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In silico analysis of novel mutations determined within RRDR region of rpoB gene of *M. leprae*: A possible cause of Rifampicin resistance among leprosy patients

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ABSTRACT:-*In silico* methods help in understanding the role of mutations in gene sequences through bioinformatics tools and can help in the identification of therapeutic targets. This study determined, the binding interactions of Rifampicin, a chemotherapeutic drug commonly used in Multidrug Therapy (MDT) for treatment of leprosy, against the rpoB models bearing point mutations. Using Molegro Virtual Docker we analyzed 4 novel mutations within Rifampicin Resistance Determining Region (RRDR) of rpoB gene of *M.leprae* which could confer resistance to Rifampicin, revealed that mutations causing structural modifications modulated the drug binding energies with receptor.

Keywords:- Rifampicin resistance, Mutation analysis, Molecular Docking

Abbreviations:
MVD: Molegro Virtual Docker;MDT: Multi drug therapy;RIF: Rifampicin;RRDR: Rifampicin resistance determining region, rpoB: RNA polymerase beta subunit, DRDR: Drug Resistance Determining Region,NM: Novel mutation,PCR: polymerase chain reaction

I. INTRODUCTION

Leprosy, a bacterial disease caused by *M. leprae* affects mainly skin, peripheral nerves and the mucous membranes especially the epithelial lining of the nose**.** The World Health Organization (WHO) recommended multidrug therapy (MDT) for leprosy has been effective in reducing both the prevalence and incidence of leprosy globally [1]. Rifampicin (RIF), a bactericidal drug, is used to treat Mycobacterium infections, including tuberculosis and leprosy. RIF has a molecular mechanism of antibacterial activity involving the inhibition of DNA-dependent RNA polymerase of the organism [2]**.** High-level resistance to RIF can be caused by single point mutations in rpoB, the gene coding for the β subunit of RNA polymerase [3]. Most of these mutations change amino acids in or surrounding the RIF binding pocket and probably cause resistance by interfering with RIF binding to RNA polymerase [4]. The mechanism of action for Rifampicin previously discussed was determined through study of mutations found in a relatively small number of clinical isolates [5]**.** Mutations within rpoB decrease the affinity of the protein for the rpoB-rifampicin adduct via either structural changes that alter the architecture of active site or mutations within the active site itself [6]**.**

To understand the possible role of structural variation (non-synonymous substitution) of rpoB we carried out the homology modeling of the novel proteins and its interaction with the drug molecule, RIF using molecular docking *in silico* experiments. The novel DNA sequences for *M. leprae* rpoB were deposited in public database (NCBI accession numbers shown in **table 1**). This study can lead us towards the better and faster screening of these mutations and reduce the cost of further experimental analysis.

II. EXPERIMENTAL PROCEDURES AND RESULTS

PCR amplification and DNA sequencing

The detection of mutations in drug resistance determining region (DRDR) of the gene associated with drug resistance was performed with the help of PCR followed by DNA sequencing. PCR based gene amplification was done using primers according to the Guidelines of WHO "Global Surveillance of Drug Resistance in Leprosy 2008" for detection of mutation in rpoB [7]. *M. leprae* genome sequences were obtained from a total of 195 samples obtained from relapsed leprosy patients reported at The Leprosy Mission (TLM) hospitals during the period between 2009 and 2014. Among these 195 sequenced samples 3.6% were showing

mutation in rpoB gene responsible for resistance to RIF [8]. Four novel mutations were selected to perform homology modeling and further docking with RIF to compare the changes within novel mutations in comparison to wild type (Thai 53) and known high level resistant mutant strain [(at position Ser456Leu; Z-4), kind gift from Dr. Masanori Matsuoka, Japan]

Protein Homology Modeling and Secondary Structure Prediction

The Standard protein-protein BLAST (blastp) was used for both identifying a query amino acid sequence and for finding similar sequences in protein databases. RpoB of *M.leprae*, encoded by ML1891c has ~1179 amino acids in its protein sequence and upon sequence comparison using BLASTp of rpoB protein, it was found that in query sequence, the mutation occurred at codon positions 427 (Ile427Phe, NM 1), 424 and 442 (Val424Gly; Gln442His, NM 2), 434 (Ser434Gly, NM 3) and 437 (Ser437Gln, NM 4) as compared to wild type (WT). Homology modeling with I-Tasser [9]was used for modeling the three-dimensional structure of rpoB. More than 1 probable model was generated for each of the 6 input sequences. The best model with maximum C Score of each sequence was used for docking. The I-Tasser also gives information about the secondary structure and solvent accessibility. Structural refinement and energy minimization of the predicted models were carried out using Molegro Virtual Docker 2011.5.0 (MVD). The refined model reliability was assessed through Rampage.

