Development and evaluation of a novel multiple-primer PCR amplification refractory mutation system for the rapid detection of mutations conferring rifampicin resistance in codon 425 of the rpoB gene of Mycobacterium leprae

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Rifampicin-resistant Mycobacterium leprae is regularly reported and drug resistance is a major threat for the elimination of leprosy. There is an urgent need for a simple method that can detect rifampicin resistance in clinical isolates. This study developed a multiple-primer PCR amplification refractory mutation system, a simple, reliable and economical method for clinical specimens that allowed the rapid detection of mutations in the nucleotides of the codon for Ser425 of the M. leprae rpoB gene, mutation of which to Leu, Met or Phe is associated with rifampicin resistance. The approach involved a multiple-primer PCR in which both mutant-specific and normal sets of primers were included in the reaction. The mutant-specific primer was complementary to the corresponding sequence of the wild-type gene except for one additional deliberate mismatch at the fourth nucleotide from the 3'-OH terminus. A single mismatch has little influence on the yield of PCR products, but if there are two mismatches as a result of mutation at the position being tested, the mutant-specific primer will not function in PCR under appropriate conditions, leading to no yield of PCR product from the mutant allele. The assay was evaluated successfully using a panel of plasmids and M. leprae reference strains carrying the wild-type or known rpoB mutations. The assay was subsequently applied to M. leprae DNA extracts from skin biopsies taken from patients. In all biopsy samples, the wild-type allele was detected for Ser425. The PCR results correlated with rifampicin susceptibility, as also measured by the traditional in vivo mouse footpad technique.

INTRODUCTION

Leprosy is a chronic infectious disease caused by Mycobacterium leprae and is of importance to public health because of the disabilities it causes. Globally, Nepal is one of the six major endemic countries which accounted for 23% of all new cases detected during 2005 and 24% of registered cases at the beginning of 2006 (World Health Organization; http://www.who.int).

Although the World Health Organization recommended multi-drug therapy (MDT) in 1981, the threat of drug resistance remains, as regimen recommendations and diagnostic criteria have been modified several times (Scollard et al., 2006). Several authors have reported resistance after MDT to various concentrations of dapsone, rifampicin or clofazimine using different methods (Ebenezer et al., 2002; Maeda, 2004; Maeda et al., 2001; Norman et al., 2003).

Drug monitoring of rifampicin resistance is done using the mouse footpad (MFP) technique, but this method has a number of limitations: the viable bacteria must be preserved in inocula for long periods of time (MFP inoculation must be done within 72 h as the organism cannot be grown in artificial medium) and the method requires a large number of organisms (5 × 10^6) and is expensive and time-consuming (taking up to 12 months). It would be highly desirable to have a rapid, simple technique for monitoring suspected cases of rifampicin resistance that could be applied directly to clinical specimens.

The molecular basis of rifampicin resistance has been studied extensively in Escherichia coli and has been established in M. leprae (Honoré & Cole, 1993; Honoré...
et al., 1993). Mutations for rifampicin resistance in *M. leprae* are located in a short, highly conserved region called the rifampicin resistance-determining region, affecting codons 401–427 (equivalent to codons 507–533 of *E. coli* and *Mycobacterium tuberculosis*), predominantly involving substitution of Leu for Ser425; substitution of Met and Phe for Ser425 has also been reported (Cambau et al., 2002; Honoré & Cole, 1993; Maeda et al., 2001; Matsuoka et al., 2000; Williams & Gillis, 2004).

Although several different genotyping methods (Sapkota et al., 2006; Suzuki & Matsuoka, 2006; Williams & Gillis, 2004) have been used for analysis of *rpoB* gene mutations associated with rifampicin resistance, these procedures have their own limitations with requirements for sophisticated laboratories and reagents and highly trained personnel.

Here, we describe the development and evaluation of a novel multiple-primer PCR amplification refractory mutation system (MARS) for the rapid detection of mutations in the nucleotides of the Ser425 codon, resulting in mutation to Leu, Met or Phe, within the 81 bp rifampicin resistance-determining region of the *rpoB* gene of *M. leprae* isolates. The majority of rifampicin-resistant mutations in *M. leprae* involve codon 425. The method described here is capable of detecting mutations at this codon, but not other mutations known to be associated with rifampicin resistance, or indeed other potential mutations that could cause resistance.

