A method for rapid detection of rifampicin-resistant isolates of *Mycobacterium leprae*

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Summary A genotypic method for predicting rifampicin resistance in *Mycobacterium leprae* has been developed and rigorously tested on mouse footpad-derived and clinical specimens. A series of immobilized oligonucleotide capture probes can discriminate between wild type and mutant *rpoB* alleles, and positive controls are available for the most frequent mutation affecting Ser425. Two different non-radioactive detection formats have been tested with comparable success in both an industrialized and a developing country. The standardized procedure could now be used in a prospective study of potential rifampicin resistance among multibacillary patients.

Introduction

Rifampicin is the backbone of the multidrug therapy currently used to treat leprosy^{1,2} and it, or a related rifamycin, will almost certainly be included in any new regimens that may be developed.³ Fortunately, resistance to rifampicin is rare and has only been documented in a limited number of lepromatous patients, mostly receiving monotherapy or other inadequate treatment.⁴ On characterization, all rifampicin resistant isolates of *Mycobacterium leprae* were found to harbour mis-sense mutations in a short segment of the *rpoB* gene, encoding the β -subunit of DNA-dependent RNA polymerase.^{5–8} The same region, termed RIF-RDR has been shown to be associated with rifampicin resistance in almost all resistant isolates, of *Mycobacterium tuberculosis*,^{7,9–12} and in a range of other bacteria.^{13,14}

Testing *M. leprae* isolates for rifampicin susceptibility is generally performed after inoculation of mice¹⁵ but this is expensive and requires specialized resources that are gradually disappearing from leprosy control programmes. Radio-respirometry is a useful, more rapid alternative¹⁶ but this also needs a sophisticated laboratory infrastructure and

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relatively large numbers of metabolically active organisms that can only be obtained by passage and growth in experimental animals. In recent years, several groups have established predictive molecular methods for detecting rifampicin resistance that are based on the *rpoB* genotype. The first such approach involved single strand conformation polymorphism (SSCP)-PCR,⁶ yielding excellent results within 48 h and giving perfect agreement with those obtained from the mouse. Similar results were subsequently obtained using PCR-facilitated heteroduplex analysis (HDA) of *rpoB*⁷ and this method has also been applied to detecting dapsone resistance.¹⁷ However, both PCR-SSCP and PCR-HDA require gel electrophoresis and sophisticated laboratory equipment and may not be suitable for resource-poor settings. Here we describe the development of a simpler method for predicting rifampicin resistance, involving solid-phase hybridization to oligonucleotide capture probes, and its application in the field.

Materials and methods

MEMBRANE PREPARATION

Oligonucleotides corresponding to both strands of the RIF-RDR region were designed (Figure 1, Table 1) then tested empirically for use as capture probes. The best results were obtained with probes corresponding to the complementary strand so these were then used exclusively. Capture probes were synthesized with a 5'-amino group to facilitate covalent linkage to Biotin C membranes and diluted to the optimized concentrations in 350 µl of 500 mM NaHCO₃, pH 8.4. Membranes (9.5 × 7 cm) were activated by incubation in 20 ml freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), with shaking at room temperature for 10 min, rinsed with water and placed in a clean miniblotted system. Diluted oligonucleotide solutions (350 µl) were added to the slots and left for 5 min before removal. The membrane was removed from the miniblotted, inactivated in 100 mM NaOH for 10 min, then washed in 2 × SSPE/0.1% SDS for 5 min at 60°C. After washing in 20 mM EDTA pH 8, for 15 min, membranes were stored at 4°C in Saranwrap, then cut into appropriate strips before use.

Figure 1. Organisation and sequence of the RIF-RDR of *rpoB* from *M. leprae* and location of capture probes. The upper part shows the DNA sequence and predicted protein sequence together with mutations known or suspected to confer rifampicin resistance. The lower part shows the position of the probes discussed in the text; their sequences may be found in Table 1.

Table 1. Oligonucleotides used in this study*

For PCR and sequencing	
Brpo22	bCAGGACGTCGAGGCGATCAC
rpo32	TCCTCGTCAGCGGTCAAGTA
rpo46	TCGATCGGGCACATCCGGC
Capture probes	
S1	aCCATGAACTGCGACAGCTGGCTG
S2	aGGGTTGTTCTGATCCATGAACTG
S3	aTGGGTCAGGCCCGACAGAGGGT
S4	aGACAGCCGGCGCTTGTGGGTCA
A27	aAAAGGGCCCAGCGCCGACAG
A30R1	aAAAGGGCCCAGCGCCAACAG
A32R3	aAAAGGGCCCAGCGCCATCAG
A33R9	aAAAGGGCCCAGCGCGAACAG
A34	aAAACGACAGCCGGCGCTTGT
A35	aAAAGCTTGTGGGTCAGGCC
A36	aAAAAGGCCCGACAGAGGGTT
A37	aAAAAGGGTTGTTCTGATCCA
A38	aAAAGATCCATGAACTGCGAC
A39	aAAATGCGACAGCTGGCTGGT

a, denotes the position of the 5'-amino link group; b, denotes biotin.

