Lepr Rev (2012) **83,** 269–281

Drug-resistant leprosy: Monitoring and current status

DIANA L. WILLIAMS & THOMAS P. GILLIS
HRSA, BPHC, National Hansen's Disease Programs, Laboratory Research Branch

Accepted for publication 30 August 2012

Introduction

Leprosy control depends solely on case detection and treatment with multi-drug therapy (MDT).[1–3] This strategy is based on the principle that identifying and treating chronic infectious diseases with combinations of effective antibiotics limits the emergence and spread of new or existing antibiotic resistant pathogens.[2] According to the World Health Organization (WHO), MDT formulated for leprosy has been effective at reducing both the prevalence and incidence of leprosy globally.[3–5] According to official reports from 130 countries and territories, the global registered prevalence of leprosy at the beginning of 2011 was 192,246 cases, while the number of new cases detected during 2010 was 228,474.[5]

The most important indicator for the effectiveness of a chemotherapeutic regimen is the rate of relapse following successful completion of the scheduled course of treatment. Information from a number of leprosy control programmes suggests that the relapse rate is very low for both paucibacillary (PB) leprosy (0·1% per year) and multibacillary (MB) leprosy (0·06% per year).[5] Lessons learned from tuberculosis strongly suggest that relapse cases are at risk for drug resistance and can undermine existing control measures.[6,7] Therefore establishing the success of a strategy like MDT for leprosy control requires thorough evaluation of treatment failures, including drug susceptibility testing. Several studies have documented relapses after MDT[8–14] and drug-resistant strains of *Mycobacterium leprae* have been identified.[15–26] In contrast to what we know for tuberculosis, the current prevalence of primary and secondary resistance to rifampicin, dapsone, and clofazimine is virtually unknown for leprosy. Therefore, surveillance of drug resistance globally is a key factor in monitoring MDT effectiveness and preventing the spread of drug resistance.

Over the past two decades, rapid DNA-based molecular assays for detection of drug-resistant *M. leprae* directly from clinical specimens have been developed [Reviewed in[22,23]]. Even though these assays are based on sophisticated, modern, molecular biology techniques, many reference laboratories in leprosy endemic countries have the capability of utilizing

Correspondence to: Diana L. Williams, HRSA, BPHC, National Hansen's Disease Programs, Laboratory Research Branch, Molecular Biology Research Dept., LSU-SVM, Rm 3517W, Skip Bertman, Dr., Baton Rouge, LA 70803 USA (Tel: +1 225 578 9839; Fax: + 1 225 578 9856; e-mail: dwilliams2@hrsa.gov)

0305-7518/12/064053+13 $1.00 © Lepra

these tools for detection of drug resistance. Information gained from their implementation can now be used as an integral component of an overall public health strategy for better patient care as well as monitoring the spread of drug-resistant *M. leprae*. In this review we describe the antibiotics used to treat leprosy and, where known, the mechanism of resistance for each in *M. leprae.* We also describe current DNA-based assays for drug susceptibility testing and surveillance studies aimed at quantifying the global burden of drug-resistant leprosy.

ANTI-LEPROSY DRUGS AND RESISTANCE MECHANISMS

The WHO Study Group on Chemotherapy of Leprosy for Control Programmes recommended the introduction of Multi-Drug Therapy (MDT) in 1982[2] in response to the serious threat to leprosy control posed by the widespread emergence of dapsone resistance.[15,16] Concern has also been expressed about the development of drug resistance to rifampicin, as it is the most important component of the MDT regimen.[17–23] As with tuberculosis, the emergence of multi-drug resistant strains of *M. leprae* would pose a serious threat to leprosy control efforts.

FIRST LINE DRUGS

The drugs used in WHO-MDT are a combination of rifampicin, clofazimine and dapsone for MB leprosy patients and rifampicin and dapsone for PB leprosy patients. Among these drugs, rifampicin is the most important anti-leprosy drug and, therefore, is included in the treatment of both types of leprosy. Experience strongly suggest that treatment of leprosy with either dapsone[15,16] or rifampicin alone[27] will result in the development of resistance to the respective drug and therefore should be discouraged.

In the 1950 s, dapsone was introduced as standard chemotherapy for leprosy[28] and was used worldwide for treating both MB and PB forms of the disease. The use of dapsone required long-term, often life-long, treatment to control infections because of its slow bacteriostatic effect on *M. leprae*. Long-term monotherapy with dapsone resulted in poor compliance in many areas ultimately leading to treatment failures and the emergence of dapsone-resistant strains of *M. leprae* in the 1970 s.[15,16] This presented serious problems for leprosy control programmes as resistance levels were reported as high as 40% in some areas of the world.[29,30] By the mid-1970 s it was clear that life-long dapsone monotherapy was failing. Between the 1960 s and 1970 s, additional antimicrobial agents such as rifampicin[31,32] and clofazimine[33] were introduced for treating leprosy. Rifampicin proved to be a powerful anti-leprosy drug however; using rifampicin alone resulted in relapses.[27] In addition, clofazimine proved to be only weakly bactericidal against *M. leprae* and, therefore, was not a suitable single drug therapy for leprosy.[33]

To overcome the threat posed by the worldwide spread of dapsone resistance and to improve treatment efficacy the WHO recommended MDT for leprosy in 1982.[2] The current WHO recommendations for adults are: daily dapsone (100 mg) and clofazimine (50 mg), with once monthly rifampin (600 mg) and clofazimine (300 mg) for a duration of 1 year in the treatment of MB leprosy (skin smears with a bacterial index of $\geq 2+$); and daily dapsone (100 mg) and once monthly rifampicin (600 mg) used for a duration of 6 months to treat patients with PB leprosy (skin smears with a bacterial index of $< 2+$). A simple scheme to define disease type by number of lesions is applied in peripheral clinics, where microscope BI testing is not available. MB leprosy patients are those with more than five skin lesions and PB leprosy patients are those with up to five skin lesions.[34] These drug formulations are

incorporated into blister packs that can be stored at room temperature. This has made it possible to distribute drugs to patients in rural or hard to reach locations sufficient for several months of treatment, thereby improving treatment completion rates.[5]

DAPSONE

The first effective treatment for leprosy was promin (diamino-azobenzene4′-sulfonamide) introduced in 1941 and given intravenously. Six years later a more effective oral sulphone, dapsone (diamino-diphenylsulphone), replaced promin and is still a fundamental part of MDT for leprosy.[28] Sulphone drugs target the dihydropteroate synthase (DHPS), a key enzyme in the folate biosynthesis pathway in bacteria including *M. leprae*, by acting as a competitive inhibitor of p-aminobenzoic acid (PABA).[35–39] Missense mutations within codons 53 and 55 of the drug resistance determining region (DRDR) of the *folP1*gene, encoding the DHPS of *M. leprae,* have been observed in dapsone-resistant strains (Table 1, Figure 1).

