

Enriched whole genome sequencing identified compensatory mutations in the RNA polymerase gene of rifampicin-resistant *Mycobacterium leprae* strains

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Abstract: Despite more than three decades of multidrug therapy (MDT), leprosy remains a major public health issue in several endemic countries, including India. The emergence of drug resistance in *Mycobacterium leprae* (*M. leprae*) is a cause of concern and poses a threat to the leprosy-control program, which might ultimately dampen the achievement of the elimination program of the country. Rifampicin resistance in clinical strains of *M. leprae* are supposed to arise from harboring bacterial strains with mutations in the 81-bp rifampicin resistance determining region (RRDR) of the *rpoB* gene. However, complete dynamics of rifampicin resistance are not explained only by this mutation in leprosy strains. To understand the role of other compensatory mutations and transmission dynamics of drug-resistant leprosy, a genome-wide sequencing of 11 *M. leprae* strains – comprising five rifampicin-resistant strains, five sensitive strains, and one reference strain – was done in this study. We observed the presence of compensatory mutations in two rifampicin-resistant strains in *rpoC* and *mmpL7* genes, along with *rpoB*, that may additionally be responsible for conferring resistance in those strains. Our findings support the role for compensatory mutation(s) in RNA polymerase gene(s), resulting in rifampicin resistance in relapsed leprosy patients.

Keywords: leprosy, rifampicin resistance, compensatory mutations, next generation sequencing, relapsed, MDT, India

Introduction

Since the introduction of effective multidrug therapy (MDT) in 1985, India is still inhabited by more than 60% of the leprosy patients of the world – a situation that has remained unchanged despite massive decline in the global prevalence of the disease. However, the attainment of the prevalence rate (PR) of 0.66 per 10,000 population indicated the progress of elimination.¹ At this crucial stage of elimination, the emergence of drug resistance in *Mycobacterium leprae* (*M. leprae*) to rifampicin is being reported from different endemic populations of India.^{2,3} If this emergence of rifampicin resistance is not checked immediately with the administration of an alternative drug regimen, the elimination program, which relies on rifampicin-based MDT, will delay the achievement of the leprosy control program of the country. Although rifampicin-based MDT, recommended by the World Health Organization (WHO; 1982), has been successful in reducing the number of leprosy cases around the world, the global registered prevalence of leprosy at the end of 2015 was still 176,176, and the new cases reported globally in 2015 was 211,973. The number of registered cases and new cases almost remained steady for many years.⁴ MDT was originally designed to prevent the emergence and spread of drug-resistant strains and contained rifampicin,

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dapsone, and clofazimine. Rifampicin, the backbone of MDT, is a bactericidal drug, which binds to the beta subunit of the RNA polymerase encoded by *rpoB* and inhibits transcription. Recent reports have, however, indicated the occurrence of rifampicin resistance in several endemic areas such as Brazil, India, and People's Republic of China.^{2,3,5-7} At this present stage, it is important to monitor the emergence of rifampicin-resistant mutants in endemic populations where transmission of *M. leprae* infection is continuing. On the other hand, resistance to dapsone has been reported since the late 1960s. Moreover, a strain showing resistance to both dapsone and rifampicin was reported in 1993.⁸ At present, further reports indicating the emergence of *M. leprae* strains resistant to multiple drugs are also emerging.^{2,9,10}

As *M. leprae* cannot be cultured in vitro, molecular drug susceptibility testing, including PCR-sequencing, for determining resistant strains provides a practical alternative. At present, the rapid detection and control of such drug-resistant strains is essential in countries approaching leprosy elimination levels, such as India. As new cases are continuing, which is considered a major indicator for disease transmission, there is still a need for better measures to control leprosy. Furthermore, relapsed cases with drug-resistant strains could be a new source of disease transmission. Therefore, at this juncture of elimination, it will be essential to conduct a thorough investigation of relapsed multibacillary (MB) cases and all new cases to identify the pattern of existence of primary and secondary resistance to anti-leprosy drugs such as rifampicin and dapsone. Whole genome sequencing (WGS) of *M. leprae* directly from clinical samples is a good tool for identifying mutations that cause drug resistance, especially those that are not known or well characterized. In biopsy samples, *M. leprae* genome copies can vary in number, but the relative proportion of bacterial DNA is minute in comparison to host DNA. Direct sequencing of mixed human and bacterial DNA does not yield significant proportions of sequence reads that map to the bacterial genome. For this reason, we used a capture probe-based bacterial DNA enrichment method which selectively isolates bacterial DNA from host DNA prior to sequencing. With the help of this approach, the present study focuses on identifying compensatory mutations in genes other than, but related to, *rpoB* in rifampicin-resistant strains of *M. leprae*.

