



RIFAMPICIN RESISTANCE IN LEPROSY: MUTATIONAL ANALYSIS OF *rpoB* GENE

¹Partha Sarathi Mohanty, ²Shivangi Mishra, ^{1*}Farah Naaz, ¹Devendra Singh Chauhan, ¹Mamta Arora, ¹Haris Sagar, ¹Avi Kumar Bansal & ¹Umesh Datt Gupta

¹National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Tajganj, Agra, India

²Banasthali Bidyapeeth, Nawai, Jaipur, India

Corresponding author email: farah.ferry@gmail.com

ABSTRACT

Leprosy is a slow chronic infection caused by *Mycobacterium leprae*, which is a slow-growing intracellular bacillus that infiltrates the skin, the peripheral nerves, the nasal and other mucosa, and the eyes. India achieved leprosy elimination in Dec 2005 but still some endemic pockets exist in the country and still responsible for continue transmission of leprosy. In the present study, we examined the frequency of mutations in the RRDR (Rifampicin Resistant Determining Region) in *rpoB* gene in relapse and poor responder cases of leprosy to elucidate the drug resistant pattern in *rpoB* gene region of *M. leprae* genome. Slit-skin smears (SSS) samples were taken from patients with leprosy those were failing in treatment after receipt of informed consent. *rpoB* gene codes for RNA polymerase and were responsible for drug resistant in relapse leprosy cases were amplified by WHO recommended primers and sequenced. The mutation rate in *rpoB* gene of relapse cases was found to be 2 in 20 samples which were found to be 10%. Moreover, it was shown that many of the patients with relapse who had rifampicin-resistant mutations had histories of treatment with rifampicin monotherapy.

KEY WORDS: Leprosy, Drug resistance, Rifampicin.

INTRODUCTION

Leprosy is a neurotic, chronic and granulomatous communicable disease affecting skin, peripheral nerves, mucosa of the upper respiratory tract and several other organs of the human body. The disease is caused by *Mycobacterium leprae*, is a slow growing and strongly acid-fast, rod-shaped bacilli. The incubation period between infection and appearance of disease is normally 2 to 10 years but may be as long as 20 years (WHO, 1998). Most of the untreated leprosy cases are progressive in nature and can cause permanent damage to affected organ and capable enough to disseminate the disease. The disease affected 1.15 million people around the World in 2013 including Brazil, India, Indonesia, Myanmar, Nigeria and several other countries (WHO, 2013). In India several endemic pockets still exist where the leprosy is a major health problem. Current treatment for leprosy is based on standard MDT which consists of Dapsone, Rifampicin and Clofazimine (WHO, 1982; Malathi and Thapa, 2013). However other antibiotics like Ofloxacin and Minocycline are also administered to patients if required (Kumar *et al.*, 2015). Dapsone monotherapy was used for treatment of leprosy prior to the introduction of MDT. Long term monotherapy with Dapsone resulted in poor compliance, treatment failures and emergence of Dapsone resistant strains of *M. leprae* (Guinto *et al.*, 1981; Cruz *et al.*, 1996). Between the 1960's and 1970's additional antimicrobial agents like Ampicillin and Clofazimine were introduced for the treatment of leprosy (Browne *et al.*, 1962; Levy *et al.*, 1976). MDT takes 6 months for paucibacillary (PB) and 1 year or more for multibacillary (MB) leprosy to cure depending on strength of leprosy

infection. Worldwide leprosy prevalence was reduced due to the introduction of MDT. India achieved the elimination phase in 31st December 2005 (Singal and Sonthalia, 2013). The rate of reduction in leprosy cases in India has slow down over the years and put a hurdle in total elimination of the disease from the country. Still leprosy cases are continuously being detected from the endemic pockets of India. Apart from new case detection several MB patients showed drug resistant or non-respondents' to standard MDT. Some of the non-responders had mono resistance to rifampicin or to ofloxacin or to dapsone while some patients were multi drug resistant. In the present study, we examined the frequency of mutations in the RRDR (Rifampicin Resistant Determining Region) of *rpoB* gene in relapse and poor responder cases of leprosy to elucidate the drug resistant pattern in the specified region of the respective gene.