Table 1. Mutations within drug target genes that confer resistance to *M. leprae*

Drug Target Gene	Drug Susceptibility MFP assay[1]	Substituted Amino Acid[2]	Number Resistant Strains[3](%)	Reference
Rifampin/*rpoB*	R	Gly401Ser; His420Asp	1(1)	[10]
	R	Gln407Val	1(1)	[10]
	R	Phe408(Lys + Phe insert) Met409	1(1)	[75]
	NC	Asp410Tyr	2(2)	[23,66]
	NC	Asp410Asn	1(1)	[77]
	NC	Asp410Asn; Leu427Pro	1(1)	[76]
	R	Ser416Cys	1(1)	[77]
	R	His420Asp	2(2)	[77,66]
	R	His420Tyr	11(13)	[76]
	R	*Ser425Leu*	55(63)	[10,23,75,78,76,79,66,67,80,81]
	R	Ser425Met	4(5)	[75,80]
	R	Ser425Met; Leu427Val	1(1)	[10]
	R	Ser425Phe	4(5)	[75,77,80]
	NC	Ser425Trp	1(1)	[76]
	NC	Gly428Ser	1(1)	[67]
Dapsone/*folP1*	R	Thr53Ala	14(18)	[82,83,76,84]
	NC	Thr53Ala; Pro55Leu	1(1)	[76]
	R	Thr53Arg	2(3)	[66,83]
	R	Thr53Ile	14(18)	[39,82,76,83]
	NC	Thr53Val	2(3)	[84,66]
	NC	Pro55Ala	1(2)	[84]
	R	Pro55Arg	19(24)	[39,83,66,67,80,84]
	R	*Pro55Leu*	23(30)	[82,83,76,66,68,84]
	R	Pro55Ser	2(3)	[22]
Ofloxacin/*gyrA*	NC	Gly89Cys	1(8)	[76]
	R	Ala91Val	11(92)	[23,76,79,67,80,81]

[1] R=resistance in mouse footpad assay; NC=no confirmation in mouse footpad assay.

[2] Substituted amino acid in drug target protein; Bold and *italic* mutants are highest frequency mutations for *M. leprae* drug resistance.

[3] Number of *M. leprae* strains with substituted amino acid. (%) derived from: 87 rifampin-resistant strains tested; 78 dapsone-resistant strains tested; and 12 ofloxacin-resistant strains tested.

```
A
WHO M. leprae rpoB DRDR region
gtc gag gcg atc acg ccg cag acg ctg atc aat atc cgt ccg gtg gtc gcc

gct atc aag gaa ttc ttc ggc acc agc cag ctg tcg cag ttc atg gat cag

aac aac cct ctg tcg ggc ctg acc cac aag cgc cgg ctg tcg gcg ctg ggc
                             H420                 S425
ccg ggt ggt ttg tcg cgt gag cgt gcc ggg cta gag gtc cgt gac gtg cac

cct tcg cac tac ggc cgg atg tgc ccg atc gag act ccg gag ggc ccg aac

ata ggt ctg atc ggt tca ttg tcg

WHO M. leprae folP1 DRDR region
ctt gat cct gac gat gct gtc cag cac ggc ctg gca atg gtc gcg gaa ggc

gcg gcg att gtc gac gtc ggt ggc gaa tcg acc cgg ccc ggt gcc att agg
                                       T53     P55
acc gat cct cga gtt gaa ctc tct cgt atc gtt cct gtc gta aaa gaa ctt

gca gca cag ggg att aca gta agt atc gat act acg cgc gct gat gtt gca

cgg gcg gcg ctg caa agc ggc gca cgg atc gtc aac gat gtg tct ggt gg

WHO M. leprae gyrA DRDR region
 at ggt ctc aaa ccg gta cat cgt cgg gtc ttg tac gcg atg tta gac tcc

ggt ttc cgc ccg gac cgt agc cac gct aag tca gca cgg tca gtc gct gag

acg atg ggc aat tac cat ccg cac ggc gac gca tcg att tat gac acg tta
                             G89     A91
gtg cgc atg gcg cag ccg tgg tcg ctg cgg tat ccc ttg gtt gat ggg caa

ggc aat ttc ggt tcg ccg ggt a


B
Rifampin-rpoB
M. leprae RMP-S   ctg acc cac aag cgc cgg ctg tcg gcg ctg ggc ccg ggt
M. leprae RMP-R   ctg acc cac aag cgc cgg ctg ttg gcg ctg ggc ccg ggt
                  *** *** *** *** *** *** *** * * *** *** *** *** ***
                                              S425L
Dapsone-folP1
M. leprae DDS-S   gtc gac gtc ggt ggc gaa tcg acc cgg ccc ggt gcc att
M. Leprae DDS-R   gtc gac gtc ggt ggc gaa tcg acc cgg ctc ggt gcc att
                  *** *** *** *** *** *** *** *** *** * * *** *** ***
                                                       P55L
Ofloxacin-gyrA
M. leprae OFX-S   aat tac cat ccg cac ggc gac gca tcg att tat gac acg
M. leprae OFX-R   aat tac cat ccg cac ggc gac gta tcg att tat gac acg
                  *** *** *** *** *** *** *** * * *** *** *** *** ***
                                              A91V
```

Figure 1. DNA sequences for PCR/direct DNA sequencing assays for surveillance of *M. leprae* drug resistance.[65] A) DNA sequence of the drug resistance determining regions (DRDRs) of *rpoB* (ML1891c), *folP1* (ML0224) and *gyrA* (ML0006) in the *M. leprae* TN genome. Underlined bases represent primers for PCR amplification and DNA sequencing of amplicons. Boxes represent codons most commonly mutated yielding rifampicin (RMP)-, dapsone (DDS)- and ofloxacin (OFX)-resistant *M. leprae,* respectively. B) Alignments of partial drug susceptible DRDRs from *M. leprae* TN strain with those obtained from PCR/direct DNA sequencing of clinical *M. leprae* strains containing mutations most frequently found associated with RMP, DDS and OFX resistance. Asterisk (*) denotes identical nucleotide in both sequences. Single letter amino acid code used to denote the resultant amino acid change in the target proteins of *M. leprae.*

In addition, the majority of these patient biopsies were confirmed to harbour *M. leprae* with moderate to high-levels of dapsone resistance as demonstrated by the mouse footpad (MFP) drug susceptibility assay.