PREPARATION OF BIOTINYLATED PROBES AND DNA SEQUENCING

PCR was performed using the primers B-rpo22 and rpo32 (Table 1), where B-rpo22 is biotinylated, with samples of *M. leprae* DNA prepared by the freeze-boiling method,¹⁸ as described previously.⁵ For control purposes, plasmids carrying cloned RIF-RDR from wild type and three rifampicin resistant isolates of *M. leprae* were used in PCR reactions. In some experiments, DNA sequencing was performed to confirm the presence of mutations predicted by the kit. Primers B-rpo22 and rpo32 were used to generate a PCR fragment spanning the RIF-RDR, which was then sequenced using primer rpoB46 (Table 1) and fluorescent big dye terminators. Sequences were obtained using an ABI373 automated sequencer.

HYBRIDIZATION AND DETECTION

This requires the following prewarmed buffers (20°C, unless specified otherwise): denaturation buffer (DB) 2 M NaOH/2 mM EDTA; hybridization buffer (HB) 6×SSPE/0.1% SDS, 50°C; washing buffer (WB1) 4×SSPE/0.1% SDS, 55°C, and 42°C; washing buffer (WB2) 2×SSPE/0.1% SDS; washing buffer (WB3) 2×SSPE; staining buffer (SB) 0.1 M Tris-buffer pH 9.5/0.05 M MgCl₂, 0.1 M NaCl. PCR fragments were denatured in an equal volume of DB for 5 min then added to prewarmed HB (1 ml, 55°C), and incubated with the strips, in a shaking water-bath at 50°C, for 30–60 min. After discarding the hybridization solution and washing the strips with 2 ml of prewarmed WB1 for 15 min, the appropriate streptavidin-conjugate in WB1 (2 ml, 42°C) was added and incubated for 30–60 min. Streptavidin-peroxidase conjugate was diluted 1/4000 for chemiluminescent detection and streptavidin-alkaline phosphatase conjugate was diluted 1/1000 for colorimetric detection. After discarding the conjugate solution, strips were washed with 2 ml of WB2 for 10 min

before adding 2 ml of WB3 and washing for 10 min. For chemiluminescent detection, strips were incubated for 2 min in 2 ml ECL detection liquid (Amersham), washed, then covered with Saran-wrap and exposed to X-ray film for 1 min. For colorimetric detection, 2 ml of diluted NBT/BCIP stock solution in SB (200 μ l/10 ml), was added and the strips incubated for 15–60 min.

Results

STANDARDIZING THE METHOD

The aim of this work was to construct and standardize a kit that allows mutations to be detected in the *rpoB* gene of *M. leprae*, and hence to predict whether the corresponding isolates would be sensitive or resistant to rifampicin. The approach involved the synthesis of a series of oligonucleotide capture probes spanning the lower strand of the RIF-RDR, their immobilization on a nylon membrane and use in reverse hybridization experiments. Many oligonucleotide probes of different lengths spanning RIF-RDR were synthesized, bound to the membrane then tested with biotinylated probes prepared from wild type and mutant *rpoB* alleles by PCR. The optimal combination of oligonucleotides, their sequence and location is shown in Figure 1. We found that the best results were obtained by combining four 23-mer probes with six (or seven) 17-mer probes for the bottom strand. Hybridization results were uneven in initial experiments with the shorter probes, probably due to steric hindrance from the membrane resulting in less of the capture probe being available to form hybrids. Consequently, these probes were resynthesized with three additional deoxyadenosine residues at the 5'-end thereby allowing better access of the biotinylated PCR fragment to the 17-mer capture sequence.

To date, only a limited number of mutations responsible for rifampicin resistance have been reported in *M. leprae*.^{5,7} The amino acid substitutions involved are His420Asp (position 525 in *Escherichia coli*), Ser425Leu, Ser425Met and Ser425Phe. For control purposes, 17-mer capture probes (with 5'-adenosine) corresponding to mutant *rpoB* alleles of Ser425 were also synthesized and included on the membranes. This proved particularly useful as the DNA sequence encompassing Ser425 is very GC-rich and readily adopts stable secondary structures that often preclude hybridization.

Typical results obtained with this method are shown in Figure 2. Hybridization to capture probes 1–4 is seen in all cases except when a mutation affecting the codon for His420 is present as probe 4 then displayed no signal (column 5). Mutations affecting this codon can also be detected with probe A35. Probe 4 also covers part of the mutational hot-spot corresponding to codon 425 but does not detect mutations affecting this position probably because the site is too close to the 5'-end of the capture probe for heteroduplex formation to be sufficiently destabilized. However, the shorter probe A27, in combination with the mutant probes A30R1, A32R3 and A33R9, allows all known mutations affecting codon 425 to be detected efficiently (Figure 2, columns 2, 3 and 4). The mutant probes displayed excellent specificity as no signals due to crosshybridization with the wild type *rpoB* gene were obtained. Probes A34 and A37 sometimes displayed weaker signals than the others but the corresponding region are well covered by probes A27 and 4, or 2 and 3, respectively (Figure 1). Mutations were correctly revealed under standard laboratory conditions using either colorimetric or chemiluminescent detection methods.

