Mutation at codon 442 in the rpoB gene of *Mycobacterium leprae* does not confer resistance to rifampicin


#Equally Contributed

*Stanley Browne Laboratory, TLM Community Hospital, Shahdara, New Delhi 110093

**Department of Biochemistry, Institute of Post Graduate Medical Education & Research, 244B AJC Bose Road, Kolkata, 700020, India

***Department of Dermatology and Venerology, School of Tropical Medicine, 108, C R Avenue, Kolkata-700073, India

****Department of Animal House, National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Taj Ganj, Agra 282001, India

*****Department of Physiotherapy, TLM Champa and TLM Shahdara

******Department of Biochemistry, Pt. Jawahar Lal Nehru Memorial Medical College, Raipur 492001, Chattisgarh, India

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Summary

*Background:* Rifampicin is the major drug in the treatment of leprosy. The rifampicin resistance of *Mycobacterium leprae* results from a mutation in the rpoB gene, encoding the β subunit of RNA polymerase. As *M. leprae* is a non-cultivable organism observation of its growth using mouse food-pad (MFP) is the only Gold Standard assay used for confirmation of “in-vivo” drug resistance.

*Objective:* Any mutation at molecular level has to be verified by MFP assay for final confirmation of drug resistance in leprosy.
Material and methods: In the present study, *M. lepraee* strains showing a mutation only at codon 442 Gln-His and along with mutation either at codon 424 Val-Gly or at 438 Gln-Val within the Rifampicin Resistance Determining Region (RRDR) confirmed by DNA sequencing and by high resolution melting (HRM) analysis were subjected for its growth in MFP.

Result and Conclusion: The *M. lepraee* strain having the new mutation at codon 442 Gln-His was found to be sensitive to all the three drugs and strains having additional mutations at 424 Val-Gly and 438 Gln-Val were conferring resistance with Multi drug therapy (MDT) in MFP.

These results indicate that MFP is the gold standard method for confirming the mutations detected by molecular techniques.

Keywords: Drug Resistance, Leprosy, Multi Drug Therapy, RRDR

Introduction

Currently, leprosy treatment and control is based on World Health Organization (WHO)-recommended multidrug therapy (MDT) and is in use for treatment of leprosy for the last 29 years. Using MDT the prevalence of leprosy has come down drastically all over the world. It has been noted from earlier experience that any therapeutic control measure for prevention of disease with antibiotics ultimately leads to emergence of drug resistance.\(^1\sim4\) Therefore, a surveillance mechanism should function as a ‘watch dog’ for identification of drug resistance. India had been the home for >60% of the leprosy patients worldwide and since the implementation of effective MDT in 1985 despite the massive decline in global prevalence of the disease the scenario during elimination remained unchanged. At this juncture, if the emergence of mutation in *M. lepraee* to rifampicin, the only bactericidal drug, is not critically checked by clinical, bacteriological and laboratory investigations of relapsed or MDT unresponsive patients then the control measure with the present MDT regimen will be completely defeated. Although MDT has been successful in reducing the huge number of leprosy cases in the world however, the number of registered cases and new cases amounting to 210,000 and 250,000 respectively, has remained almost steady.\(^5\) In addition, it is being noted that new cases are appearing almost at the same rate as earlier.\(^6\)

Rifampicin, a major drug used for treatment of tuberculosis and leprosy, has a molecular mechanism of antibacterial activity involving the inhibition of DNA-dependent RNA polymerase of the organism.\(^7\) This enzyme is a complex oligomer comprised of four subunits \(\alpha, \beta, \beta', \text{and} \sigma\) encoded by \(rpoA, rpoB, rpoC,\) and \(rpoD\) regions of gene respectively. Mutations in the \(rpoB\) gene, encoding the \(\beta\) subunit of RNA polymerase result in resistance to rifampin in several mycobacterial species including *M. lepraee* and *M. tuberculosis*.\(^1\sim2\) As *M. lepraee* is uncultivable *in vitro*, the laboratory test for confirmation is based on growth of the bacilli in mouse footpad (MFP). However, MFP assay to determine the susceptibility of *M. lepraee* to drug takes 6 months to one year.\(^8\)

The major mutations that confer rifampicin resistance to *M. lepraee* are located in the 81-bp core region of the \(rpoB\) gene, known as the Rifampicin Resistance Determining Region (RRDR).\(^1\) To date, a total of nine point mutations (401, 407, 408, 409, 410, 416, 420, 425 and 427) within the RRDR \(rpoB\) gene of *M. lepraee* have been reported.\(^9\) Mutations at codon 407, 410, 420, 425 (456 now) and insertions between 408 and 409 have been confirmed to be
associated with rifampicin resistance and were also supported by MFP studies.\textsuperscript{1,3,8–13} However, some other mutations at codons 401, 416, and 427 in the 81-bp region have not yet been confirmed by MFP assay and hence are not considered responsible for rifampicin resistance.\textsuperscript{8,14} Although molecular determination of rifampicin resistance in \textit{M. leprae} in clinically relapse leprosy patients would be a prerequisite, but the relationship between the mutations and resistance must be established strongly by \textit{in vivo} growth in MFP of mice treated with anti-leprosy drugs (rifampicin, dapsone and clofazimine).