**METHODS**

*M. leprae rpoB* plasmids, reference strains and clinical specimens. Plasmids carrying the cloned rifampicin resistance-determining region from wild-type and three rifampicin-resistant isolates of *M. leprae* were obtained from the Pasteur Institute (Paris, France). Nudemouse-derived *M. leprae* reference strains were obtained from the Leprosy Research Center (National Institute of Infectious Diseases, Tokyo, Japan). Armadillo-derived whole *M. leprae* and genomic DNA of *M. leprae* were obtained from Colorado State University (USA) (Table 1).

Skin biopsies (3 x 4 mm) were obtained from the most representative new/active lesions of untreated, relapsed and MDT defaulter leprosy patients attending Anandaban Hospital (Kathmandu, Nepal). In all cases, the specimen was taken with informed consent and was approved by the local ethics committee in Nepal. The clinical details of the study population are summarized in Table 2.

**Drug susceptibility testing using the MFP assay.** Biopsies were processed immediately using standard techniques (Ji, 1987; Shepard, 1960). The biopsy sample was cut, minced and hand-ground in 2 ml sterile 0.1% BSA in PBS. Acid-fast bacilli (AFB) in the suspension were counted after staining using the Ziehl–Neelsen hot method on a four-ring spot slide and the total number of AFB per ml of suspension was calculated (Shepard & McRae, 1968). A suspension containing $10^8$ cells in 30 μl was prepared by appropriate dilution of the initial suspension and inoculated into both hind footpads of 20 Swiss mice (Ji, 1987; Shepard, 1960). One loopful of this suspension was inoculated on a Löwenstein–Jensen agar slant to confirm that the AFB were *M. leprae* and not *M. tuberculosis*. Inoculated mice were divided into two groups of ten mice each: an untreated control group and the rifampicin-treated group. Two concentrations of freshly prepared rifampicin (Sigma) were assessed: 5 and 10 mg rifampicin kg$^{-1}$ (five mice in each group). Treatment started 1 week after inoculation and was administered in a volume of 0.4 ml once a week by gavage until harvesting (Grosset et al., 1989).

After 6, 9 and 12 months, control mice were killed and the soft tissue under the skin of the footpad was prepared for AFB enumeration (Shepard, 1960) to evaluate the growth of bacilli. A cut-off value was established to confirm drug resistance. *M. leprae* bacilli were considered to have multiplied when $>10^5$ AFB were observed per footpad in control mice. If no multiplication was observed after 12 months, the drug susceptibility test was deemed to be unsuccessful. Isolates were defined as sensitive when they multiplied in untreated mice but not in any treated mouse, and as resistant when they multiplied in untreated mice and in at least one treated mouse (Grosset et al., 1989; Ji, 2002; Levy & Ji, 2006).

**MARS assay**

**Crude *M. leprae* DNA preparation.** A 0.1 ml aliquot of the biopsy homogenate was transferred to a 1.5 ml Eppendorf tube and subjected to five cold and heat shocks, achieved by snap freezing in

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**Table 1. Evaluation of the MARS assay in reference controls**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Test DNA</th>
<th>Reference control</th>
<th>References</th>
<th>MARS result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Plasmid 65</td>
<td>Wild-type</td>
<td>Honoré et al. (2001)</td>
<td>Wild-type</td>
</tr>
<tr>
<td>2</td>
<td>Plasmid 66</td>
<td>TCG425TTG (S425L)</td>
<td>Honoré et al. (2001)</td>
<td>Mutant</td>
</tr>
<tr>
<td>3</td>
<td>Plasmid 67</td>
<td>TCG425ATG (S425M)</td>
<td>Honoré et al. (2001)</td>
<td>Mutant</td>
</tr>
<tr>
<td>4</td>
<td>Plasmid 68</td>
<td>TCG425TTT (S425F)</td>
<td>Honoré et al. (2001)</td>
<td>Mutant</td>
</tr>
<tr>
<td>6</td>
<td>Japan – 8: Zenso-15</td>
<td>TCG425TTG (S425L)</td>
<td>Matsuoka, NIH, Japan*</td>
<td>Mutant</td>
</tr>
<tr>
<td>7</td>
<td>Japan – 9: Zenso-4</td>
<td>TCG425TTG (S425L)</td>
<td>Maeda et al. (2001)</td>
<td>Mutant</td>
</tr>
<tr>
<td>8</td>
<td>Japan – 3: Izumi</td>
<td>Wild-type</td>
<td>Matsuoka, NIH, Japan*</td>
<td>Wild-type</td>
</tr>
<tr>
<td>9</td>
<td>Japan – 4: Keifu-4</td>
<td>Wild-type</td>
<td>Matsuoka, NIH, Japan*</td>
<td>Wild-type</td>
</tr>
<tr>
<td>10</td>
<td>Armadillo-derived <em>M. leprae</em></td>
<td>Wild-type</td>
<td>Brennan, CSU, NIH Contract†</td>
<td>Wild-type</td>
</tr>
<tr>
<td>11</td>
<td>Genomic <em>M. leprae</em></td>
<td>Wild-type</td>
<td>Brennan, CSU, NIH Contract†</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>