Materials and methods

Ethical approval

Ethical clearance for this study was approved by the The Leprosy Mission Trust India Ethical Committee, held on August 29, 2014, under the regulations of the Indian Council

of Medical Research. Written informed consent was obtained from all subjects before collection of biological samples.

Source of biopsies for DNA extraction

A total of ten relapsed leprosy patients from hospitals of The Leprosy Mission Trust India (TLMTI) and one reference control strain (Br4923) from BEI Resources, USA, were included in this study. Among these ten strains, five were resistant to rifampicin, characterized by molecular methods and PCR-sequencing. The remaining five strains were drug sensitive. *M. leprae* DNA was extracted from these ten clinical biopsy specimens using the Blood and Tissue DNA extraction kit (QIAGEN, USA). Single-nucleotide polymorphism (SNP) subtyping data was generated by PCR sequencing¹¹ from DNA before carrying out WGS for the remaining DNA.

Library preparation and sequencing

Target capture sequencing libraries were prepared with Illumina-compatible SureSelect QXT Library Prep Reagent Kit (Agilent Technologies, Santa Clara, CA, USA) at Genotypic Technology Pvt. Ltd., Bangalore, India. Briefly, 50 ng Qubit-quantified genomic DNA was fragmented and adapter-tagged using SureSelect QXT Enzyme (Table S1). The fragmented and adapter-tagged DNA was purified with HighPrep magnetic beads and then amplified by eight cycles of PCR. The PCR-enriched products were purified with HighPrep beads, followed by library quality control check using the Agilent 2100 Bioanalyzer.

Target enrichment was undertaken according to the manufacturer's instructions using SureSelect RNA capture baits. In-solution hybridization was done as per the SureSelect QXT hybridization method. After hybridization, the captured biotinylated probe-target hybrids were pulled down by using streptavidin-coated magnetic beads (Dynabeads MyOne Strep-tavidin T1, ThermoFisher Scientific, Waltham, MA, USA). The magnetic beads were washed according to the manufacturer's instructions and resuspended in 15 μ l nuclease-free water. The captured DNA libraries were amplified by 12 cycles of PCR with the inclusion of the appropriate indexing primer for each sample.

The final PCR product (sequencing library) was purified with HighPrep beads, followed by quantification by a Qubit fluorometer (ThermoFisher Scientific, MA, USA), and fragment size distribution was analyzed on an Agilent 2100 Bioanalyzer.

Sequencing was carried out on an Illumina Hi-Seq 2500 instrument and the Illumina paired-end raw reads were quality checked using FastQC. Illumina raw reads were processed for adapters and trimming of low-quality bases. The processed

reads were aligned with *M. leprae* TN reference using a Burrows–Wheeler Aligner (BWA) 0.7.5 algorithm, and the variants were identified with Samtools 1.2 and Bcftools 1.2 filtered for a read depth threshold of greater than 20, and a quality threshold of more than Q30. Filtered variants were further annotated with SnpEff to get the information on the genes, protein change, and the impact of the variation.

Results

Demographics and diagnosis

Among the five rifampicin-resistant patients, one patient was a defaulter who took MDT for 2 months only and remaining four patients were those who relapsed after completion of a full course of MDT. One of the patients (1027) had a dual infection of leprosy and tuberculosis. The demographic and clinical details of these five resistant patients together with the five sensitive ones are mentioned in Table 1A and B.