Protein Structure Validation-Rampage

The validity of the modeled structures was assessed using Rampage. Rampage validates the structure of the input protein and is based on Ramachandran Plot. Each of the residues was designated to be present either in Favored or Allowed or Outlier region. The residues of Allowed and Outlier regions were shown in evaluation of validation and the remaining residues were said to be in favored region. The Ramachandran plots provided by the Rampage full model analysis reported that 86.2%, 82.7%, 92.1%, 86.6%, 83.6% and 79.2% of the residues fell within the most favorable regions according to generated structures: a, b, c, d, e and f respectively. **(Figure 1)**

Cavity Prediction

In order to identify the energetically most favorable pose (also referred to as 'pose prediction'), each pose was evaluated ('scored') based on its complementarity to the target in terms of shape and properties such as dock score. Potential binding sites (also referred to as cavities or active sites) were identified using the built-in cavity detection algorithm. The cavities found were then ranked according to their volume. A good score for a given molecule indicates that it is potentially a good binder. This process was repeated for all molecules in the collection. The energy of the resulting pose (fitness) consists of three terms: (1) hydrogen-bonding energy, (2) internal energy of the ligand, and (3) steric interaction energy [10]. The MolDock algorithm goes through repeated cycles of Monte Carlo simulations followed by energy minimization in order to generate and refine an ensemble of low-energy ligand poses. The geometry of binding pockets shows that NM 1 and 4 binds in the cavity of maximum volume among the predicted binding cavities along with mutation Z-4, while NM 2 and 3 showed that the binding cavity volume decreased and the cavity became rigid. This did not permit proper alignment of RIF inside the cavity and hampered hydrogen bond formation indicating a weak interaction with RIF at the active site **(Table 1).**

Protein-Ligand Docking

The ligand RIF used in this study against rpoB was retrieved from the PubChem database [11] PubChem CID 5381226, in mol2 format, and the PyMol molecular graphics system (http://www.pymol.org) was used to convert it into Protein data base (PDB) format. When all the molecules have been prepared, the docking was commenced using MVD. MVD includes MolDock [12] for evaluating docking solutions. Flexible docking protocols were used in which both protein and ligand were kept flexible and a grid was generated around the active site residues of the protein molecule **(Figure 2)**. The docking complexes of RIF with wild and mutant rpoB were subjected to molecular dynamic (MD) simulation using the MolDock. **Table 2** shows the interaction energies including the total, electrostatic and steric for all the mutants and wild type sequences in the active site of the rpoB-RIF complex (Z-4 is a known high level resistant strain and taken as reference in this study). This study determined not only the conformation of the mutated rpoB proteins but also identified significant changes in the docking score, free binding energies, hydrogen bond energies by molecular docking [13]. Based on these studies, it was found that wild type rpoB-RIF complex exhibited total interaction energy of -185.1308 kcal/mol, while much lower interaction energy has been noted in all the mutant forms (Z-4 -118.986, NM1-132.649, NM 2 -146.238, NM 3 -126.058, NM 4 -102.835 kcal/mol). This was also reflected by changes in energies of hydrogen bonds and decrease in internal energies in the mutant models expect for NM 1 with almost similar