*Dr Masanori Matsuoka, The Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan.
†Professor Dr Patrick Brennan, Colorado State University, National Institute of Health Contract, USA.
liquid nitrogen for 1 min followed by boiling for 1 min at 100 °C in a
dry heating block. The crude lysate was stored in the refrigerator at
4 °C for use as the M. leprae template DNA in PCRs.

**Primers and PCR.** Primers used in this study were designed to
amplify specific products of 390 and 260 bp and flanked the rpoB
sequence of M. leprae (GenBank accession no. AL583923; gene
ML1891: 3537 bp, complementary to nt 274319–277855). The
primers used were: control forward primer (CFP), 5'-CAGGACGTC-
GAGGCGATCAC-3' (nt 1219–1238); M-425 forward primer, 5'-
ACCCACAAGCGCCGGCAGTC-3' (positions 1348–1367); and com-
mon reverse primer (CRP), 5'-TCCTCGTCAGCGGTCAAGTA-3'.

**Table 2. Evaluation of the MARS assay in clinical specimens**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Study no.</th>
<th>BI</th>
<th>Clinical details at the time of biopsy</th>
<th>MFP</th>
<th>MARS assay</th>
<th>Seq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MD-566</td>
<td>4+</td>
<td>1 month MB MDT-treated and defaulter</td>
<td>S</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>DR-625</td>
<td>4+</td>
<td>144 months DDS-treated relapse</td>
<td>S</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>MR-056</td>
<td>1.25+</td>
<td>17 months MB MDT-treated relapse</td>
<td>ND</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>4</td>
<td>DMR-404</td>
<td>6+</td>
<td>29 months DDS- and 13 months MB MDT-treated relapse</td>
<td>S</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>5</td>
<td>MR-768</td>
<td>4+</td>
<td>49 months MB MDT-treated relapse</td>
<td>S</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>6</td>
<td>PR-873</td>
<td>4.5+</td>
<td>5 months PB MDT-treated relapse</td>
<td>S</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

**Rapid detection of rpoB mutation in M. leprae by MARS**

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was stained with ethidium bromide (0.56 μg/ml). DNA served as negative and positive controls, respectively, and *M. leprae* was run at least three times to check the reproducibility of the result for each clinical specimen. Amplicons were analysed by 1.5% agarose gel electrophoresis in 1× Tris/borate/EDTA buffer for 50 min at 145 V. The amplified product was stained with ethidium bromide (0.56 μg/ml), visualized with a UV transilluminator and photographed.

**PCR product sequencing.** In some clinical samples, DNA sequencing was performed to confirm the results. Primers CFP and CRP were used to generate a PCR fragment and for sequencing. All sequencing was done at the Pasteur Institute.

### RESULTS AND DISCUSSION

It is demanding to carry out susceptibility testing before specific chemotherapy in any bacterial infection, but MDT for leprosy is applied without any information on drug resistance, as *M. leprae* has not yet been cultivated on artificial medium. Thus the present work aimed to construct and standardize a rapid PCR assay that allowed mutations to be detected in the nucleotides of the codon for Ser425 of the *rpoB* gene of *M. leprae*, which result in substitution with Leu, Met or Phe (Fig. 1), and to predict whether the corresponding isolates would be sensitive or resistant to rifampicin. The PCR results were compared with those from a conventional MFP assay, which is performed routinely for all untreated and relapsed bacterial-index-positive patients at Anandaban Hospital.

#### Drug susceptibility testing using the MFP assay

The MFP assay was successful (multiplication of *M. leprae* bacilli) for 40/44 biopsies. In the remaining biopsies, obtained from multibacillary (MB) MDT-treated relapsed cases, two showed no growth in the control mice, even after 12 months of inoculation. The MFP assay was not done for the remaining two MB MDT-treated relapsed cases, as these were not obtained within 72 h in the laboratory. The remaining samples were processed for the MFP assay within 30 h of the biopsy being taken from the patient. None of the samples was positive for *M. tuberculosis* in Löwenstein–Jensen slant cultures.