All resistant strains were characterized for drug-susceptibility testing by PCR sequencing. We found the already published *rpoB* mutations in three patients (507, 42, and 80) at codon positions Leu436Meth, Asp441Tyr, and Ser456Leu, except in two (1027 and 439) who had mutations at codon positions Gln442His and Ser437Pro (Table 2). These two patients did not respond to the treatment. Gln442His mutation in patient 1027 and Ser437Pro in patient 439 of *rpoB* gene did not confer resistance to rifampicin, which was confirmed by mouse foot pad growth earlier.¹² The patient 1027 continuously visited the hospital with recurrent Type 2 reaction and other complications despite being administered thalidomide. He was later diagnosed with a pulmonary tuberculosis infection along with leprosy. He was started on anti-tuberculosis treatment (ATT) as well, but the infection was unalleviated. We tested the same specimen with *Mycobacterium tuberculosis*-specific *rpoB* primers, undertook DNA sequencing, and

Table 1 Demographic and clinical details of rifampicin-resistant cases (A). Demographic and clinical details of rifampicin-sensitive cases (B).

A					
Patients' identification	507	42	80	1027	439
Age at diagnosis (years)	23	31	18	18	18
Sex	Male	Male	Female	Male	Male
Start of MDT (month/year)	10/2011	05/2013	02/2012	11/2011	2013
Completion of MDT (month/year)	10/2011	05/2014	02/2013	11/2012	2014
Relapse date	08/2016	05/2015	12/2015	01/2015	07/2015
RJ Classification					
At diagnosis	BB	BT	BL	BL	BL
At relapse	BB	BL	BL	LL	LL
Bacillary index					
First diagnosis	0.66+	4.33+	3.0+	2.66+	NA
Relapse	2.0+	3.0+	3.0+	2.0+	6.0+
Clinical complication at the time of relapse	None	Type 1 reaction	Type 2 reaction	Type 2 reaction with concomitant pulmonary tuberculosis infection	None
B					
Patients' identification	1,335	834	730	SM	728
Age at diagnosis (years)	38	19	33	40	34
Sex	Male	Male	Male	Male	Female
Start of MDT (month/year)	11/2008	2005	04/2013	2005	2008
Completion of MDT (month/year)	10/2009	2007	04/2014	2007	2009
Relapse date	12/2015	10/2010	12/2015	04/2017	10/2010
RJ Classification					
At diagnosis	BL	LL		NA	LL
At relapse	LL	LL	BL	BL	LL
Bacillary index					
First diagnosis	3.0+	2.0+	1.0+	2.0+	4.33+
Relapse	4.0+	2.66+	2.0+	3.0+	4.33+
Clinical complication at the time of relapse	Infiltration with raised reddish lesions	Infiltration all over the body	Infiltration with Type 1 reaction	Infiltration with new lesions	Multiple ENLs on face

Abbreviations: BB, borderline leprosy; BL, borderline lepromatous; BT, borderline tuberculoid; LL, lepromatous leprosy; MDT, multidrug therapy; NA, not available; RJ, Ridley–Jopling.

Table 2 Mutations in *rpoB* and *rpoC* genes by WGS in molecularly characterized rifampicin-resistant strains

Patient ID	Resistance	Geographical area	<i>rpoB</i> mutations (WGS)*	<i>rpoC</i> mutations (WGS)
1027	RIF	Delhi	Arg1168Leu; Arg505Trp	Val517Leu
507	RIF	Chhattisgarh	Iso433Ser; Leu436 Met	NM
42	RIF	Delhi	Leu458Phe	NM
80	RIF	Delhi	Tyr-Cys	NM
439	RIF	Chhattisgarh	Tyr-Cys	Leu807Met; Leu244Phe

Note: *Amino acid.

Abbreviations: RIF, rifampicin; NM, no mutation; WGS, whole genome sequencing.

found him susceptible for the TB *rpoB* gene. This led us to consider the possibility that other mutations at other genes play a role in conferring resistance in such cases.