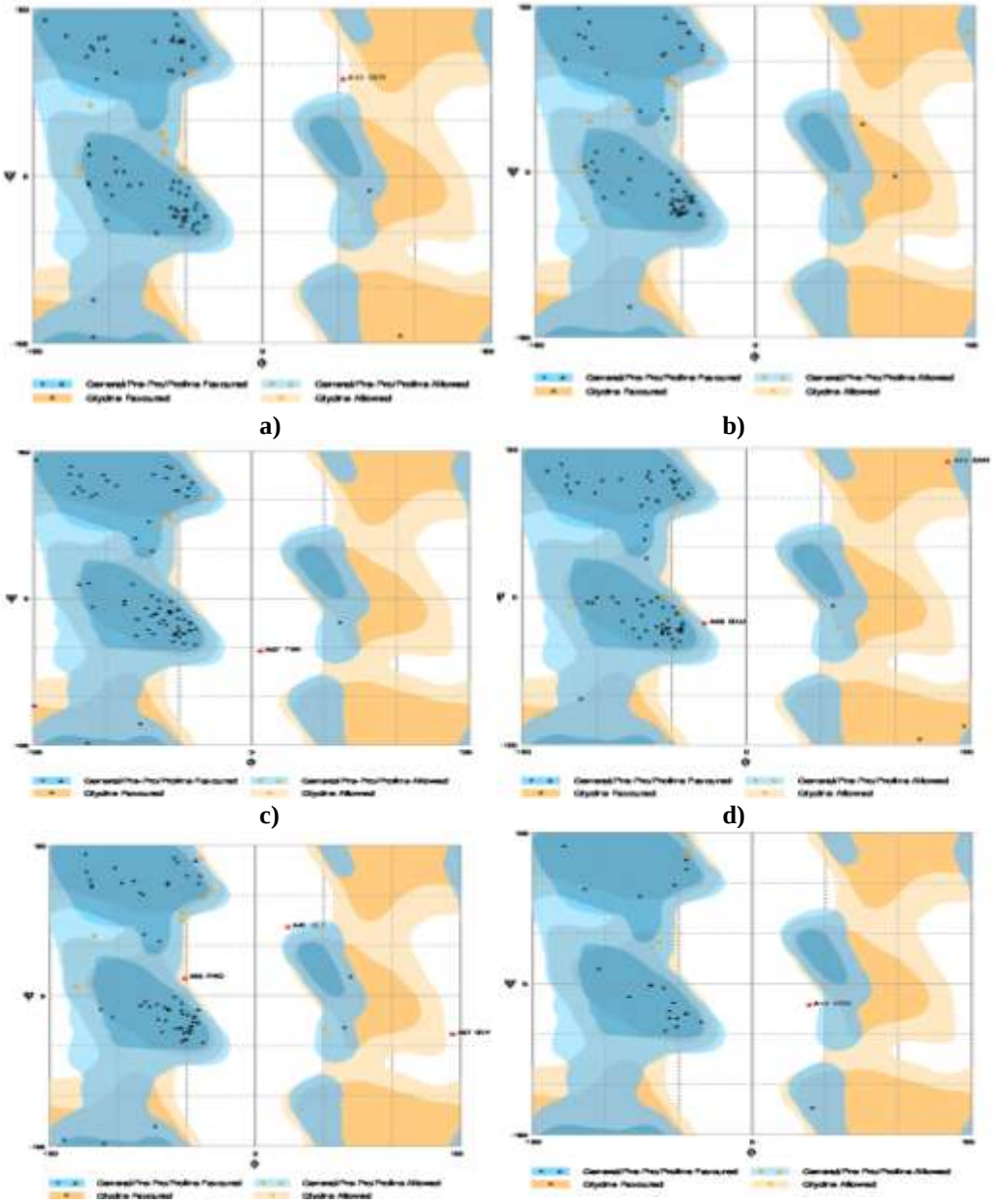
internal energy change as WT **(Figure 3 and 4).** Our study showed that average energy required for binding for the mutated rpoB structures was lesser than that for the wild type structure.

III.DISCUSSION

Until the advent of molecular tools mouse foot-pad experiments was the only technique for the detection of drug resistance in *M. leprae* in vivo [14]. Since *M. leprae* cannot be cultivated in *in-vitro* system, it is not possible to measure resistance rates at a large scale in endemic countries. One of the major aims of this work was to explore the ability of structural bioinformatics based mutational analysis to determine rifampicin resistance in leprosy patients, the commonly used chemotherapy drug for treatment of leprosy whose mechanism of action is to inhibit bacterial protein synthesis. This drug interacts with, and is substrate of gene product like β-subunit of RNA polymerase [15]. In this study, using 4 novel mutations in the rpoB gene we have performed several analyses to understand the mechanism of resistance including docking studies, binding pocket analysis and molecular dynamics of both native and mutant rpoB. We used several bioinformatics tools to proceed with *in silico*studies and the results showed that the variations in the structure of the targets due to single amino acid change influences the drug-binding capacity of these novel mutations and therefore affects drug toxicity. The MD simulations are known to provide the dynamic conformational changes in the interaction of protein and ligand [16]. The results obtained in our study inferred that the drug resistance could be due to the alteration in 3-D conformation of the protein by mobility of the binding residues in the mutant. Further, binding pocket of rpoB, as predicted by MVD along with their amino acid residues showed that change in single amino acid may affect the binding of drug with the receptor. Binding of RIF is extremely sensitive to shape modification of binding pocket due to its rigid conformation [4]. In our generated models, the geometry of binding pockets shows that NM 1 and 4 binds in the cavity of maximum volume among the predicted binding cavities as mutation Z-4, while NM 2 and 3 showed that the binding cavity volume decreased and the cavity became rigid. This did not permit proper alignment of RIF inside the cavity and hampered hydrogen bond formation indicating a weak interaction with RIF at the active site. Another reason for these weak interactions might be the orientation of the residues of side chains in mutant type orient away from the binding pocket and were not observed to possess direct interactions with RIF. The single amino acid mutation at codon 456 previously 425,i.e. Ser to Leu (TCG to TTG) of the rpoB gene is reported to be the most widespread mutation, associated with RIF resistance. Thus, the interactions of all docked novel mutations were compared and analyzed with docked structure of Ser456Leu (Z-4) mutation. The docking results of NM 2 are also in line with and are comparable with the mouse footpad results [17] confirming high level of resistance for RIF among leprosy patients.