MATERIALS & METHODS

Collection of sample

Slit-skin smears (SSS) samples were taken from patients with leprosy after receipt of informed consent. Samples were collected from OPD of National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Tajganj, Agra. The samples were classified as relapse cases. Relapse was defined as development of new skin lesions after completion of MDT and increase in bacterial index by >2 log units in any lesion. In the present study only relapse cases were taken. A total of 20 samples were collected. All the samples were collected from ear lobe using sterile blade and was dipped in 1 ml of TE buffer in a micro centrifuge tube. All the samples were labelled properly for

patient ID, village name, date of relapse and date of start of MDT and samples were stored at -20°C for further experimentation.

Isolation of DNA

DNA from samples was isolated using the protocol described by van Soolingen *et al.* (1991). Briefly the method is as follows. The samples were taken from the earlobe and dipped in $200\mu\text{l}$ TE buffer. Suspensions of slit scraping were boiled for 5 to 10 minutes and then snap chilled in ice. This make the cell wall rupture. Lysozyme (20mg/ml) was added in each tube at a volume of $40\mu\text{l}$ and incubated at 37°C for 2 hours in shaking water bath. After incubation $56\mu\text{l}$ of 10% SDS and $5\mu\text{l}$ of proteinase k (19mg/ml) was added in each tube and mixed by vortexing followed by incubation at 65°C for 30 minutes in shaking waterbath. Then $80\mu\text{l}$ of CTAB and $64\mu\text{l}$ of NaCl was added in each tube, vortexed and incubated at 65°C for 30 minutes. Equal volume of chloroform and isoamylalcohol mixture (24:1) was added in each tube and centrifuged at $10,000\text{ rpm}$ for 5 minutes. Protein and carbohydrate were settled down and supernatant of each tube was transferred in another respective tube. DNA was precipitated by adding 0.6 volume of isopropanol in each tube and incubated at -20°C for overnight for DNA precipitation. Next day tubes centrifuged for 15 minutes at $10,000\text{ rpm}$, supernatant of each tube was discarded. The DNA pellets were washed in 70% ethanol and air dried. Pellets were dissolved in $25\mu\text{l}$ of TE buffer and stored at -20°C .

Amplification of rpoB gene

rpoB gene which codes for RNA Polymerase beta chain and is responsible for rifampicin resistant in relapse leprosy cases was amplified using primers 5'GTCGAGGCGA TCACGCCGCA3' (forward primer) and 5'CGACAAT GAACCGATCAGAC 3' (reverse primer). The amplification was done at initial temperature of 94°C for 2 minutes followed by 40 cycles of initial temperature 94°C for 45 seconds, primer annealing temperature at 58°C for 1 minute, extension temperature of 72°C for 1 minute and a final extension temperature at 72°C for 8 minutes. The

amplified bands were resolved in 2% agarose gel at a voltage of 50 mAmp.

Sequencing of amplified products

PCR products were purified using PCR clean up kit (Quigen, 28053). All the PCR products were sequenced directly using the forward primer by 3130XL ABI Big Dye Terminator sequencer.

Detection of mutation through sequence alignment

Sequences were aligned by MEGA-4. Multiple alignment was done with the parameters, *viz.*, gap opening [0-100]=15, gap extension [0-100]=6.66, delay divergent sequences %=30, DNA transition weight [0-1]=0.5 and DNA matrix weight set to IUB for scoring (Tamura *et al.*, 2007). The mutations in the DNA sequences and corresponding protein sequences were determined visually by seeing the alignment files. The corresponding triplet code and amino acid change position was recorded accordingly. To predict the point mutation in a particular sequence the reference rpoB gene sequence of *M. leprae* TN isolate, NCBI accession number AL583923 was included in the alignment.

RESULTS

Molecular techniques are becoming increasingly popular in detecting and tracing the drug resistance in different genes caused by point mutations in a particular species. In this study the identification such point mutations in rpoB genes were enumerated for rifampicin resistance *M. leprae*. DNA was extracted from the SSS samples collected in 1ml of TE buffer. Total genomic DNA was extracted, electrophoresed through 0.7% (w/v) agarose gel. The DNA extracted was of good quality showing no shearing and the ratio of DNA and protein ($260\text{nm}/280\text{nm}$) was found to be between 1.9 to 2.1.

Amplification of rpoB gene

rpoB gene which is responsible for the rifampicin resistant was amplified for 20 strains of *M. leprae* using the primers described in materials and methods. All 20 strains yielded an amplified product of approximately 300bp when resolved in 2% agarose gel.