Genetic polymorphisms

Whole genome read coverage was adequately generated from the 10 *M. leprae* isolates, and the reference strain for comparative genomic analysis. Among these ten samples, 11,791 variants were identified, including 10,833 (92 %) SNPs and 958 (8.0%) Indels. As there is no previous database to filter out common variants, we focused on *rpoB* and its associated genes. After analyzing the data of the five rifampicin-resistant strains, we found that two strains showed mutation in the *rpoC* gene (Patient ID 1027 and 439) along with the *rpoB* gene (Table 2) as well as a non-synonymous mutation in the resistance nodulation division (RND) family transporter efflux pump gene *mmpL 7*. These two genes are mainly responsible for causing resistance among the rest of the SNPs present in these strains.

Analysis of rifampicin-resistant strains revealed 1,308 variants, of which there were 44 synonymous, 101 non-synonymous, 112 intergenic, and 12 frame-shift mutations. On genome comparison of all ten strains, we observed seven unique SNPs. While comparing resistant strains with other sensitive strains, it revealed 16 unique SNPs that were present only in these strains (Table 3). An insertion of eight nucleotides (TTTCTTAT at position 508802) was found in ML0411, encoding the Pro-Pro-Glu (PPE) serine-rich antigen.

Discussion

The emergence of rifampicin resistance in *M. leprae* after more than 30 years of MDT is not an unexpected outcome. The occurrence of dapsone resistance also took nearly the same duration. However, the emergence of rifampicin-resistant *M. leprae* during the phase of elimination may cause a major setback to the public health program. Drug-resistant leprosy infection can be caused by transmission of already resistant strains (primary resistance) or by selection of resistance-conferring mutations during inadequate therapy (secondary resis-

tance). Our own work^{2,3} has shown the presence of rifampicin, dapsone, and ofloxacin resistance cases in highly endemic areas in several states of India. *M. leprae* isolates resistant to single and multiple drugs have already been encountered.^{2,13} Although the genome of *M. leprae* has undergone massive gene decay,¹⁴ the rifampicin resistance determining region (RRDR) region of the organism and the associated genes are still functional. In order to monitor the transmission dynamics of drug-resistant leprosy, a comparison of *M. leprae* strains was carried out by WGS of strains derived from rifampicin-resistant leprosy patients in this study.

Rifampicin inhibits bacterial RNA synthesis by binding strongly to RNA polymerase, preventing attachment of the enzyme to DNA and thus blocking the initiation of transcription. Bacterial resistance to rifampicin is caused by mutations leading to a change in the structure of the beta subunit of RNA polymerase. Resistance to rifampicin is one of the main causes of treatment failure.¹⁵ Various studies have shown that resistance to rifampicin in *M. tuberculosis* as well as in *M. leprae* is mainly due to a single mutation in an 81-bp region in the *rpoB* gene.^{16,17} Furthermore, it has been reported earlier that there are some alternative mechanisms of rifampicin resistance in various bacterial species such as *rox* gene-mediated mono-oxygenation of rifampicin¹⁸ and duplication of the *rpoB* gene as *rpoB2* in *Nocardia*.¹⁹ In our study, all strains belonged to the SNP subtype 1D, which is the predominant genotype prevalent in many countries such as India, Bangladesh, Nepal, Madagascar, Malawi, and the French West Indies.^{11,20,21}

The coexistence of the two mycobacteria has been demonstrated in archaeological samples by Donoghue et al,²² who identified DNA from both tuberculosis and leprosy in the archeological samples from the Roman period to the 13th century from several sites around the world. On review of data from three leprosy referral centers in Hyderabad, India, from 2000 to 2013, Rawson et al²³ identified three cases of concomitant disease. Similarly, we observed in one of our samples a patient (Pt1027) who is suffering from both TB and leprosy.