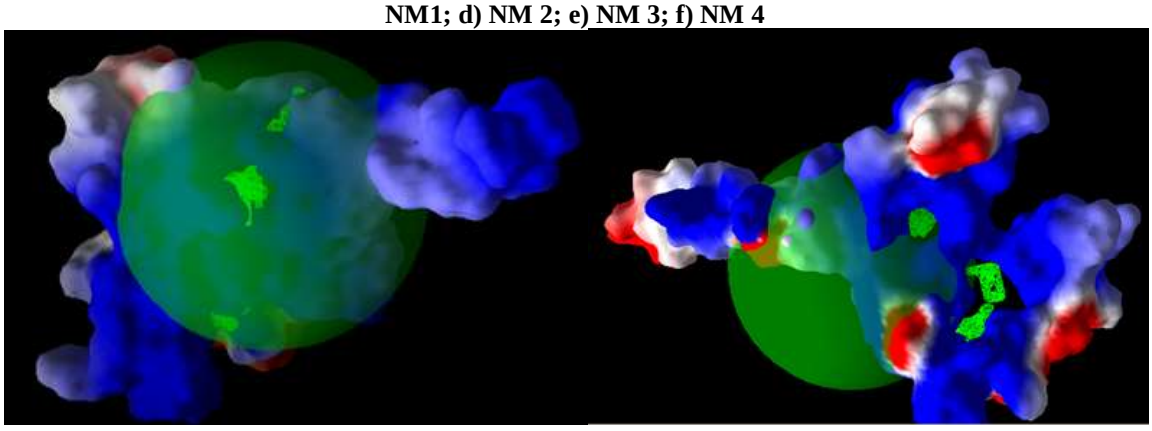
Apart from the novel point mutations a second site mutation in NM 2 was also noticed. This "novel" mutation was paired with mutation 442 previously reported to confer resistance[18](later confirmed by mouse footpad assay as sensitive[17]. Therefore the role of this second site mutation in determining the overall resistance has yet to be established. Of the four "novel" mutations Gln442His is thought to be the only insignificant change, because the amino acid change is a conservative substitution, but mutation at position 424, (Val424Gly) in the same patient determined resistance which was confirmed by MFP assay[17]. The other three mutations (NM 1, 3 and 4) have resulted possible structural changes in the rpoB active site. A ligand binding interaction always influences the stability of the receptor protein, this justifies that the sustainability of the rpoB-RIF interaction and wild-type rpoB is more acceptable over the mutant[19]. Most importantly, these bindings were also described via interaction energies (IEs). The sum of short-range Columbic and van der Waals interaction energies are taken as the total IE between the protein and ligand [20]. This clearly indicates a big difference in IEs between the two complexes, the one is showing mutations will require higher IEs than the wild-type rpoB, which possesses comparatively high IE and hence show more affinity towards RIF. This study reveals that because of the changes in structure due to the polymorphism (point mutations) in the structure of rpoB, the drug binding capacities changed and due to this the response to RIF may vary in patients.

