

Drug-resistant leprosy: Monitoring and current status

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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

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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 1982² 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 alone²⁷ 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 leprosy²⁸ 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 clofazimine³³ 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-azobenzene^{4'}-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/ <i>rpoB</i>	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	<i>Ser425Leu</i>	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/ <i>folP1</i>	R	Thr53Ala	14(18)
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		<i>Pro55Leu</i>	23(30)	[82,83,76,66,68,84]
R		Pro55Ser	2(3)	[22]
Ofloxacin/ <i>gyrA</i>		NC	Gly89Cys	1(8)
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 cgc 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 tog 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 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
 ccg gcg gcg ctg caa agc ggc gca ccg atc gtc aac gat gtg tct ggt gg

WHO *M. leprae* gyrA DRDR region

at ggt ctc aaa ccg gta cat cgt ccg gtc ttg tac gcg atg tta gac tcc
 ggt ttc cgc ccg gac cgt agc cac gct aag tca gca ccg 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 ccg 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 1994⁵³ 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 23S rRNA 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 dapson 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 dapson-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 10⁷ and 10⁸,⁶ 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 (10¹¹ *M. leprae*), it is feasible that a patient could contain up to 105 dapson-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 dapson 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 dapson 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}C -CO₂ 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 hybridize 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.

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