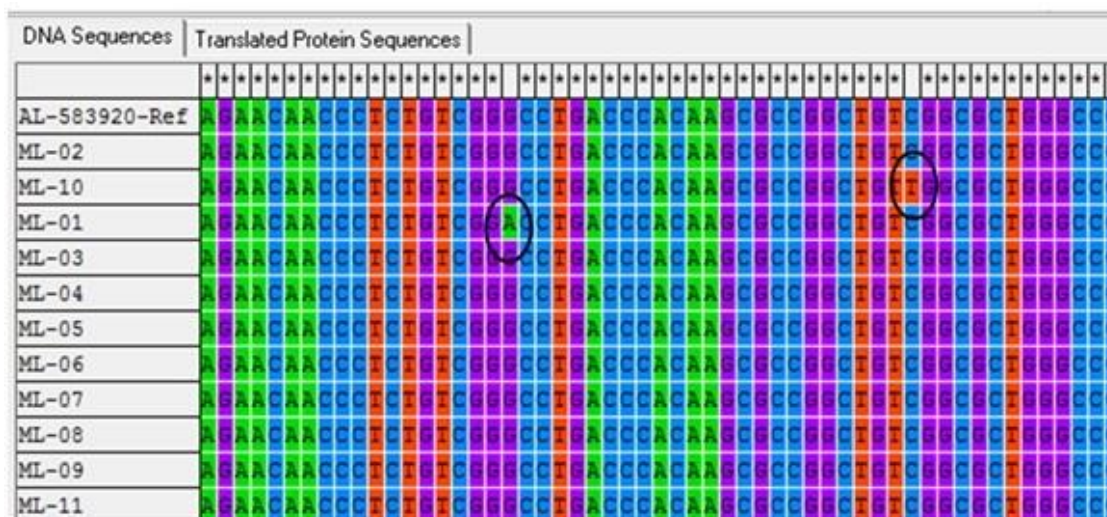


FIGURE 1a: DNA sequence alignment for 11 *M. leprae* strains along with reference strain TN-emb-AL583917 showing mutational position at nucleotide position in circle

Sequencing of rpoB gene

Purified DNA bands of rpoB gene were sequenced and carefully examined for the presence of junk DNA (sequencing error). No trace of junk DNA was found in any of the sequences. The annotation results showed that the sequenced fragment of rpoB gene region is about 300bp in length.

Detection of drug resistant mutation in rpoB gene

The unambiguous sequence length of the amplified rpoB gene region was 298bp to 300bp. For alignment and

analysis of rpoB gene region, 298 characters were included. Out of 298 sites only 2 variable sites were detected in 1 strain viz ML-01 and ML-10. The variable sites were in the nucleotide position 1343 and 1376, which were falling in second codon. The triplet codon changed from GGC to GAC and from TCG to TTG. The corresponding amino acid positions were 448 and 456. The amino acid changed from Glycin to Asparatic Acid (Gly--Asp) and Serine to Leucine (Ser--Leu) (Fig 1a, b).

DNA Sequences	Translated Protein Sequences																													
AL-583920-Ref	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-02	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-10	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-01	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-03	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-04	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-05	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-06	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-07	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-08	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-09	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V
ML-11	L	S	F	M	D	D	N	N	L	S	G	L	H	R	F	L	S	A	L	G	G	G	L	S	E	A	G	L	E	V

FIGURE 1b: Protein sequence alignment for 11 *M. leprae* strains along with reference strain TN-emb-AL583917 showing mutational position at nucleotide positions in circle

DISCUSSION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* and is still a health problem in India. Leprosy exists in several endemic pockets of our country and still imposes a problem in leprosy elimination programme. Moreover the emergence of drug resistance *M. leprae* in some patients is a major concern in treatment of leprosy patients. To prevent the emergence and transmission of multi drug resistant (MDR) leprosy and to identify and treat existing cases of MDR leprosy, it is necessary to establish rapid methods for detection of drug resistance in *M. leprae*. In the present study an attempt has been made to explicate the point mutations those are responsible for the drug resistant in non relapse cases.