Figures And Tables

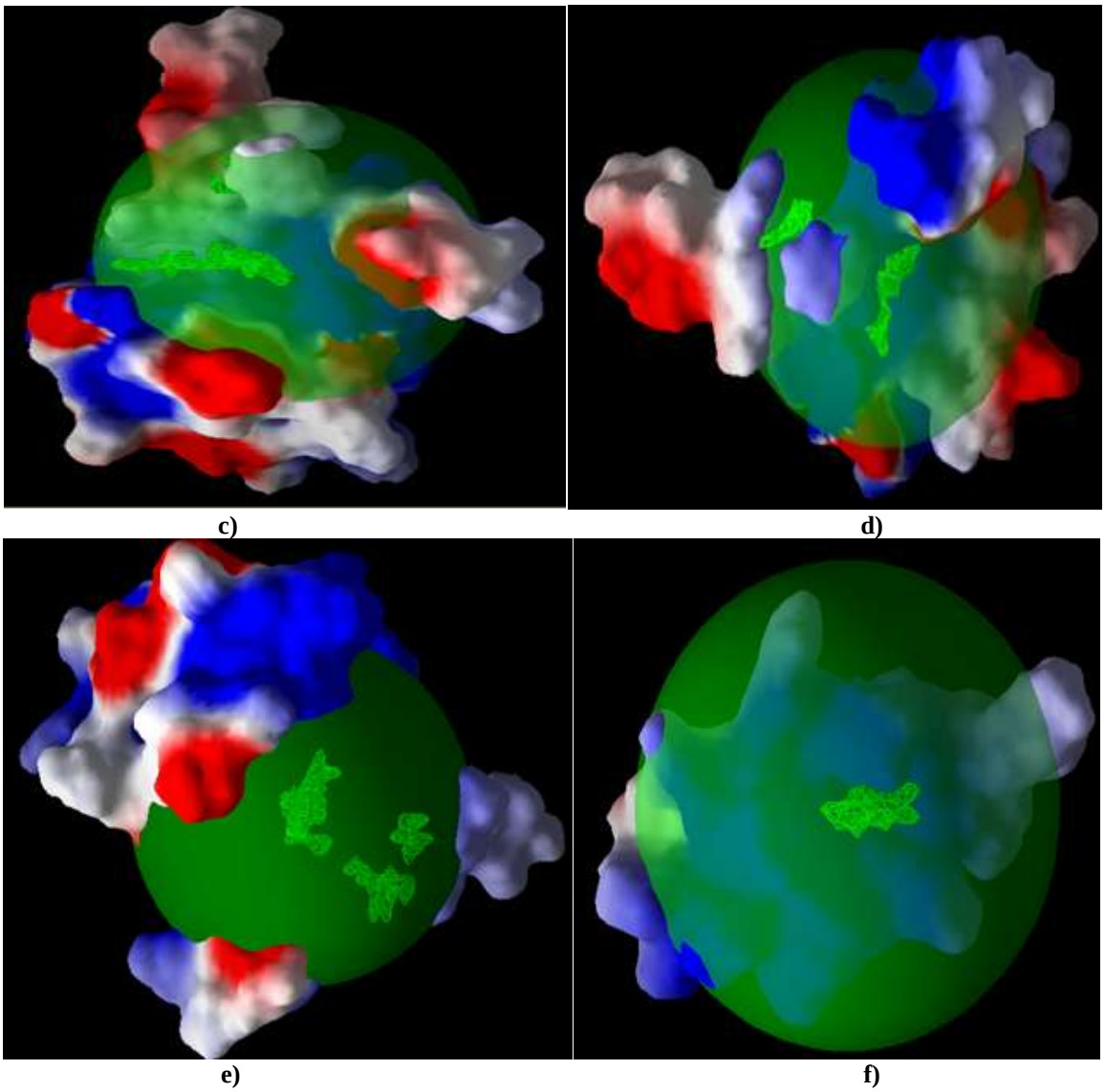


a) **b)** **c)** **d)** **e)** **f)**

Figure I: Ramachandran plot of predicted rpoB model, Validation using RAMPAGE: a) Z-4; b) WT; c) NM1; d) NM 2; e) NM 3; f) NM 4



