Public Health 31: 493–497.
40. Woodall P, Scollard D, Rajan L (2011) Hansen disease among Micronesian and Marshallese persons living in the United States. Emerg Infect Dis 17: 1202–1208.
41. Truman RW, Singh P, Sharma R, Busso P, Rougemont J, et al. Probable zoonotic leprosy in the southern United States. N Engl J Med 364: 1626–1633.

Jpn J Lepr 81, 191-198(2012)

Genotyping of *Mycobacterium leprae* in Myanmar and supposed transmission mode

Khin Saw Aye[1)], Yin Thet Nu Oo[1)], Kyaw Kyaw[2)], Aye Aye Win[1)], Masanori MATSUOKA[3)] *

1) Immunology Research Division, Department of Medical Research (Lower Myanmar), Yangon, Myanmar
2) Central Special Skin Clinic, Yangon General Hospital, Yangon, Myanmar
3) Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

[Received: 7 May, 2012 / Accepted: 28 May, 2012]

Key words : Genotyping, Leprosy, *Mycobacterium leprae*, Transmission mode, TTC repeats

The polymorphism of TTC repeats in *Mycobacterium leprae* was examined using bacilli from slit skin samples of leprosy patients attending at Central Special Skin Clinic, Yangon General Hospital and nasal swabs of their contacts to elucidate the possible mode of leprosy transmission. It was found that bacilli with different TTC genotypes were distributed among same household contacts and also harbored bacilli in patients were different TTC genotype from that harbored on the nasal mucus of the healthy contacts. Genotypes of TTC repeats were found to differ between husband under treatment and his wife and also mother under treatment and her sons living in same house. This study revealed that TTC genotype of bacilli harbored by household contacts was different with the TTC genotype by index cases. These results indicate that the family members get transmission from outside the dwellings rather than from commonly supposed their MB index cases. There might have been some infectious sources to which the populace had been commonly exposed outside the dwellings.

Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* infection. It has long being believed that the source of infection is untreated multibacillary leprosy patients. It has also been predicted that multidrug therapy (MDT) with strong bactericidal antibiotics (such as rifampicin) would reduce the source of infection and consequently interrupt further transmission to others. However, the number of new cases has shown no substantial decline especially other than India. It is reported that about 200,000 to 30,000 new cases are continuously found in the world every year (1), which

*Corresponding author :
Leprosy Research Center, National Institute of Infectious Diseases
4-2-1, Aoba-Cho, Higashimurayama, Tokyo, 189-0002, Japan
TEL : +81-423-91-8211 FAX : +81-423-94-9092
E-mail : matsuoka@nih.go.jp

suggests that the transmission of leprosy bacilli still occurs, especially in countries of endemicity. Elucidation of the mode of transmission would be essential to reduce newly transmitted cases. The differentiation of strains of leprosy bacilli by genomic polymorphism might be of great value in efforts to understand the mode of transmission of the disease. The range of molecular techniques for epidemiological analysis has expanded in recent years, and there are now many genotypic methods that allow a high level of discrimination between bacterial strains (references 2, 3, 4). Shin *et al.* discovered a genomic divergence of *M. leprae* by the variation of TTC repeats (5) and subdivided 34 isolates into 15 subtypes. Genotyping according to the TTC repeats for fragments amplified by PCR seemed to be feasible for molecular epidemiological analysis of leprosy transmission. A previous study by Saeki *et al.* revealed that *M. leprae* existed on the surface of nasal cavities of residents in areas with high prevalence (6). Here, we report the distribution of different TTC genotypes of *M. leprae* among family members of each household and inconsistent genotypes obtained from patients and their family members in the same dwelling. The results strongly supported the previously proposed hypotheses (7) on the existence of an infectious source(s) other than that of patients living with family members.

Materials and Methods

Samples from patients

To clarify whether the TTC genotype in one patient varies or not, genotypes of the bacilli obtained from various lesions of one patient were compared. Slit-skin smear samples (SSS) from 45 lesions of 22 patients, two SSS from 21 patients and 3 SSS from one patient, were obtained at Central Special Skin Clinic (CSSC), Yangon General Hospital. Samples were collected in the same manner as is used for routine slit-skin smear testing for bacterial index examination. The sample on the disposable surgical blade was soaked in 70% ethanol and kept at room temperature until test.