RIFAMPICIN

Rifampicin (3-{[(4-methyl-1-piperazinyl)-imino]-methyl}rifamycin) is the key bactericidal component of all recommended MDT regimens. A single dose of 1,200 mg can reduce the number of viable bacilli in a patient's skin to undetectable levels within a few days.[32] This study also showed that a single dose of 600 mg had the same effect as 1200 mg in approximately 7 days. The target for rifampicin in bacteria is the β-subunit of the DNA-dependent RNA polymerase encoded by *rpoB*.[40] *M. tuberculosis* resistance to rifampicin correlates with changes in the structure of the β-subunit of the RNA polymerase, primarily due to missense mutations that occur within a highly conserved region of the *rpoB* gene referred to the rifampicin resistance determining region (RRDR).[6,41] Rifampicin resistance in *M. leprae* also correlates with missense mutations within the *rpoB* RRDR (Table 1, Figure 1). Substitutions within codon Ser456 have been shown to be the most frequent mutations associated with the development of the rifampicin-resistant phenotype in *M. leprae* (Table 1, Figure 1B).

CLOFAZIMINE

Clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10]-dihydro-2-(isopropylimino)-phenazine] is a lipophilic riminophenazine antibiotic that possesses antimycobacterial activities[1,42,43] for which the mechanism has not been fully elucidated. Clofazimine attains high intracellular levels in mononuclear phagocytic cells, its metabolic elimination is slow, it has an anti-inflammatory effect, and the occurrence of resistance in *M. leprae* is extremely low.[1,22,23] Clofazimine is highly lipophilic and appears to bind preferentially to mycobacterial DNA.[1] Binding of the drug to DNA appears to occur principally at base sequences containing guanine, which may explain clofazimine's preference for the G + C-rich genomes of mycobacteria over human DNA. The accumulation of lysophospholipids (detergent-like agents with membrane-disruptive properties in bacterial cells) appears to mediate the activity of clofazimine in some gram-positive bacteria.[44] However, it is unclear whether this mechanism of action is operational in *M. leprae*. Since clofazimine may act through several different mechanisms, it is not difficult to understand why only a few cases of clofazimine-resistant leprosy have been reported over the years.

OTHER ANTI-LEPROSY DRUGS

OFLOXACIN

Ofloxacin (4-fluoroquinolone) is a fluorinated carboxyquinolone that has moderate bactericidal activity for *M. leprae*.[45–50] The mechanism of action of ofloxacin on *M. leprae* is unknown, but in other bacteria it appears to inhibit DNA replication by inactivating the DNA gyrase, a tetramer containing two β-subunits (GyrA) and two β-subunits (GyrB).[51] Mutations within a highly conserved region of *gyrA*, the quinolone resistance-determining region (QRDR), are associated with the development of ofloxacin resistance in most resistant strains

of *M. tuberculosis*.[52] The first ofloxacin-resistant *M. leprae* was described in 1994[53] and subsequently other cases have been found. The DRDR of *M. leprae gyrA* is highly homologous to that of *M. tuberculosis*, and missense mutations within codon Ala91 of this region have been found in the majority of ofloxacin-resistant strains of *M. leprae* (Table 1, Figure 1).

CLARITHROMYCIN

Clarithromycin is a semisynthetic macrolide that differs from erythromycin in its methyl substitution at the number six position of the macrolide ring. This drug displays significant bactericidal activity against *M. leprae* in humans.[54–56] In patients with lepromatous leprosy, daily administration of 500 mg of clarithromycin kills 99% of viable *M. leprae* within 28 days and 99·9% by 56 days. Although the mechanism of action against *M. leprae* is unknown, it is thought to be similar to that of erythromycin, which acts by inhibiting protein synthesis by binding to the ribosome.[57] Clarithromycin resistance in bacteria and mycobacteria appears to be due to a decrease in binding of the drug to ribosomes and is associated with missense mutations within the 23SrRNA gene.[57,58,59] This has not been fully investigated in *M. leprae* due to the lack of well characterised resistant strains.[60]

MINOCYCLINE

Minocycline (7-dimethylamino-6-demethyl-6-deoxytetracycline) is the only member of the tetracycline group of antibiotics to demonstrate significant activity against *M. leprae*, presumably due to its lipophilic nature which may enhance cell wall penetration.[1,61] Minocycline is bactericidal for *M. leprae* and its activity is additive when it is combined with dapsone and rifampicin. The mechanism of action of minocycline against *M. leprae* is unknown but is thought to be similar to that of all tetracyclines which act by inhibiting protein synthesis. Tetracyclines bind reversibly to the 30S ribosomal subunit blocking the binding of aminoacyl-tRNA to the mRNA ribosome complex.[62] Resistance to tetracyclines may be mediated by three different mechanisms: an energy-dependent efflux of tetracycline brought about by an integral membrane protein; ribosomal protection by a soluble protein;[62] or enzymatic inactivation of tetracyclines. The molecular mechanism of minocycline resistance has not been studied in *M. leprae* due to the lack of resistant mutants, presumably because minocycline has been primarily used to treat single-lesion PB leprosy in combination with rifampin and ofloxacin.

RIFAMPICIN, OFLOXACIN AND MINOCYCLINE (ROM) COMBINATION THERAPY

MDT for leprosy has been very practical and successful for both MB and PB leprosy and the overall prevalence rates of leprosy in the world have fallen dramatically.[5] However, noncompliance is a primary reason that drug-resistant strains develop and relapses with resistant strains may occur posing a potential problem for MDT in the control of leprosy. As new drugs have been shown to be active against *M. leprae*, new combinations have been tried in attempts to shorten the duration of therapy and improve therapeutic efficacy. For example, in 1998 a single dose of a combination of rifampicin (600 mg), ofloxacin (400 mg) and minocycline (100 mg) (ROM) was evaluated for treating single lesion, PB leprosy.[63] A recent review of ROM therapy in leprosy concluded that, while ROM therapy has inherent advantages (potential for improved compliance, absence of skin pigmentation and severe

reactions) it was less protective than WHO MDT in single lesion, PB patients.[64] The authors also concluded that current published data are insufficient to make meaningful comparisons of monthly ROM therapy vis a vis standard MDT for treating MB leprosy. Future studies with these drugs and others should be encouraged in an attempt to improve compliance and cure rates while maintaining a focus on holding drug resistance to a minimum and reducing the incidence of severe reactions.