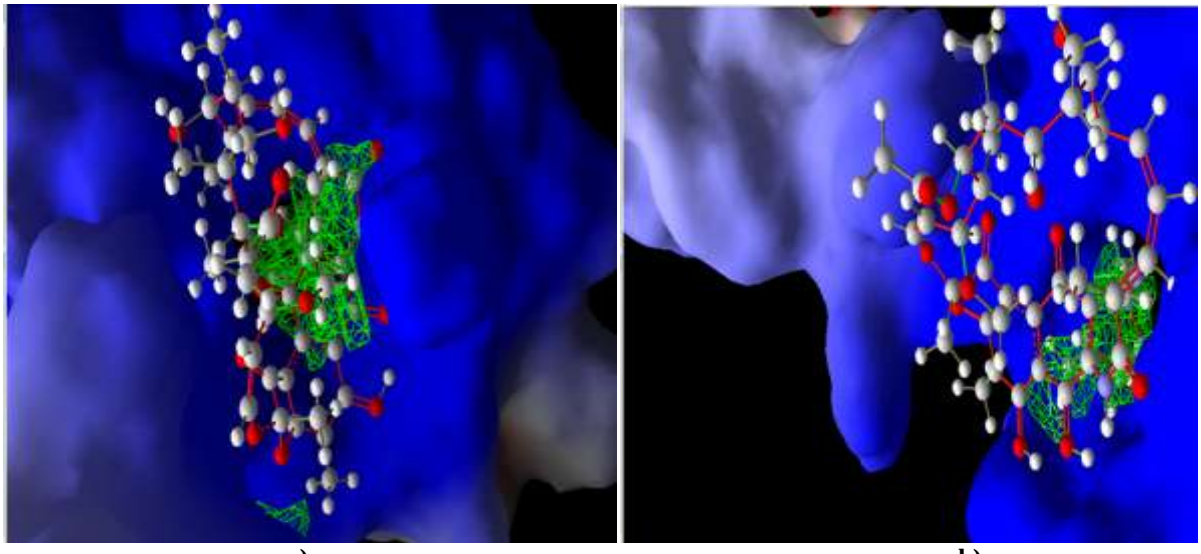
a) **b)**



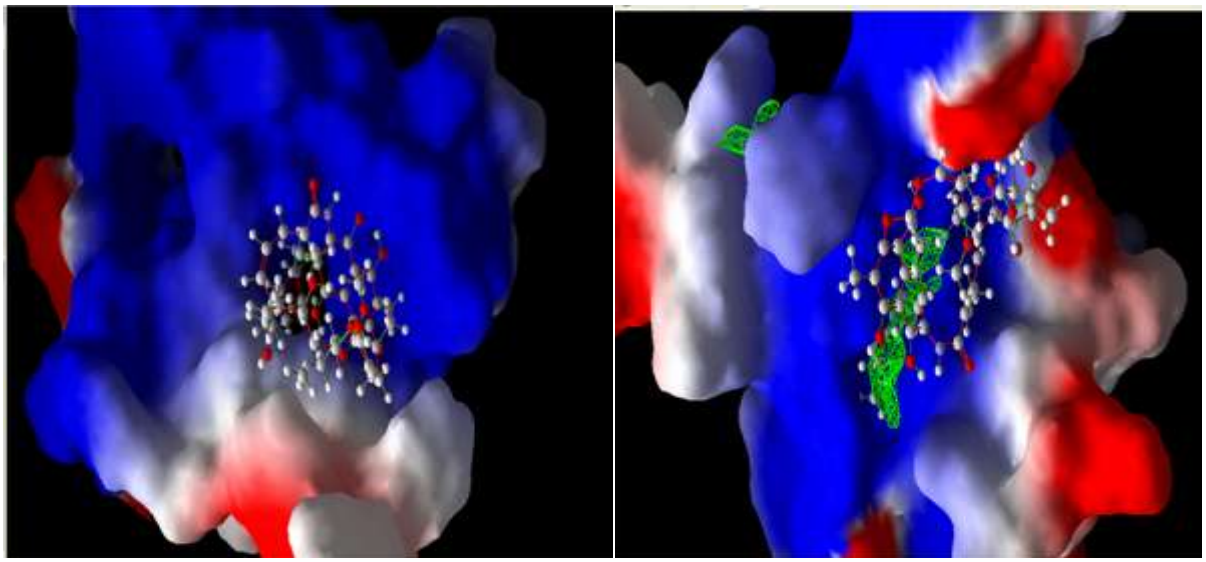
c) **d)**

e) **f)**

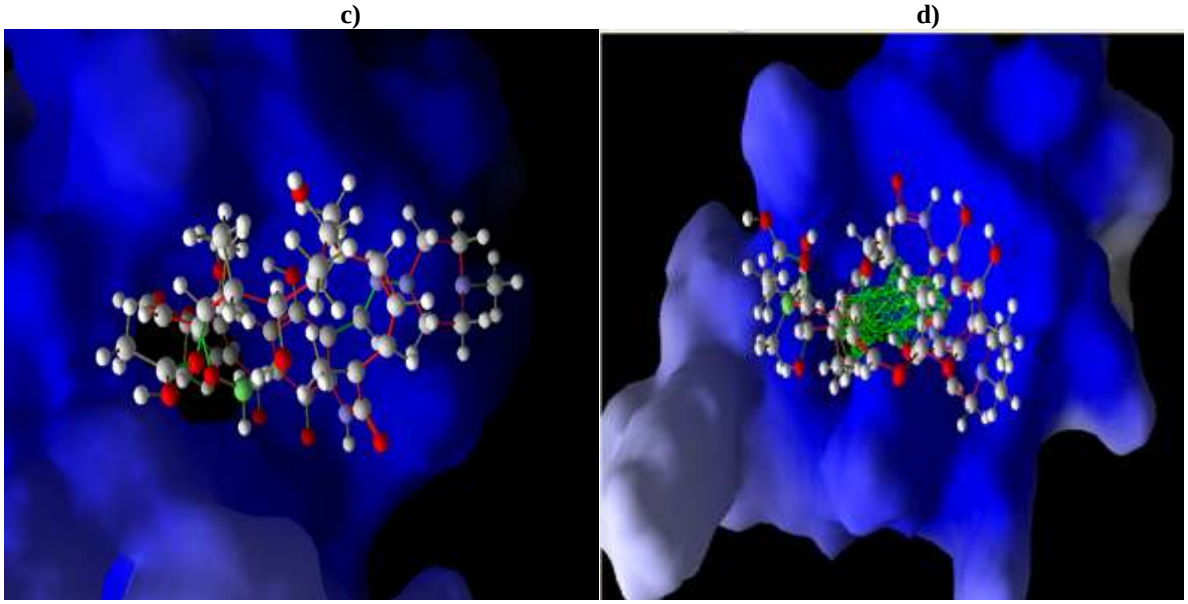
Figure II: The possible binding–sites of rpoB model and the grid formation around the cavities before start of docking. The active site region is represented in green. a) Z-4; b) WT; c) NM1; d) NM 2; e) NM 3; f) NM 4