Samples from patients and their contacts (who develop new case later) in the same dwelling

TTC genotypes of the bacilli from the lesion of four multi-family cases, multiple leprosy case among family member or living in the same dwellings, were compared. Skin slit samples were collected from at least two lesions of each patient. The genotype of each isolate was examined as described below.

Case 1: A MB case supposed to be a index case and his son developed the disease later.

Case 2: The same as Case 1 in another house.

Case 3: Supposed index MB case and after 10 months of MDT, his daughter developed the disease.

Case 4: MB case and after 9 months of MDT, his brother developed the disease

Samples from household contacts

TTC genotypes of the bacilli from nasal swab specimen of 92 household contacts (HC) in 18 dwellings with 22 patients diagnosed at CSSC were examined. HC were defined as persons sleeping during the night under the same roof. Nasal swabs were taken by introducing cotton tip swabs (sterilized *JCB* MENTIP, Japan) 2-3cm into each nostril successively, and rubbing gently on the lateral and median sides of each cavity. Swabs were immediately chilled (kept in ice box) and transported to the Immunology Research Division, DMR (Lower Myanmar) and analyzed.

Preparation of template DNA and sequencing analysis

The sample obtained from slit skin was removed

from the blade and collected as a pellet by centrifugation at 10,000 rpm for 20 min in 70% ethanol and then washed with phosphate-buffered saline. The template was prepared by treatment with lysis buffer according to the method described by Klatser *et al*, (8), and then the TTC genotype was examined.

Templates from nasal swab materials and slit-skin samples were prepared by treatment with lysis buffer at 60°C overnight as described elsewhere (8), TTC repeats regions were amplified by PCR with the primers indicated by Shin *et al*. (5). Copy numbers of TTC repeats were examined by the direct sequencing of the PCR products. Briefly, the regions flanking TTC repeats were amplified using a G mixture and a FailSafe PCR system (EPICENTRE, Madison, Wis. USA). DNA samples for sequencing were recovered with a MinElute gel extraction kit (QIAGEN GmbH, Hilden, Germany) after 1.2% Seakem agarosegel electrophoresis of PCR products. Samples were sequenced with a BigDye terminator cycle sequencing FS Ready Reaction kit Ver. 1.1 (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) and an ABI Prism 310 genetic analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed using DNASIS software (Hitachi Software Engineering, Yokohama, Japan).

Ethical approval

The study was approved by the Institutional Ethical Review Committee of Department of Medical Research (Lower Myanmar). Informed consent was obtained from all subjects. Bacillary samples of nasal swabs and slit-skin smears were collected after informed consent was obtained.

Results

Genotype of the bacilli from the nasal swab samples

Of 92 HC in 18 dwellings, there were 30 (33%) HC individuals carried the bacilli on the surface of their nasal cavities. TTC genotype of the bacilli from nasal mucus of HC in 14 dwellings out of 18 dwellings was identical. Genotype of the bacilli obtained in 4 multi-family cases different among family members (Table 1). Residents in these houses harbored different TTC genotypes from each other; their TTC genotypes were 9, 11, 12, 13, 14, 15, 16, 17, 21, and 22 repeats. The TTC repeats of the bacilli from the skin lesion of new MB case consisted of 11 copies and the TTC repeats of the bacilli from his nasal cavities was 15, on the other hand the bacilli from his family contacts, wife and son, showed 14 and 17 copies respectively. The TTC repeats genotype of the bacilli from PB patient showed 21 copies but bacilli from his household contact (HC) nasal mucus showed 15 copies. The TTC repeats of the bacilli from another new MB case consisted of 13 copies, but the bacilli from his family contacts, two daughters and a son, showed 13, 16 and 9 copies (Table.1). The frequency of each TTC genotype of the bacilli obtained from 45 skin lesion and 52 nasal samples from 22 patients and HC were shown in Table 2. The most predominant genotype was 16 copies of TTC repeats and the 2nd dominate type was 14 copies of TTC repeats.