A previous \textit{in silico} study reported from our laboratory on mutation at codon 442\textsuperscript{15} revealed that this mutation affects the interaction of 441 with rifampicin due to change in the loop conformation, a consequence to this mutation, but later on clinical follow up, the patient was found to respond to MDT. Upon gathering this information we compared our observations of molecular \textit{in silico} analysis with MFP results for confirmation of rifampicin resistance.

**Material and Methods**

**COLLECTION AND PROCESSING OF SKIN BIOPSIES**

Skin biopsies were obtained from five relapsed leprosy patients attending the Out Patient Department of The Leprosy Mission (TLM) Hospitals and The Institute of Post Graduate Institute of Medical Education and Research, Kolkata. These cases were diagnosed and classified by standard clinical criteria based on the guidelines of WHO.\textsuperscript{5} \textit{M. leprae} DNAs were isolated from three samples by overnight lysis with proteinase K.\textsuperscript{16} The reaction was terminated at 97°C for 15 minutes. These lysate preparations were further used for PCR.

**MOUSE FOOTPAD (MFP) ASSAY**

Out of these five biopsies, three were processed for inoculation into MFP within 48–72 hours of collection at the National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra, India. Skin biopsies were processed following the method of Shepard.\textsuperscript{17} Each hind foot pad of outbred three BALB/c mice was inoculated subcutaneously with 0.03 ml of \textit{M. leprae} suspension (2 × 10\textsuperscript{3} bacilli). Drug sensitivity of \textit{M. leprae} to all the three drugs obtained from SIGMA, USA was carried out by administration of drugs through feed at the concentration of 0.01% for both DDS and clofazimine and 0.03% for rifampicin by gavage. The harvests from the foot pads were done after 10 months of inoculation.\textsuperscript{17}

**ETHICAL APPROVAL**

Informed consent was obtained from all the patients and the study was approved by the Institutional Animal Ethical Committee of National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra and TLM Ethical Committee, The Leprosy Mission Trust, India.

**Real time PCR and High Resolution Melting (HRM) analysis**

Initially all the samples were screened by HRM analysis. The real-time PCR (20 μl) was composed of 10 μl of 2 × HRM PCR mastermix (Type-it HRM PCR Kit, Qiagen), forward
and reverse primers (0.5 μl each of 10 μM working stocks) were used for amplification of rpoB gene. DNAs extracted from clinical and reference materials (2 μl) were used for wild type and mutant type. The reactions were set up in a Rotor Gene Q real-time PCR system (Qiagen). Rotor Gene Q software (Qiagen) was utilised to set up the sample arrangement to define PCR conditions for monitoring the amplification in real time, for viewing melting curves and to calculate DNA concentrations and other PCR parameters.

The cycling parameters of PCR were as follows: 95°C for 2 min followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s and then a hetero-duplex formation step, including 95°C for 10s and 60°C for 1 minute. After the PCR amplification steps, melt curves for the products were generated by heating in 0.2°C increments at a rate of 10 s/step for the temperature range 65 to 95°C.

DETECTION OF MUTATIONS BY PCR TARGETING rpoB

Amplification was done using primers according to the guidelines of WHO5 for detection of mutation in rpoB gene in M. leprae genome. PCR mastermix contained 12.5 μl Hot Start Taq polymerase PCR master mix (2X) (Qiagen), 1.25 of μl forward primer and reverse primer at final concentration 0.5 μM, 15.0 μl of nuclease free water and made up the final volume to 25 μl which had 5 μl of template DNA. Each experiment contained one negative and one positive control. These reference controls were received from Dr Masanori Matsuoka, Leprosy Research Centre, National Institute for Infectious Disease, Tokyo, Japan. PCR products of 279bp were detected on 2% agarose gels and amplicons were excised from the gel and were purified by using the Qiagen Gel extraction Kit. Eluted products were sent for commercial sequencing (An Xplorigen Technologies Pvt Limited, Delhi, India). Sequence data were analysed using FinchTV and MEGA 5-1.