a) **b)**

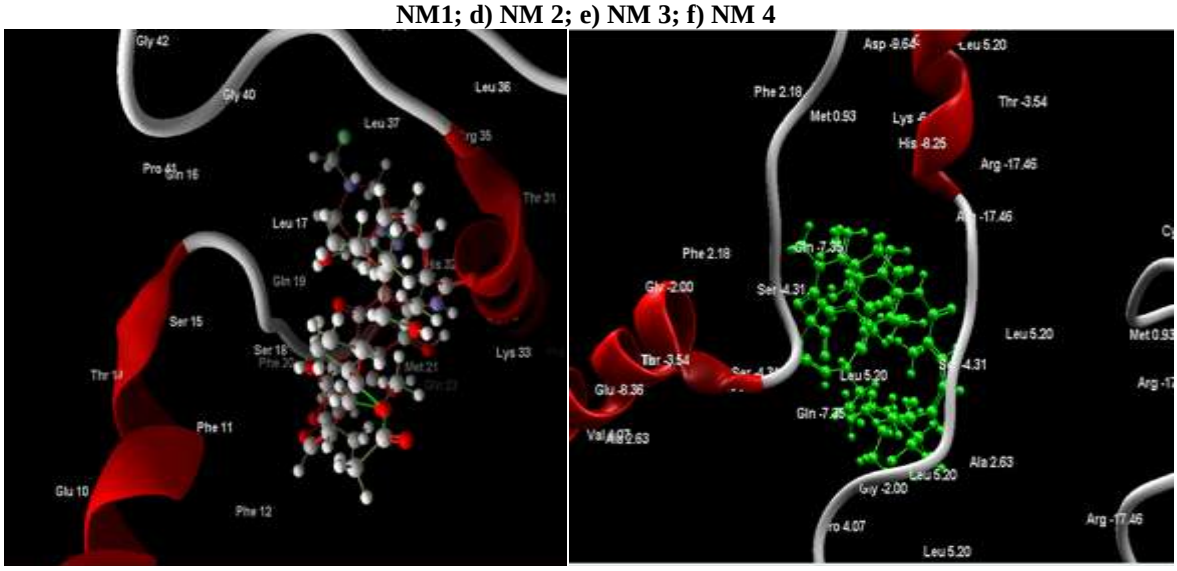


c) **d)**



e) **f)**

Figure III: Docking of Rifampicin in the cavity of receptor (rpoB) with: a) Z-4; b) WT; c) NM1; d) NM 2; e) NM 3; f) NM 4