DEVELOPMENT OF DRUG RESISTANCE IN *M. LEPRAE*

Lacking direct evidence for the mechanisms of *M. leprae*'s resistance to most of the anti-leprosy drugs, our current understanding is based on studies carried out in *M. tuberculosis,*[6] other bacteria, and a few studies with *M. leprae* genes in surrogate hosts. From these studies one can predict that drug resistance in *M. leprae* is attributable to: 1) chromosomal mutations in genes encoding drug targets; 2) these mutations occur spontaneously as a result of errors in DNA replication; and 3) these mutants are enriched in a population of susceptible M. *leprae* by inappropriate drug therapy. Drug-resistant *M. leprae* mutants can be acquired during the initial infection from an infection source containing drug-resistant leprosy (primary drug resistance) or from inadequate treatment (secondary drug resistance).

Because *M. leprae* cannot be cultivated *in vitro*, the frequency of drug-resistant mutants in a population of bacteria is also inferred from studies with *M. tuberculosis* or other cultivable mycobacteria. For example, the frequency of dapsone-resistant mutants in a population of *M. leprae* is estimated to be 106 and the frequency for rifampicin and ofloxacin resistance is estimated to be 10^7 and 10^8,[6] respectively. Rates of clofazimine resistance in *M. leprae* are unknown but appear to be extremely low. Since untreated MB patients can harbour large bacterial loads (10^{11}*M. leprae*), it is feasible that a patient could contain up to 105 dapsone-resistant organisms and thousands of rifampicin- or ofloxacin-resistant mutants in their tissues. MDT was designed to reduce the development of drug resistance and therefore these frequencies become less relevant when effective drug combinations are given. However noncompliance or inadequate therapy of MB patients with high bacterial loads has the potential to enrich the subpopulations of drug-resistant *M. leprae,* leading to the spread of one or more resistant phenotypes.

SURVEILLANCE OF DRUG RESISTANCE IN LEPROSY

The success of leprosy control programmes relies heavily upon MDT. Therefore, it is important that trends in drug resistance be monitored periodically. If resistance rates are found to be increasing new strategies should be formulated that arrest its spread. Acknowledging the growing concern of drug resistance in leprosy, the WHO issued guidelines for the global surveillance of drug resistance in leprosy using PCR-direct sequencing of *M. leprae* DRDRs from patients with characterised relapse from MDT.[65] During 2010, a total of 109 relapsed cases were diagnosed at sentinel sites in China, Colombia, India, Myanmar, Pakistan, The Philippines, Viet Nam and Yemen. Of the 109 cases identified 88 (81%) were tested for drug resistance.[4] Nine (10%) were resistant to dapsone and one (1·1%) case tested positive for resistance to rifampicin. No resistance to ofloxacin was reported and no MDR cases were detected in this cohort.

Other studies have found similar results for dapsone and rifampicin resistance in patients who had relapsed with active disease after completion of, or premature termination of,

MDT.[66–69] In addition, very low levels of ofloxacin-resistant and MDR cases were observed. Three of these studies also evaluated anti-leprosy drug resistance in newly diagnosed patients.[67–69] In 565 new cases tested 1·7% of cases were resistant to dapsone and 1% of cases were resistant to rifampicin. Ofloxacin-resistant and MDR cases were not detected in newly diagnosed patients in these studies. As more sites in more countries participate in future surveillance studies, it should be possible to formulate an accurate view of drug-resistant leprosy and thereby assess the success of current control strategies.

DETECTION OF DRUG-RESISTANT LEPROSY

Leprosy presents a very special problem for detecting drug resistance because *M. leprae* cannot be cultured axenically. Accordingly, drug susceptibility testing was non-existent until 1962 when Shepard developed the MFP assay for determining *M. leprae's* susceptibility to anti-leprosy drugs.[31] Since its development, the MFP assay has been the 'gold standard' for leprosy drug susceptibility testing. This method requires the recovery of a sufficient number of viable organisms from a patient to inoculate the footpads of 20 to 40 mice (depending on the number of drugs to be tested) with each footpad receiving 5×10^3 organisms. Infected mice are treated with the appropriate drug(s) orally. Mice are sacrificed after a defined period of time (usually 6 months or longer) and the numbers of bacilli in the footpads of treated mice and untreated mice are compared.

The MFP method is the only bacteriological assay for drug susceptibility testing for *M. leprae* and presently is the standard for characterising the association of mutations in target genes with drug resistance in *M. leprae*. While the MFP gives definitive information pertaining to the susceptibility of an *M. leprae* isolate to anti-leprosy drugs, it is laborious, expensive and often fails due to the need for significant numbers of viable *M. leprae* in a patient's biopsy. Because of the need for special resources to conduct this assay, only a few facilities in the world have retained high quality mouse footpad laboratories. Their support is critical as new drug-resistant mutants may evolve requiring corroboration in this model.

The first rapid drug-screening assays for *M. leprae* were developed based on radiorespirometry techniques[70,71] and have been used successfully to identify new anti-leprosy drugs.[71,46] Both assays are based on detection of 14-CO_2 production from *M. leprae's* metabolism of 14-C-labelled palmitic acid in 7H12 medium in the presence and absence of anti-leprosy drugs. However, the use of these techniques for drug susceptibility testing in leprosy biopsies is limited by a stringent requirement for very large numbers (2×10^7) of viable bacteria from a patient and the use of radioactivity, often restricted in many countries.