Results

MOUSE FOOTPAD RESULTS

In vivo analysis of M. leprae susceptibility to rifampicin for first three cases (PAT 1, PAT 2 and PAT 3) which showed mutations at codons 424:GAA-GGA (Val-Gly) +442:CAA – CAC (Gln – His), 438: CAG - GAG (Gln-Val) +442:CAA – CAC (Gln – His) and only at codon 442:CAA – CAC (Gln – His) respectively were performed in MFP assay. After 10 months the foot pads of mice inoculated with 442 Gln – His of M. leprae isolate (PAT 3) did not show any growth of M. leprae with all the three drugs of multidrug regimen. On the other hand, the other two isolates having mutations at 424 Val-Gly and 438 Gln-Val in addition to 442 Gln-His mutation showed 5 to 11-fold growth in MFP with drugs compared to that of 14 to70-fold growth in control mice (Table 1). As M. leprae isolates from (PAT 1 and PAT 2) grew 5 to 11-fold in spite of drug administration these isolates were considered resistant to MDT (Table 1).

MUTATION DETECTION BY PCR AND DNA SEQUENCING

PCR was amplified targeting rpoB gene region of M. leprae in all the samples. These samples after amplification of fragment size 279bp were sent for DNA sequencing to a commercial company (An Xplorigen India Pvt Ltd, Delhi). All these five relapse cases showed mutation at
codon position 442 Gln-His (Table 2, Figure 1). PAT 1, 2 and 3 were from Kolkata, PAT 4 was from Delhi, and PAT 5 was from Champa. All three cases were clinically found to have high BI (2.6+ to 5+) at the time of relapse. These cases were earlier treated with WHO MDT.

**Table 1.** Foot pad growth of three patients at codon position 442 at 10 months in mice

<table>
<thead>
<tr>
<th>Patient code</th>
<th>PAT 1</th>
<th>PAT 2</th>
<th>PAT 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutation in rpoB:</strong> Codon position</td>
<td>424 Val-Gly; 442 Gln-His</td>
<td>438 Gln-Val; 442 Gln-His</td>
<td>442 Gln-His</td>
</tr>
<tr>
<td><strong>Mutation in folP:</strong> Codon position</td>
<td>53 Ala-Asp</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
<tr>
<td><strong>Initial Incoulum</strong></td>
<td>$2 \times 10^3$</td>
<td>$2 \times 10^3$</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>$1.4 \times 10^5 \pm 0.3$</td>
<td>$2.9 \times 10^4 \pm 0.9$</td>
<td>$3.0 \times 10^4 \pm 0.8$</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>$2.1 \times 10^4 \pm 0.6$</td>
<td>$2.1 \times 10^4 \pm 0.7$</td>
<td>0</td>
</tr>
<tr>
<td><strong>Dapsone</strong></td>
<td>$1.17 \times 10^4 \pm 0.8$</td>
<td>$1.17 \times 10^4 \pm 0.8$</td>
<td>0</td>
</tr>
<tr>
<td>*<strong>Clofazimine</strong></td>
<td>$1.17 \times 10^4 \pm 0.8$</td>
<td>$2.0 \times 10^3 \pm 0.5$</td>
<td>0</td>
</tr>
</tbody>
</table>

*As there is no specific identified gene for the determination of Clofazimine resistance, we are unable to show the mutation pattern for the bacterium for the above mentioned case.

REAL TIME PCR- HRM ANALYSIS OF RIFAMPICIN RESISTANT AT CODON 442 SAMPLES

For the detection of mutations at codon within RRDR of *rpoB* for rifampicin, real-time PCR-HRM analysis was performed. Wild-type mouse footpad-derived strain (Thai 53) was included in a reference panel and was subjected to HRM analysis as control. Real Time-PCR-HRM correctly distinguished the wild type (with straight line) from the mutant strains (negative slopes). Variations in the melting curve patterns were observed between the mutant strains (at codons 424+442, 438+442, 442) and sensitive samples as shown in Figure 2.

**Discussion**

Rifampicin is used mainly to treat mycobacterial infections, including tuberculosis and leprosy. It can be also used to treat post BCG vaccinated abscesses which is an uncommon complication of BCG vaccination. In MDT regimen of leprosy rifampicin is always used in

**Table 2.** Details of the cases bearing mutation at codon 442, 424 and 438 in the rpoB gene region of *M. leprae*

<table>
<thead>
<tr>
<th>Patient Code</th>
<th>Location</th>
<th>Age/Sex</th>
<th>Current Bacteriological Index</th>
<th>Amino Acid Substitutions$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAT1</td>
<td>Kolkata</td>
<td>35/M</td>
<td>5+</td>
<td>424 Val-Gly+ 442 Gln- His</td>
</tr>
<tr>
<td>PAT2</td>
<td>Kolkata</td>
<td>40/M</td>
<td>3+</td>
<td>438 Gln-Val+ 442 Gln- His</td>
</tr>
<tr>
<td>PAT 3</td>
<td>Kolkata</td>
<td>35/M</td>
<td>5+</td>
<td>442 Gln- His</td>
</tr>
<tr>
<td>*PAT 4</td>
<td>Delhi/ Shahdara</td>
<td>20/M</td>
<td>2.6+</td>
<td>442 Gln- His</td>
</tr>
<tr>
<td>*PAT 5</td>
<td>Champa</td>
<td>36/M</td>
<td>5+</td>
<td>424 Val-Gly+ 442 Gln- His</td>
</tr>
</tbody>
</table>

*Not inoculated in mice.*
combination with dapsone in PB cases and dapsone and clofazimine in MB cases to avoid
development of drug resistance.