Genotype of the bacilli in the lesions

From all 22 patients, 45 samples of different lesions showed identical genotypes. The most dominant genotype has 16 copies of TTC repeats in these patients. The other genotypes (number 9, 11, 12, 13, 14, 15, 16, 17, 21 and 22 copies of TTC repeats) were detected. The frequency of each TTC genotype observed in samples from lesions of the patients and the nasal cavities of the residents is shown in Table 2.

Comparison of TTC genotypes among patients in a dwelling

The TTC genotypes of *M. leprae* of supposed index and secondary cases were compared. The genotypes of index case patients in two multi-family cases harbored the bacilli with 13 and 22, and their son (secondary case) showed 9 and 17copies of TTC repeats respectively. In case 3 who was MB case harbored bacilli with 11 copies of TTC repeats, after 10 months of MDT his daughter developed as secondary case and harbored bacilli with 14 TTC repeats. Another case 4 of household cases of two brothers showed different TTC genotypes (15 and 16 TTC repeats) within the family (Table 3).

Discussion

Elucidation and understanding of the transmission mode, the source and the routes of transmission, of *M. leprae* are essential in developing drastic measures to prevent an infection. Previous sero-epidemiological studies indicated widespread *M. leprae* infections within a population (9, 10, 11, 12), and studies by PCR on the distribution of the bacilli also found that many individuals in areas in which leprosy is endemic carried *M. leprae* on the surface of their nasal cavities (6, 12, 13). These studies suggested the presence of an infectious source other than that of a patient within the same dwelling. The aim of this study was to clarify

Table 1. TTC genotypes of *M. leprae* detected from the skin and surfaces of nasal mucosa of patients and surfaces of nasal mucosa residents living in the same house.

Multi-family case	Leprosy patient (Type of patients)	Contacts (Relationship)	TTC genotype (Slit skin)	TTC genotype (Nasal swabs)
A	MB*		11	15
		Wife	-	14
		Son	-	17
B	PB**		21	18
		Grand mother	-	15
C	MB*		16	15
		Son	-	16
		Son	-	15
D	MB*		13	16
		Daughter	-	13
		Daughter	-	16
		Son	-	9

* MB; Multibacilary

**PB; Paucibacillary

microbiologically whether or not MB cases in the same dwelling represent the main source of infection. Establishing a methodology to discriminate the isolates of *M. leprae* is fundamental for these purposes. Although no useful genotyping methods for epidemiological analysis have been available until in 2000, two genomic divergence of *M. leprae* successfully found based on variable number tandem repeats (VNTRs) (5, 14). One of the authors (M. Matsuoka) discovered that *M. leprae* isolates could be divided into two subtypes on the basis of the polymorphism in the *rpoT* gene. The geographical distribution of each genotype in the world was biased and seemed to be related to prehistoric movement of the human race (14). Nevertheless, the genomic diversity of the *rpoT* cannot be used for epidemiological tracing of the transmission of leprosy bacilli. Genotyping by Single Nucleotide Polymorphisms (SNPs) is applicable to analyze movement of the human race but neither useful for analyzing transmission. (15). Genotyping to compare diversity of short-tandem-repeat loci on the basis of PCR is feasible for community based molecular epidemiological analysis, since *M. leprae* is not cultivable and shows very low levels of diversion in genomic DNA (16). Variety in the copy numbers of TTC repeats can be used to classify *M. leprae* into a considerable number of subtypes and discriminate isolates for each leprosy case. It is reasonable to assume that if the index case in the same dwelling is the source of infection, the genotypes detected in the house should be identical among the household members. In this study, various types of TTC genotypes were detected from nasal mucosa of the healthy HC.

Table 2. Frequency of each genotype observed in patients and household contacts.