a) **b)**

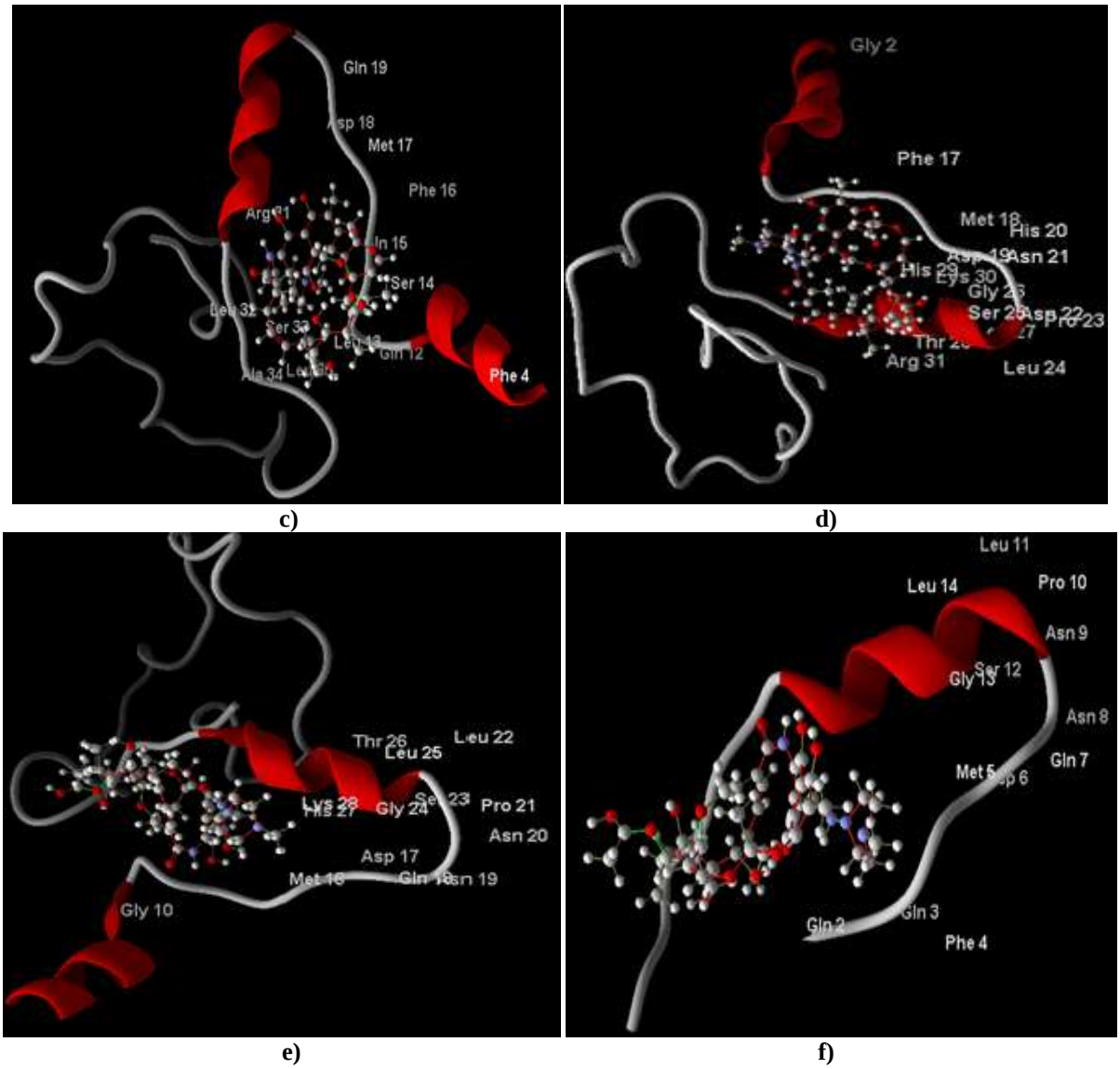


Figure IV: Receptor ligand interaction in Binding cavity with amino acid residues of: a) Z-4; b) WT; c) NM1; d) NM 2; e) NM 3; f) NM 4

Table I: Geometry of Binding cavities detected

S. No.	Sequence ID	Type of Mutation	Sequence Name	Gene Bank Accession No.	No. of Binding Pockets detected for each predicted structure of RpoB	Volume of the Binding Pocket(in Å)
1.	Z-4	Known High Level Resistant Mutant	Ser456Leu	Multidrug Resistant Reference Strain	3	44.032
2.	WT	Wild Type	Wild Type	Sensitive Reference Strain	3	20.48
3.	NM 1	Novel Mutation 1	Ile427Phe	SAMN04124504	4	56.32
4.	NM 2	Novel Mutation 2	Val424Gly; Gln442His	SAMN04124505	3	10.24
5.	NM 3	Novel Mutation 3	Ser434Gly	SAMN04124506	3	10.752
6.	NM 4	Novel Mutation 4	Ser437Gln	SAMN04124507	1	42.528

Table: II Measuring Parameters for molecular docking of Rifampicin with Native and Mutant RpoB

S.No	Sequence ID	Seq. Name	Ligand	Measuring Parameters (Energy in Kcal/mol)			
				Interaction Energy	Internal Energy	H Bond	Docking Scores
1.	Z 4	Ser456Leu	Rifampicin	-118.986	-17.9461	-6.75515	-147.483
2.	WT	Thai 53	Rifampicin	-185.1308	-21.4881	-3.19193	-115.799
3.	NM 1	Ile427Phe	Rifampicin	-132.649	-23.3363	-5.35977	-168.648
4.	NM 2	Val424Gly; Gln442His	Rifampicin	-146.238	-11.07	-7.118	-163.851
5.	NM 3	Ser434Gly	Rifampicin	-126.058	-19.8588	-10.0898	-148.989
6.	NM 4	Ser437Gln	Rifampicin	-102.835	-15.8771	-9.2623	-140.098

IV. CONCLUSION

We have used computational approach to study the interaction between RIF and rpoB in native and mutant models. Our *in silico* docking study revealed that novel mutation in rpoB at amino acid positions Ile427 Phe, Ser434Gly, Ser437Gln, might be involved in drug resistance. In wild-type rpoB, the RIF bind more effectively with rpoB with low binding energy and thus inhibits rpoB protein. These novel mutations in rpoB need to be explored further for understanding the resistance mechanism in *M.leprae*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

This study was approved by the Ethical Committee of The Leprosy Mission Trust India (TLMTI) and written informed consent was obtained from all patients and control subjects.

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