Figure 2. Detection of rifampicin resistance by reverse hybridization. The results were obtained using the protocol described in Materials and methods with biotinylated probes generated by PCR using *M. leprae* DNA from the following sources: column 1, wild type *M. leprae*; column 2, positive control *rpoB*Ser425Leu; column 3, positive control *rpoB*Ser425Met; column 4, positive control *rpoB*Ser425Phe; column 5, strain 92041 with *rpoB*His420Asp mutation. The capture probes are indicated on the right, for further details see Table 1 or Figure 1.

BLIND TESTING

Having established that the method reproducibly detected the common *rpoB* mutations at our disposal, it was then evaluated blindly on a set of six uncharacterized *M. leprae* isolates. Five of these proved to be wild type for *rpoB* (represented in column 2, Figure 3), but one of them appeared to harbour an *rpoB* mutation as no signal was obtained with capture probes 3 and A36 (column 1, Figure 3). These results were confirmed by DNA sequencing of PCR fragments spanning the RIF-RDR as the missense mutation Ser416Cys (TCG→TGT) was uncovered. Mutation of this residue to Leu and Ser has been reported in rifampicin resistant isolates of *M. tuberculosis* and *E. coli*, respectively.^{9,11,13}

FIELD TESTING

Kits for detecting rifampicin resistance were established in Paris then tested in Kathmandu at the Anandaban Leprosy Hospital. The results obtained there were of comparable quality to those generated in France as were those obtained with membranes produced locally. A potential case of rifampicin resistance was successfully detected in a mouse footpad-derived specimen. The corresponding strain was predicted to harbour a Ser425Phe mutation by solid-phase hybridization and this was confirmed by DNA sequencing. An *M. leprae* isolate harboring the corresponding allele has previously been shown to be drug resistant in the

Figure 3. Detection of rifampicin resistance by reverse hybridization. As stated in legend to Figure 2 except: column 1, strain 25200 with *rpoB*Ser416Cys mutation; column 2, wild type *M. leprae*; column 3, positive control *rpoB*Ser425Leu; column 4, positive control *rpoB*Ser425Met; column 5, positive control *rpoB*Ser425Phe.

mouse model.^{4,5} Having validated the system, attempts were then made to apply it to clinical specimens obtained from leprosy patients.

Skin biopsies were collected from ~60 lepromatous leprosy patients in Nepal and India, stored in 70% ethanol, then transported to the Anandaban Leprosy Hospital for further analysis. Most of these patients had either relapsed or not responded to treatment. After rehydration and mincing, the biopsies were prepared for PCR analysis by freeze-boiling. Only five samples reproducibly yielded PCR fragments and these were used successfully with the chemiluminescent detection system. Two samples from patients who had not responded to treatment were found to be resistant by both methods and harboured the Ser425Phe mutation. The remaining three samples were found to be wild type, or rifampicin-susceptible, by both methods.

Discussion

This study has convincingly demonstrated that detection of rifampicin resistance by molecular methods is a feasible and practical alternative to the mouse footpad assay. Equivalent results were obtained using two different non-radioactive detection systems in laboratories in France and Nepal. There is thus no technical reason why a kit such as that described here should not find more extensive application in developing countries. A similar method has been developed for detecting rifampicin resistant *M. tuberculosis* and is now commercially available.¹⁹ It has yielded satisfactory results in several settings.¹⁰ The availability of the *M. leprae* genome sequence also provides us with means to develop genotypic methods for detecting resistance to other drugs. This could now be done for dapsone and fluoroquinolones and, in the near future for other therapeutic agents as information about the molecular basis of resistance becomes available.

We believe that the time is now right to extend the study and to learn from the present work as this approach has much to offer leprosy control programmes. In the next phase, a much larger number of specimens should be tested. Although every available mutation has been detected by the reverse hybridization system, the number of sites within *rpoB* that can be monitored by positive controls is limited by the restricted set of base changes described.⁵⁻⁷ As a result, the faint possibility that new mutations might be missed by the system cannot yet be excluded and more extensive evaluation is required.

This could be done in the framework of a prospective study of rifampicin resistance in which skin biopsies are examined from ex-multibacillary leprosy patients who have not responded properly to the treatment of MDT. Combined with PCR-based, DNA sequence analysis of the *rpoB* gene from *M. leprae* extracted from the biopsies rigorous appraisal of the reverse hybridization kit would be obtained and valuable information about the level of rifampicin resistance generated. Such a study would also enable us to evaluate, in a systematic manner, the long-term efficacy of multidrug therapy for multidrug-resistant leprosy.

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