The most popular PCR method for detection of drug resistance in *M. leprae* is the molecular method viz., sequencing of particular genes and their comparison with reference strain. Drug sensitivity test could not be applied to access the drug resistance in *M. leprae* due the organism's inability to grow in artificial medium. In the present study we used WHO recommended primers for the detection of drug resistance in rpoB (rifampicin resistant). The mutation rate in rpoB gene of relapse cases was found to be 2 in 20 samples which is 10% and is less prominent as compared to previous studies (Cruz *et al.*, 1996; Maeda *et al.*, 2001; Wahyuni *et al.*, 2012). Two possible reasons were conceived regarding the rifampicin resistance in patients with relapse: (1) reinfection by the primary rugresistant strain (2) reactivation of rifampicin resistant strains capable of persisting after chemotherapy. Although it is still unclear whether the relapses are caused by

reinfection by *M. leprae* or by reactivation of persistent *M. leprae*, close correlation between drug resistance and relapse have been recognized in several studies (Lopez-Roa *et al.*, 2006; Kaimal and Thappa, 2009).

There was no mutation in the major sites for drug resistance on the rpoB gene. However, we observed mutations at 2 position, codons 448 and 456, which has not been associated with rifampicin resistance. These mutations in the rpoB gene are finding needs further clarification by mouse footpad technique.

CONCLUSION

Overall, our study indicated a low ratio of rifampicin resistance in patients with relapse, compared with the other patients with leprosy. Moreover, it was shown that many of the patients with relapse who had rifampicin-resistant mutations had histories of treatment with rifampicin monotherapy.

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REFERENCES

Browne, S.G. & Hogerzeil, L.M. (1962) "B 663" in the treatment of leprosy. Preliminary report of a pilot trial. *Lepr Rev.* 33: 6-10.

- De la Cruz, E., Cellona, R.V., Balagon, M.V.F., Villahermosa, L.G., Fajardo, T.T. Jr., Abalos, R.M., Tan, E.V. and Walsh, G.P. (1996) Primary dapsone resistance in Cebu, The Philippines; cause for concern. *Int J Lepr Other Mycobact Dis.* 64: 253-267.
- Guinto, R.S., Cellona, R.V., Fajardo, T.T. and de Cruz, E.C. (1981) Primary dapsone-resistant leprosy in Cebu, Philippines. *Int J Lepr Other Mycobact Dis.* 49: 427-430.
- Kaimal, S. & Thappa, D.M. (2009) Relapse in leprosy. *Indian J Dermatol Venereol Leprol.* 75:126-135.
- Kumar, A., Girdhar, A., Chakma, J.K. & Girdhar, B.K. (2015) WHO multidrug therapy for leprosy: epidemiology of default in treatment in Agra district, Uttar Pradesh, India. *BioMed Res Int.* dx.doi.org/10.1155/2015/705804.
- Levy, G.H., Shepard, C.C. & Fasal, P. (1976) The bactericidal effect of rifampicin on *M. leprae in man*: a) single doses of 600, 900 and 1,200 mg, and b) daily doses of 300 mg. *Int J Lepr.* 44: 183-187.
- Maeda, S., Matsuoka, M., Nakata, N., Kai, M., Maeda, Y., Hashimoto, K., Kimura, H., Kobayashi, K. & Kashiwabara, Y. (2001) Multidrug resistant *Mycobacterium leprae* from patients with leprosy. *Antimicrob Agents Chemother.* 45: 3635-3639.
- Malathi, M. & Thappa, D.M. (2013) Fixed-duration therapy in leprosy: Limitations and opportunities. *Indian J Dermatol.* 58: 93-100.
- Singal, A. & Sonthalia, S. (2013) Leprosy in post-elimination era in India: difficult journey ahead. *Indian J Dermatol.* 58:443-446.
- Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) *MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0*, *Mol Biol Evol.* 24: 1596-1599
- Van Soolingen, D., Hermans, P.W., de Haas, P.E., Soll, D.R. and van Embden, J.D.A. (1991) The occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol.* 29: 2578-2586.
- Wahyuni, R., Adriati, D. & Iswahyudi, I., Prakoeswa, C.R.S., Agusni, I. and Izumi, S. (2012) Profile of mutation on drug resistance *Mycobacterium leprae* isolates in Indonesia collected during 2003-2011. *Microbiol.* 6: 135-138.
- World Health Organization (1982) Chemotherapy of leprosy for control programmes, WHO Technical Report Series, 675.
- World Health Organization (1998) WHO model prescribing information. Drugs used in leprosy. Geneva, pp32.
- World Health Organization (2013) Leprosy fact sheet. WHO media center, Geneva. Available at: <http://www.who.int/mediacentre/factsheets/fs101/en/>