The availability of genomic sequence of *M. leprae*[72,73] and an improved understanding of the genetic basis of drug resistance in mycobacteria led to the development of molecular methods for detection of mutations associated with dapsone, rifampicin and fluoroquinolone resistance [reviewed in[22,23]]. These molecular methods have proven valuable in the rapid and efficient detection of drug-resistant *M. leprae* derived directly from clinical specimens. All of the current molecular methods for drug susceptibility testing of *M. leprae* are based on PCR amplification of *M. leprae* DNA regions containing the DRDRs of gene targets (*folP1, rpoB* and *gyrA*) for subsequent mutation detection (Figure 1A). Assays can be performed on purified DNA or crude biological specimens (e.g., skin biopsy specimens or skin slit smears). Most laboratories use direct DNA sequencing of PCR amplicons containing DRDRs (described below) to detect mutations causing resistance. Other non DNA sequencing-based assays have been also developed[24–26] and are described below.

MUTATION DETECTION BY PCR/DIRECT DNA SEQUENCING

Several laboratories have shown the association of mutations in the *M. leprae* DRDRs of anti-leprosy drug targets *folP1, rpoB,* and *gyrA* using PCR/direct DNA sequencing (Table 1). In addition, the majority of these sequenced mutants have been confirmed with the MFP assay.

In 2008 WHO recommended guidelines for global surveillance of drug-resistant *M. leprae* using PCR-direct sequencing.[65] These guidelines included: 1) DNA isolation from skin biopsy of MB relapse patients using DNeasy Kit (Qiagen, Germantown, MD); 2) PCR amplification of the appropriate target DNA fragments containing DRDRs of *M. leprae* using specific primers (Figure 1A); 3) automated DNA sequencing of these fragments with both forward and reverse primers; and 4) alignment of generated sequences to that of reference DRDR sequences in the *M. leprae* TN strain (NC002677GenBank) to determine the presence of drug-resistant mutations.

Figure 1B demonstrates representative partial alignments of sequences of the genomic *M. leprae* TN strain and mutant strains showing the mutations most frequently found associated with rifampicin-, dapsone- and ofloxacin-resistant *M. leprae* (Table 1). For example, the most frequently detected mutation associated with rifampin resistance in *M. leprae* is TCG → TTG in codon 425 of *rpoB,* resulting in the substitution of a leucine amino acid residue for a serine residue (Ser425Leu) in the β-subunit of the RNA polymerase (Table 1, Figure 1B). The most frequently detected mutation associated with dapsone resistance in *M. leprae* is CCC → CTC in codon 55 of *folP1* resulting in the substitution of leucine for a proline residue (Pro55Leu) in the DHPS. The most frequently detected mutation associated with ofloxacin resistance in *M. leprae* is GCA → GTA in codon 91 of *gyrA* resulting in the substitution of valine for alanine (Ala91Val) in the α-subunit of the DNA gyrase.

As DNA sequencing has become routine in more laboratories around the world it has become the new 'gold standard' for drug susceptibility testing for leprosy. However, other assays have been developed recently for laboratories unable to perform DNA sequencing.

MUTATION DETECTION BY LEPROSY DRUG SUSCEPTIBILITY-DNA ARRAY (LDS-DA)

DNA array technology has been exploited to develop a reverse hybridisation method LDS- DA to detect mutations in the genome of *M. leprae* that confer resistance to key drugs for leprosy.[24] Briefly, this assay is performed using the following steps: 1) multiplex PCR to simultaneously amplify the DRDRs of target genes from DNA of clinical specimens and label the amplicons with biotin; 2) PCR amplicons are heat denatured and quickly chilled; 3) the chilled mixture is hybridized to the LDS-DA slide containing a series of bound oligonucleotide probes corresponding to each mutation in the *folP1, rpoB* and *gyrA* genes for dapsone, rifampicin and ofloxacin resistance, respectively; and 4) biotin-labelled DNA fragments hybridize15 to the capture probes on the LDS-DA and are detected by avidin-biotin horseradish peroxidase.

Feasibility studies were conducted to evaluate the performance of the LDS-DA in Myanmar and the Philippines.[24] The results of 305 isolates studied showed a high correlation with that of PCR/direct DNA sequencing. Therefore, the LDS-DA is a rapid method for the simultaneous susceptibility testing of two of the three front-line drugs for leprosy and ofloxacin, sometimes used to treat leprosy as well as other common infections.

MUTATION DETECTION BY REAL TIME-PCR- HIGH RESOLUTION MELT (RT-PCR HRM)

To enable wider implementation of molecular drug resistance analyses in leprosy a novel RT- PCR-HRM assay without the need for allele-specific primers, probes or post-PCR sample handling has been developed.[26] This method is based on real-time PCR using primer sets for amplification of the DRDRs in *rpoB, folP1* and *gyrA* with subsequent mutation detection using HRM analysis. Briefly, PCR primers generate labelled products <200 bp for each DRDR RT-PCR. After PCR amplification there is a hetero-duplex formation step and a melt curve for each product generated. Post-PCR HRM analysis of the melt curves is performed identifying wild-type and mutant *M. leprae*. In addition to identifying homologous susceptible or resistant *M. leprae* populations, RT-PCR-HRM analyses aided in recognising samples with mixed or minor alleles. When tested in 121 sequence-characterised clinical strains, HRM identified all the *folP1* mutants representing two mutation types, including one not within the reference panel but associated with dapsone resistance.[26] False positives ($<5\%$) were attributed to low DNA concentrations or PCR inhibition. The authors concluded that the RT-PCR-HRM is a sensitive, simple, rapid, and high-throughput tool for routine screening of new and relapsed cases and may aid in the detection of minor mutant alleles associated with drug resistance in a population of *M. leprae* that are fully susceptible.

MUTATION DETECTION BY GENOTYPE LEPRAE-DR

The new commercially available **DNA·**STRIP® test (GenoType Leprae-DR from Hain Lifescience, Nehren, Germany) permits the simultaneous detection of *M. leprae* and its resistance to rifampin, dapsone and ofloxacin.[25,74] This assay is performed as follows: 1) DNA is isolated; 2) DRDRs of *M. leprae* target genes are amplified by PCR; 3) amplicons are chemically denatured; 4) single-stranded amplicons are bound to the complementary analogue probes during hybridisation with a **DNA·**STRIP® coated with specific mutant and wild type probes; 5) unbound amplicons are removed by washing; 6) a conjugate reaction is performed during which bound amplicons are marked with the enzyme alkaline phosphatase; and 7) wild-type or mutant DRDRs are then made visible in a colorimetric detection reaction.