### Evaluation of the MARS assay for analysis of *rpoB* gene mutations in *M. leprae*

Each primer was complementary to the corresponding sequence of the wild-type gene except for one additional deliberate mismatch at the fourth nucleotide (nt 1364 of *rpoB*) from the 3′-OH terminus of the M-425 forward primer. However, if the DNA template contained a mutation in the codon for Ser425, there would be another mismatch and the primer would not bind under appropriate conditions. Thus both fragments (390 and 260 bp) were expected from wild-type templates, whereas the shorter fragment (260 bp) would not be obtained from a mutant. As positive controls, plasmids containing known mutations in the nucleotides of the Ser425 codon were analysed by the MARS assay and the expected results were obtained. Purified genomic DNA from *M. leprae* and DNA extracted from armadillo-derived *M. leprae* were also subjected to the MARS assay and were shown to be wild-type (Table 1 and Fig. 2). Moreover, wild-type and the Ser425 to Leu mutant nude-mouse-derived reference strains from Japan yielded the expected PCR products. This indicated that the optimized MARS assay was sensitive enough to detect mutations in the nucleotides of the Ser425 codon of *M. leprae*.

#### MARS assay in clinical specimens

In this study, patients treated for many years with dapsone monotherapy and subsequently with MDT, and MB MDT relapse patients and defaulters, along with newly diagnosed untreated cases, were evaluated. A total of 44 clinical biopsy samples were analysed using the MARS assay and none were found to carry a mutation in the Ser425 codon of the *rpoB* gene of *M. leprae* associated with rifampicin resistance. Concordant results were also found in the MFP assay for 40/44 cases. For the four remaining MB MDT-treated relapse cases for which the MFP assay was unsuccessful, the MARS assay indicated that these were wild-type, one of which was verified by DNA sequencing. The results of the MARS assay were further confirmed by DNA sequencing in 21/44 clinical cases (Table 2).

This study took advantage of the accessibility to leprosy patients attending Anandaban Hospital for diagnosis and treatment, the availability of an onsite laboratory for the PCR-based assay and a well-established animal facility for MFP assays. The MARS assay designed here was specific for the Ser425 codon, which is the most reported region associated with rifampicin resistance in *M. leprae*. The assay was found to be rapid and easy to perform with conventional PCR and agarose gel electrophoresis equipment, and the results were easy to interpret. An important practical consideration with this approach is that it is not necessary to prepare high-quality DNA. A prerequisite for MARS is the absence of a 3′-exonuclease proofreading activity associated with the DNA polymerase employed. Another requirement in the application of the assay is that
3′-OH terminal-mismatched primers are refractory to extension by the chosen DNA polymerase (Fan et al., 2003; Newton et al., 1989).

The PCR-based assay convincingly demonstrated that detection of rifampicin resistance by this method is a feasible and practical alternative to the MFP assay and has practical application in developing countries where the leprosy burden is relatively high. Although a major limitation to molecular genetic detection of drug resistance by any technique is that molecular genetic tests detect only known mutations, it provides a rapid screening tool for the majority of resistant isolates, which, in turn, allows a reduction in the amount of phenotypic drug susceptibility testing. A major limitation of the MARS assay as it is currently designed is that it is only capable of detecting mutations at codon 425 in the rpoB gene of M. leprae, and does not detect other mutations known to be associated with rifampicin resistance, or indeed other potential mutations that could cause resistance. Furthermore, as this test detects only nucleotide mutations, it cannot distinguish silent amino acid changes from those that result in amino acid substitution, although no silent mutation has been reported to date at the codon 425 position in the rpoB gene of M. leprae. However, the rapidity and ease of interpretation of this PCR assay compared with other molecular assays and the MFP assay is an important finding and supports the potential use of this assay. The results suggest that the MARS assay is rapid and simple to implement and could be performed for detecting rifampicin-resistant M. leprae.

Finally, these results indicate that this technique can reliably identify rifampicin-resistant strains of M. leprae with mutations of Ser425 to Leu, Met or Phe. The assay is relatively simple and further studies are highly recommended to address the issues of rifampicin resistance in
other reported positions and in multi-drug resistance in leprosy.

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