Table 3 List of unique 16 SNPs present in resistant strains

Position	Name of the gene	TN base (reference)	Variant	SNP effect	Amino acid change	Type
80206	<i>gltB</i>	C	T	Non-synonymous coding	T/M	Probable ferredoxin-dependent glutamate synthase
83120	<i>gltD</i>	G	A	Non-synonymous coding	G/E	Probable NADH-dependent glutamate synthase
61866	<i>esxB</i>	C	A	Non-synonymous coding	G/C	Possible 10 KDA culture filtrate antigen homolog EsxB (Ihp) (cfp10)
257506	<i>ML0192</i>	C	A	Non-synonymous coding	Q/H	Conserved membrane protein
626352	<i>ML0514</i>	C	A	Non-synonymous coding	P/Q	Probable conserved membrane protein
666171	<i>priA</i>	C	A	Non-synonymous coding	H/N	Putative primosomal protein N' PriA (Replication factor Y)
672897	<i>ribG</i>	C	A	Non-synonymous coding	L/M	Probable bifunctional riboflavin-specific deaminase/reductase
140830	<i>embA</i>	G	A	Non-synonymous coding	P/L	Probable integral membrane indolylacetyl-inositol arabinosyltransferase EmbA (arabinosylindolylacetyl-inositol synthase)
145310	<i>embC</i>	G	A	Non-synonymous coding	T/I	Probable integral membrane indolylacetyl-inositol arabinosyltransferase EmbC (arabinosylindolylacetyl-inositol synthase)
2271147	<i>rpoC</i>	C	A	Non-synonymous coding	V/L	DNA-directed RNA polymerase (beta' chain)
190301	<i>mmpL7</i>	G	A	Non-synonymous coding	S/L	Conserved transmembrane transport protein MmpL7
2600317	<i>ML2186</i>		+CAG	Codon change plus codon insertion	R/RR	Possible transcriptional regulatory protein
241842	<i>ML0175</i>	G	T	Non-synonymous coding	R/L	Probable two component sensor kinase <i>MprB</i>
148945	<i>ML0107</i>	C	A	Synonymous coding	P/P	Probable conserved transmembrane protein
3237399	<i>ponA</i>		-A	Frame shift	-	
623908	<i>alaS</i>		+ACAG	Frame shift	-/T	Probable alanyl-tRNA synthetase AlaS (Alanine-tRNA ligase) and AlaRS (Alanine transylase)

Abbreviation: SNP, single-nucleotide polymorphism.

This study provides a better explanation for the presence of the rifampicin-resistance phenotype in leprosy cases in India. It has identified unique SNPs, including non-synonymous SNPs in *gltB*, *gltD*, and *esxB* and mutation in *ctpC* and *mmpL7* transporter genes. The *mmpL7* gene in *M. tuberculosis* was studied and characterized by Pasca et al.²⁴ It encodes a protein which belongs to RND drug transporter family. The MmpL7 protein is of 920 amino acids with a predicted molecular mass of 95.1 kDa, contains 12 transmembrane domains (TMDs) having two large hydrophilic extracytoplasmic domains between TMD-1 and TMD-2 and between TMD-7 and TMD-8. All of these characteristics have been described as typical of RND efflux pumps.²⁵ The MmpL7 protein confers a high level of resistance to isoniazid.²⁴ However, a functional assay is required to determine whether either of these variant genes/transporters confers any degree of rifampicin resistance in *M. leprae*. In our study, the *rpoC* gene has a non-synonymous SNPs sequence in two patients – 1027 and 439. Mouse foot pad assay was done

for the patient 1027 to check whether the mutation in 442 codon position was conferring resistance. In our previous study, we observed that this 442 codon mutation was not conferring resistance in mice.¹² Therefore, it may be suggested that these compensatory mutations in *rpoC* and/or transporter genes play a role in conferring resistance in this strain. Thus, our study suggested the role of compensatory mutation in *rpoC* and possibly of drug transporter genes in contributing to acquired resistance in rifampicin-resistant strains in *M. leprae*.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Concentration of DNA from biopsies and after library preparation

Serial number	Sample ID	DNA concentration by Qubit (ng/ μ L)	Library concentration (ng/ μ L)	Volume
1.	570	56.7	26.7	13
2.	507	93.2	0.62	13
3.	42	85.6	0.74	13
4.	80	35.6	0.38	13
5.	1027	204	0.54	13
6.	281	33.1	0.36	13
7.	439	25.6	0.41	13
8.	4923	15.3	78.5	13
9.	395	20.5	0.32	13
10.	834	432	0.37	13
11.	588	235	0.41	13
12.	SM	45.4	0.33	13
13.	728	24.9	0.57	13
14.	1335	25.4	2.85	13
15.	730	33.2	0.34	13
16.	BH	64.2	1.33	13

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