A feasibility study was conducted to determine the effectiveness of this assay to detect antibiotic-resistant leprosy.[25] Among 120 *M. leprae* strains previously analysed for resistance by mouse footpad drug susceptibility assay, 16 were resistant to rifampin, 22 resistant to dapsone and four resistant to ofloxacin. The GenoType Leprae DR assay was 100% concordant with DNA sequencing and the MFP assay for DRDRs encoding most of the major mutations in *rpoB*, *folP1* and *gyrA*. Two of the susceptible strains, as determined by DNA sequencing and MFP assays for rifampin resistance, had discordant GenoType Leprae DR results. This was due to the presence of mutations within a codon in these strains that does not induce rifampin resistance in *M. leprae*. The authors concluded that the test is easy to perform and highly specific for detection of drug resistance in leprosy.

Conclusion

Although drug resistance among new cases appears to be rare, reports of single and multi-drug-resistant *M. leprae* among relapse patients continue to appear in the literature. Since the

magnitude of resistance at the global level remains unclear, monitoring of drug resistance in leprosy is especially important. The understanding of drug resistance in *M. leprae* has led to the development of many different assays for its detection. The PCR/direct DNA sequencing assay is currently the choice of laboratories around the world for detecting drug-resistant strains of *M. leprae*. Other molecular assays, not requiring DNA sequencing, have been developed and show promise for labs unable to perform DNA sequencing. It is anticipated that these new assays may evolve into much needed low cost, point-of-care diagnostic tools for monitoring drug resistance in leprosy.

References

[1] Ji B. Treatment of leprosy. In: Gangadharam PR, Jenkins PA (eds). *Mycobacteria:* Vol II *Chemotherapy*. Chapman & Hall Medical Microbiology Series, International Thomson Publishing Co., New York, 1998; pp. 398–424.

[2] World Health Organization Study Group. Chemotherapy of leprosy for control programmes. *WHO Technical Report Series*, 1982; **675**.

[3] World Health Organization. Global strategy for further reducing the leprosy burden and sustaining leprosy control activities: plan period 2006_2010. 2006 [Internet]: [Cited 2012 August 23]. Available at: http://www.searo.who.int/LinkFiles/Guidelines_1-Global_Strategy_Plan_period_06-10.pdf

[4] World Health Organization. Surveillance of drug resistance in leprosy: 2010. *Wkly Epidemiol Rec*, 2011; **86**: 237–240.

[5] WHO MDT Effectiveness FAQ [Internet]: [cited 2012 August 23]. Available from: http://www.who.int/lep/mdt/effectiveness/en/index.html

[6] Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clin Microbiol Rev*, 1995; **8**: 496–514.

[7] Menzies D, Benedetti A, Paydar A *et al.* Effect of duration and intermittency of rifampin on tuberculosis treatment outcomes: a systematic review and meta-analysis. *PLoS Med*, 2009; **6**: e1000146.

[8] Jamet P, Ji B *et al.* Relapse after long-term follow up of multibacillary patients treated by WHO multidrug regimen. *Int J Lepr Other Mycobact Dis*, 1995; **63**: 195–201.

[9] Chen XS, Li WZ, Jiang C, Ye GY. Studies on risk of leprosy relapses in China: relapses after treatment with multidrug therapy. *Int J Lepr Other Mycobact Dis*, 1999; **67**: 379–387.

[10] Cambau E, Bonnafous P, Perani E *et al.* Molecular detection of rifampin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. *Clin Infect Dis*, 2002; **34**: 39–45.

[11] Cellona RV, Balagon MF, dela Cruz EC *et al.* Long-term efficacy of 2 year WHO multiple drug therapy (MDT) in multibacillary (MB) leprosy patients. *Int J Lepr Other Mycobact Dis*, 2003; **71**: 308–319.

[12] Norman G, Joseph G, Richard J. Relapses in multibacillary patients treated with multi-drug therapy until smear negativity: findings after twenty years. *Int J Lepr Other Mycobact Dis*, 2004; **72**: 1–7.

[13] Gelber RH, Balagon VF, Cellona RV. The relapse rate in MB leprosy patients treated with 2-years of WHO-MDT is not low. *Int J Lepr Other Mycobact Dis*, 2004; **72**: 493–500.

[14] Poojabylaiah M, Marne RB, Varikkodan R *et al.* Relapses in multibacillary leprosy patients after multidrug therapy. *Lepr Rev*, 2008; **79**: 320–324.

[15] Pettit JHS, Rees RJW. Sulphone resistance in leprosy: an experimental and clinical study. *Lancet*, 1964; **284**: 673–674.

[16] Pearson TMH, Rees RTW, Waters MFR. Sulphone resistance in leprosy: a review of one hundred proven clinical cases. *Lancet*, 1975; **306**: 69–72.

[17] Ji BH. Drug resistance in leprosy—a review. *Lep Rev*, 1985; **56**: 265–278.

[18] Shepard CC, Rees RJ, Levy L *et al.* Susceptibility of strains of *Mycobacterium leprae* isolated prior to 1977 from patients with previously untreated lepromatous leprosy. *Int J Lepr Other Mycobact Dis*, 1986; **54**: 11–15.

[19] Butlin CR, Neupane KD, Failbus SS. Drug resistance in Nepali leprosy patients. *Int J Lepr Other Mycobact Dis*, 1996; **64**: 136–141.

[20] Namisato M, Matsuoka M, Kashiwabara Y *et al.* Drug resistance in the treatment of leprosy: study in the relapsed cases found in Japanese leprosaria. *Jpn J Natl Med Serv*, 2002; **56**: 331–337.

[21] Ebenezer GJ, Norman G, Joseph GA *et al.* Drug resistant *Mycobacterium leprae*—results of mouse footpad studies from a laboratory in south India. *Indian J Lepr*, 2002; **4**: 301–312.

[22] Scollard D, Adams LB, Gillis TP *et al.* Continuing Challenges of Leprosy. *Clin Microbiol Rev*, 2006; **19**: 338–382.

[23] Matsuoka M. Drug resistance in leprosy. *Jpn J Infect Dis*, 2010; **63**: 1–7.

[24] Matsuoka LM, Khin M, Kyaw SD *et al.* 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*, 2008; **57**: 1213–1219.