Mapping the mutations in clinical strains has been critical to the development of rapid
methods for detection of drug resistance in patients. Standard susceptibility testing of
*M. tuberculosis* generally requires 4 weeks of culture and for *M. leprae* it takes as long as 6 to
12 months because of the requirement of MFP growth. Honore and Cole\(^1\) mapped six of nine
rifampicin resistant mutations from clinical isolates of *M. leprae* to the residue corresponding
to 456. Williams *et al.* also identified the 456Ser-Leu mutation in four strains of *M. leprae*.*\(^2\) In
our study we observed mutations at codon positions 424, 438 and 442 by molecular methods.
This was also confirmed by HRM analysis (Figure 2). Our earlier study showed by homology
modeling that wild type and mutant protein were similar with slight variations in the loop
region (residue 439 to 445) near the site of mutation (442Gln-His).\(^15\) The mutation at codon

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**Figure 1.** Electrophoregram and nucleotide blast reports of mutation at codon 442 (CAG-CAC).

**Figure 2.** Real-time PCR-HRM analysis for detection of mutations at codon 442 in *M. leprae* Rif resistance-determining regions (RRDR assays). Representative real-time-PCR differential curves (A) and normalised melt curves (B) for *M. leprae* strains analysed at the DRDRs of *rpoB* is shown.
442Gln-His affected the interaction of Asp441 with rifampicin because of a change in the loop conformation as a consequence of this mutation. The interaction energy between rifampicin and wild type protein was more negative (-73.98 kcal/mol) compared to mutant protein (-71.54 kcal/mol). This magnitude of smaller degree of difference in interactions indicated that mutation 442Gln-His affected the binding of rifampicin moderately. With all these in silico findings of mutation at 442 Gln-His the strains were tested and verified in in vivo MFP assay for confirmation of resistance. The present MFP result was not able to support the molecular resistance of 442 Gln- His as the strain was not able to grow in MFP of rifampicin administered mice. Further, this strain was not able to grow in mice even with DDS and clofazimine administration. However, it was noted that M. leprae strains (PAT 1 and PAT 2) which had more mutations at codons 424 Val- Gly and 438 Gln- Val respectively in addition to 442 Gln- His were able to grow in foot pads of mice treated with all the three drugs of MDT indicating that the resistance to the strains was conferred by mutations at 424 Val-Gly and 438 Gln-Val and not by 442 Gln-His mutation. Mutation at 438 Gln-Val have earlier been shown to be responsible for drug resistance by biological experiments in MFP assay. In our present study we observed that novel mutations at 424 has shown to be responsible for conferring drug resistance by MFP. Clinically these patients demonstrated unresponsiveness to rifampicin by manifesting no reduction in bacterial load as measured by bacillary index (BI) even after completion of 24 months of multibacillary multidrug therapy (MB MDT). Similar study was earlier reported by Nakata et al. who constructed recombinant strains of M.smegmatis (disrupted of rpoB gene) carrying M. leprae or M. tuberculosis rpoB genes with or without mutation. The rifampicin and rifabutin susceptibilities of the recombinant bacteria were measured to examine the influence of the mutations. The results confirmed that several mutations (441, 451, 456 and 458) detected in clinical isolates of M. leprae can confer molecular rifampin resistance. However, Nakata et al., Kai et al. also suggested that mutations at 517 in E.coli (442 in M. leprae) detected in M. leprae strain but they did not confirm whether they are confirming rifampin resistance in MFP or not.

Thus, our study pointed out that mutation involved at codon 442Gln-His in RRDR region is not conferring resistance in vivo and therefore, patients three and four in spite of showing this M. leprae mutation were responding to treatment. Patients one and five remained refractory to MDT due to the additional mutation at 424 Val-Gly which has been supported by MFP growth. Similarly patient two was also non responsive to MDT due to the additional mutation at 438 Gln-Val which was earlier found to be associated with resistance as proved by MFP studies. Therefore, it could be concluded that mutation at 442 Gln-His is not associated with M. leprae resistance in vivo.

Competing interests

We confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this study that could have influenced its outcome.

The manuscript has been read and approved by all authors.

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