No. of repeats	Genotype frequency		
	Patients lesion	Nasal mucus	Total
9	2	1	3 (3.1%)
11	2	1	3 (3.1%)
12	6	4	10 (10.3%)
13	6	6	12 (12.3%)
14	4	9	13 (13.4%)
15	4	8	12 (12.3%)
16	11	12	23 (23.7%)
17	2	4	6 (6.2%)
21	2	3	5 (5.2%)
22	6	4	10 (10.3%)
Total	45	52	97

Table 3. TTC genotypes of *M. leprae* obtained from multi-family cases.

Case	TTC genotype of the bacilli from supposed index case	TTC genotype of patient bacilli from patient secondary case
1	Father: 13 copies	Son:9 copies
2	Father:22 copies	Son:17copies
3	Mother: 11copies	Daughter:14 copies
4	Older brother:16copies	Younger brother: 15copies

Results obtained clearly demonstrated that there were families with different TTC genotypes of *M. leprae* on the surface of nasal cavities among the residents in the same dwelling. Therefore, the results of the investigation indicate that these residents are contaminated by bacilli with different genotypes. No variations in genotype among the isolates obtained from various lesions in the same patient were shown. This result consequently enables comparisons of the genotypes of bacilli obtained from different patients. We had identified the existence of TTC genotypes of *M. leprae* that differed between the newly detected family contacts and the supposed index case patient. These results strongly suggest that the bacilli did not originate from a single patient in the dwelling and also indicate the exposure of the family members

to infectious sources out of the dwelling. Previous sero-epidemiological studies suggested that for the majority of cases, the possible source of infection might be in the environment rather than in direct contact with leprosy patients (9, 14, 17). The findings by PCR, which revealed the wide distribution of the bacilli among the residents in endemic areas, also indicated that the transmission of the bacilli was not only from the leprosy patients (6, 12, 13, 17, 18). The present study strongly supports these assumptions respecting the infectious source(s). Although many epidemiological observations indicated that the household contact was the risk factor for the development of leprosy (18, 19), on the other hand, many new cases occurred among populate without any known contact with patients (20). Therefore the source of the secondary case is not only from his/her household. The tendency seen of the accumulation of patients in some families might be attributed to other conditions such as susceptibility to leprosy infection, which is related to genetic predisposition as well as to acquired factors (21). Two groups of the household leprosy cases showed apparently different TTC genotypes between a father and his son, mother and daughter and among brothers. The inconsistency of the genotypes between *M. leprae* isolates obtained from household cases of patients living in the same dwelling clearly indicates that these patients are not always the source in infections of the other family members.

Though the members of the other groups of leprosy cases other than 4 cases shown in table 3 showed the same genotype, whether those people were truly infected by the patient in the house was unclear. The presence of the same genotype in two cases doesn't necessarily imply the infection was occurred from a patient to family contacts, for some TTC genotypes such as 16 copies were widely distributed in the areas. Other polymorphisms which can discriminate within a given TTC genotype are needed to elucidate this problem. Better epidemiological analysis could be done by the combination of various genotyping techniques. However, TTC genotyping enabled the subtyping of *M. leprae* into more types than *rpoT* or SNPs genotyping. Other short polymorphic-tandem-repeat loci exist in *M. leprae* genome (2, 22) combination with genotyping using other polymorphisms might be a useful tool for precise epidemiological analysis especially for the strains with same TTC copy numbers. Other genotyping measures depending on other short polymorphic tandem repeat loci are proposed (3).

The frequency of 24 or 25 TTC repeats was the highest in the previous study, which examined *M. leprae* isolates obtained in Cebu, Philippines (5). Bacilli with 10 copies of TTC repeats were most frequently isolated in the present study, and the bacilli with large numbers (such as 37) of TTC repeats were not detected (Table 3). It is of interest to compare the frequencies of each genotype in different areas, since the results of a previous study indicated that the spread of the bacilli with specific genotypes was consistent with migration of some human groups (14, 23). The evidence resulting from the present molecular epidemiological study indicated the existence of an infectious source other than patients in the same dwelling. Wide distribution of the bacilli among residents (6, 8, 12) and a high positive ratio of anti-PGL-1 antibody among healthy residents (10, 11) suggested that the bacilli existed in certain sources to which people were commonly exposed. Genotyping study of the bacilli obtained at the areas with high leprosy prevalence also suggested infection other than patients (17, 18) Taking these results into consideration, the environment seems to be the most likely infectious source as suggested previously (24). However, it has not been elucidated so far.