[25] Cambau E, Chaufflour-Nejevans A, Tejmar-Kolar L *et al.* Detection of Antibiotic Resistance in Leprosy Using GenoType LepraeDR, a Novel Ready-To-Use Molecular Test. *PLoS Negl Trop Dis*, 2012; **6**: e1739. Epub 2012 Jul 31.
[26] Li W, Matsuoka M, Kai M *et al.* Real-time PCR and high-resolution melt analysis for rapid detection of *Mycobacterium leprae* drug resistance mutations and strain types. *J Clin Microbiol*, 2012; **50**: 742–753.
[27] Grosset JH, Guelpa-Lauras CC, Bobin P *et al.* Study of 39 documented relapses of multibacillary leprosy after treatment with rifampin. *Int J Lepr Other Mycobact Dis*, 1989; **57**: 607–614.
[28] Lowe J. Treatment of leprosy with diamino-diphenylsulfone by mouth. *Lancet*, 1950; **1**: 145–150.
[29] Barrett DF. Lepromatous leprosy: dapsone resistance. *Proc R Soc Med*, 1976; Jun; **69**: 391–392.
[30] Pearson JM, Cap JA, Haile GS, Rees RJ. Dapsone-resistant leprosy and its implications for leprosy control programmes. *Lepr Rev*, 1977; **48**: 83–94.
[31] Shepard CC, Chang YT. Effect of several anti-leprosy drugs on multiplication of human leprosy bacilli in footpads of mice. *Proc Soc Exp Biol Med*, 1962; **109**: 636–638.
[32] Levy L, Shepard CC, Fasal P. The bactericidal effect of rifampicin on *M. leprae* in man: a) single doses of 600, 900 and 1200 mg; and b) daily doses of 300 mg. *Int J Lepr Other Mycobact Dis*, 1976; **44**: 183–187.
[33] Browne SG, Hogerzeil LM. B663 in the treatment of leprosy. Preliminary report of a pilot trial. *Lepr Rev*, 1962; **33**: 6–10.
[34] WHO expert committee on leprosy, seventh report Technical report series **874**, WHO 1998. [Internet]. [Cited 2012 August 23]. Available at: http://www.searo.who.int/LinkFiles/Reports_2-Leprosy_7th_Geneva1998.pdf
[35] Dallas WS, Gowen JE, Ray PH *et al.* Cloning, sequencing, and enhanced expression of the dihydropteroate synthase gene of *Escherichia coli* MC4100. *J Bacteriol*, 1992; **74**: 5961–5970.
[36] Richey DP, Brown GM. The biosynthesis of folic acid. IX. Purification and properties of the enzymes required for the formation of dihydropteroic acid. *J Biol Chem*, 1969; **244**: 1582–1592.
[37] Seydel JK, Richter M, Wempe E. Mechanism of action of the folate blocker diaminodiphenylsulfone (dapsone, DDS) studied in *E. coli* cell-free enzyme extracts in comparison to sulfonamides (SA). *Int J Lepr Other Mycobact Dis*, 1980; **48**: 18–29.
[38] Kulkarni VM, Seydel JD. Inhibitory activity and mode of action of diaminodiphenylsulfone in cell-free folate-synthesizing systems prepared from *Mycobacterium lufu* and *Mycobacterium leprae*. A comparison. *Chemotherapy*, 1983; **29**: 58–67.
[39] Williams DL, Spring L, Harris E *et al.* Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. *Antimicrob Agents Chemother*, 2000; **44**: 1530–1537.
[40] Jin DJ, Gross CA. Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *J Mol Biol*, 1988; **202**: 45–58.
[41] Telenti A, Imboden P, Marchesi F *et al.* Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet*, 1993; **341**: 647–650.
[42] Jamet P, Traore I, Husser JA, Ji B. Short-term trial of clofazimine in previously untreated lepromatous leprosy. *Int J Lepr Other Mycobact Dis*, 1992; **60**: 542–548.
[43] Ji B, Perani EG, Petinom C *et al.* Clinical trial of ofloxacin alone and in combination with dapsone plus clofazimine for treatment of lepromatous leprosy. *Antimicrob Agents Chemother*, 1994; **38**: 662–667.
[44] De Bruyn EE, Steel HC, Van Rensburg EF, Anderson R. The riminophenazines, clofazimine and B669, inhibit potassium transport in gram-positive bacteria by a lysophospholipid-dependent mechanism. *J Antimicrob Chemother*, 1996; **38**: 349–362.
[45] Grosset JH, Ji BH, Guelpa-Lauras CC *et al.* Clinical trial of pefloxacin and ofloxacin in the treatment of lepromatous leprosy. *Int J Lepr Other Mycobact Dis*, 1990; **58**: 281–295.
[46] Franzblau SG, White KE. Comparative in vitro activities of 20 fluoroquinolones against Mycobacterium leprae. *Antimicrob Agents Chemother*, 1990; **34**: 229–231.
[47] Ji B, Grosset J. Ofloxacin for the treatment of leprosy. *Acta Lepra*, 1991; **7**: 321–326.
[48] Gelber RH, Iranmanesh A, Murray L *et al.* Activities of various quinolone antibiotics against *Mycobacterium leprae* in infected mice. *Antimicrob Agents Chemother*, 1992; **36**: 2544–2547.
[49] Ji B, Sow S, Perani E *et al.* Bactericidal activity of a single-dose combination of ofloxacin plus minocycline, with or without rifampin, against *Mycobacterium leprae* in mice and in lepromatous patients. *Antimicrob Agents Chemother*, 1968; **42**: 1115–1120.
[50] Fajardo TT, Villahermosa LG, Cruz EC *et al.* A clinical trial of pefloxacin and ofloxacin in lepromatous leprosy. *Lepr Rev*, 2004; **75**: 389–397.
[51] Drlica K, Xu C, Wang JY *et al.* Fluoroquinolone action in mycobacteria: similarity with effects in Escherichia coli and detection by cell lysate viscosity. *Antimicrob Agents Chemother*, 1996; **40**: 1594–1599.
[52] Takiff HE, Salazar L, Guerrero C *et al.* Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. *Antimicrob Agents Chemother*, 1994; **38**: 773–780.
[53] Cambau E, Sougakoff W, Jarlier V. Amplification and nucleotide sequence of the quinolone resistance-determining region in the *gyrA* gene of mycobacteria. *FEMS Microbiol Lett*, 1994; **116**: 49–54.
[54] Ji B, Jamet P, Perani EG *et al.* Powerful bactericidal activities of clarithromycin and minocycline against *Mycobacterium leprae* in lepromatous leprosy. *J Infect Dis.*, 1993; **168**: 188–190.