Acknowledgement

We would like to thank Board of Directors, Department of Medical Research, (Lower Myanmar) for their advice and encouragements to our research. We are obliged to Dr. Masako Namisato and Dr. Yoshiko Kashiwabara, Tokyo for their support and encouragement.

References

1) World Health Organization. Leprosy global situation. Wkly. Epidemiol. Rec. 86: 389-400, 2011

2) Matsuoka M. Recent advances in the molecular epidemiology of leprosy. Jpn J Lepr 78: 67-73, 2009

3) Gillis T, Vissa V, Matsuoka M, Young S, Richardus JH, Truman R, Hall B, Brennan P, The IDEAL CONSORTIUM Partners: Characterization of Short tandem repeats for genotyping *Mycobacterium leprae*. Lepr Rev 80: 250-260, 2009

4) Monot M, Honoré N, Garnier T, Zidane N, Sherafi D, Paniz-Mondolfi A, Matsuoka M, Taylor GM, Donoghue HD, Bouwman A, Mays S, Watson C. Lockwood D, Khamispour A, Dowlati Y, Jianping S, Rea TH, Vera-Cabrera L, Stefani MM., Banu S, Macdonald M, Sapkota BR, Spencer J, Thomas J, Harshman K, Singh P, Busso P, Gattiker A, Rougemont J, Brennan PJ and Cole ST: Comparative genomic and phylogeographic analysis of *Mycobacterium leprae*. Nature Genetics 41: 1282-1289, 2009

5) Shin YC, Lee H, Lee H, Walsh GP, Kim JD, and Cho SN: Variable numbers of TTC repeats in *Mycobacterium leprae* DNA from leprosy patients and use in strain differentiation. J Clin Microbiol 38: 4535-4538, 2000

6) Saeki K, Budiawan T, Matsuoka M, Izumi S: Epidemiological significance of *M. leprae* in the residential environment: detection of *Mycobacterium leprae* on the surface of nasal cavity of inhabitants in a leprosy endemic area using the polymerase chain reaction. Jpn J Dermatol 110: 153-160, 2000 (In Japanese.)

7) Matsuoka M, Zhang L, Budiawan T, Saeki K, Izumi S: Genotyping of *Mycobacterium leprae* on the basis of polymorphism of TTC repeats for analysis of leprosy transmission. J Clin Microbiol 42: 741-745, 2004

8) Klatser PR, van Beers SM, Madjid B, Day R, de Wit MYL: Detection of *Mycobacterium leprae* nasal carriers in populations for which leprosy is endemic. J Clin Microbiol 31: 2947-2951, 1993

9) Abe M, Ozawa T, Minagawa F, Yoshino Y: Immunoepidemiological studies on subclinical infection in leprosy. II. Geographical distribution of seropositive responders with special reference to their possible source of infection. Jpn J Lepr 59: 162-168, 1990

10) Cho SN, Kim SH, Cellona RV, Chan GP, Fajardo TT, Walsh GP, Kim JD: Prevalence of IgM antibodies to phenolic glycolipid I among household contacts and controls in Korea and the Philippines. Lepr Rev 63: 12-20, 1992.

11) Izumi S, Budiawan T, Saeki K, Matsuoka M, Kawatsu K: An epidemiological study on *Mycobacterium leprae* infection and prevalence of leprosy in endemic villages by molecular biological technique. Indian J Lepr 71: 37-43, 1999.

12) van Beers SM, Izumi S, Madjid B, Maeda Y, Day R, Klatser PR: An epidemiological study of leprosy infection by serology and polymerase chain reaction. Int J Lepr Other Mycobact Dis 62: 1-9, 1994