[55] Chan GP, Garcia-Ignacio BY, Chavez VE *et al.* Clinical trial of clarithromycin for lepromatous leprosy. *Antimicrob Agents Chemother*, 1994; **38**: 515–517.
[56] Gelber RH. Successful treatment of a lepromatous patient with clarithromycin. *Int J Lepr Other Mycobact Dis*, 1995; **63**: 113–115.
[57] Vester B, Douthwaite S. Macrolide resistance conferred by base substitutions in 23S rRNA. *Antimicrob Agents Chemother*, 2001; **45**: 1–12.
[58] Wallace RJ, Meier A, Brown BA *et al.* Genetic basis for clarithromycin resistance among isolates of *Mycobacterium chelonae* and *Mycobacterium abscessus*. *Antimicrob Agents Chemother*, 1996; **40**: 1676–1681.
[59] Meier A, Heifets L, Wallace RJ *et al.* Molecular mechanisms of clarithromycin resistance in *Mycobacterium avium*: observation of multiple 23S rDNA mutations in a clonal population. *J Infect Dis*, 1996; **174**: 354–360.
[60] You EY, Kang TJ, Kim SK *et al.* Mutations in genes related to drug resistance in *Mycobacterium leprae* isolates from leprosy patients in Korea. *J Infect*, 2005; **50**: 6–11.
[61] Gelber RH. Activity of minocycline in *Mycobacterium leprae*-infected mice. *J Infect Dis*, 1987; **156**: 236–239.
[62] Taylor DE, Chau A. Tetracycline resistance mediated by ribosomal protection. *Antimicrob Agents Chemother*, 1996; **40**: 1–5.
[63] Ramu G. Single-dose rifampicin, ofloxacin and minocycline (ROM) therapy for single leprosy lesions. *Lepr Rev*, 1998; **69**: 78–82.
[64] Maninder SS, Shinde SS, Jerajani HR, Boivin JF. Is there a role for ROM therapy in the treatment of leprosy? Systematic review and meta-analysis. *J Trop Med and Int Health*, 2011; **16**: 1541–1551.
[65] World Health Organization (2009). Guidelines for Global Surveillance of Drug Resistance in Leprosy. EW-GLP 2009. [Internet]: [cited 2012 August 23]. Available from: http://www.searo.who.int/catalogue/2005-2011/pdf/leprosy/sea-glp-2009.2.pdf
[66] Matsuoka M, Budiawan T, Aye KS *et al.* The frequency of drug resistance mutations in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from Myanmar, Indonesia and the Philippines. *Lepr Rev*, 2007; **78**: 343–352.
[67] Matsuoka M, Suzuki Y, Estrada Garcia I *et al.* Possible mode of emergence for drug resistant leprosy revealed by an analysis of samples from Mexico. *Jpn J Infect Dis*, 2010; **64**: 412–416.
[68] Kai M, Nguyen NH, Nguyen HA *et al.* Analysis of drug-resistant strains of *Mycobacterium leprae* in an endemic area of Vietnam. *CID*, 2011; **52**: e127.
[69] Singh P, Busso P, Paniz-Mondolfi A *et al.* Molecular drug susceptibility testing and genotyping of *Mycobacterium leprae* strains from South America. *Antimicrob Agents Chemother*, 2011; **55**: 2971–2973.
[70] Franzblau SG. Drug susceptibility testing of *Mycobacterium leprae* in the BACTEC 460 system. *Antimicrob Agents Chemother*, 1989; **33**: 2115–2117.
[71] Franzblau SG, Biswas AN, Jenner P, Colston MJ. Double-blind evaluation of BACTEC and Buddemeyer-type radiorespirometric assays for in vitro screening of antileprosy agents. *Lepr Rev*, 1992; **63**: 125–133.
[72] Cole ST, Eiglmeier K, Parkhill J *et al.* Massive gene decay in the leprosy bacillus. *Nature*, 2001; **409**: 1007–1011.
[73] Eiglmeier K, Parkhill P, Honore N *et al.* The decaying genome of *Mycobacterium leprae*. *Lepr Rev*, 2001; **72**: 387–398.
[74] GenoType LepraeDR, Hain Lifesciences. [Internet]: [citied 2012 August 23] Available from: http://www.hain-lifescience.de/uploadfiles/file/produkte/mikrobiologie/mykobakterien/LepraeDR.pdf
[75] Honore N, Cole ST. Molecular basis of rifampin resistance in *Mycobacterium leprae*. *Antimicrob Agents Chemother*, 1993; **37**: 414–418.
[76] Maeda S, Matsuoka M, Nakata N *et al.* Multi-drug resistant *Mycobacterium leprae* from patients with leprosy. *Antimicrob Agents Chemother*, 2001; **45**: 3635–3639.
[77] Honore N, Roche P, Grosset JH, Cole ST. A method for rapid detection of rifampin resistant isolates of Mycobacterium leprae. *Lepr Rev*, 2001; **72**: 441–448.
[78] Williams DL, Waguespack C, Eisenach K *et al.* Characterization of rifampin resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother*, 1994; **38**: 2380–2386.
[79] Cambau E, Perani E, Guillemin I *et al.* Multidrug resistance to dapsone, rifampin, andofloxacin in *Mycobacterium leprae*. *Lancet*, 1997; **349**: 103–104.
[80] da Silva Rocha A, Cunha MD, Diniz LM *et al.* Drug and multiple-drug resistance among *Mycobacterium leprae* isolates from Brazilian relapsed leprosy patients. *J Clin Microbiol*, 2012; **50**: 1912–1917.
[81] Cambau E, Perani E, Guillemin I *et al.* Multi-drug resistant to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. *Lancet*, 2005; **340**: 103–104.
[82] Kai M, Matsuoka M, Nakata N *et al.* Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. *FEMS Microbiol Lett*, 1999; **177**: 231–235.
[83] Williams DL, Pittman TL, Gillis TP *et al.* Simultaneous detection of *Mycobacterium leprae* and its susceptibility to dapsone using DNA heteroduplex analysis. *J Clin Microbiol*, 2001; **39**: 2083–2088.
[84] Cambau E, Carthagena L, Chauffour AJ *et al.* Dihydroptheroate synthase mutations in the *folP1* gene predict dapsone in relapsed cases of leprosy. *CID*, 2006; **42**: 238–241.