ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Structure based assessment of *rpoB* gene from multiple-drug resistant *Mycobacterium tuberculosis* clinical isolate

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Manuscript Info

Manuscript History:

Received: 12 August 2013
Final Accepted: 24 August 2013
Published Online: September 2013

Key words:
M. tuberculosis; *rpoB*; mutations; 3D modeling; docking.

Abstract

Tuberculosis, caused by Mycobacterium tuberculosis is one of the oldest known diseases and still is a major threat to humanity. The emergence of drug resistance strains of the bacterium has further worsened the situation and also seriously affecting the preventive and control measures against TB. Rifampin is one of the major drugs used in anti-tuberculosis chemotherapies. Mutations in the β-subunit of RNA polymerase are considered to be responsible for rifampin resistance in *Mycobacterium.*DNA was isolated from MDR clinical isolates of the bacterium from pulmonary tuberculosis patients and a hot spot region was amplified by using synthetic oligonucleotide primers TR8 and TR9. The amplified PCR product was sequenced and mutations were observed by comparing with H37Rv (NP_215181) reference strain. Nine mutant sequences, associated with rifampin resistance were submitted to DNA Data Bank of Japan (DDBJ). Structure based assessment was systematically carried out in this study to investigate how the sequence variations affect structure and function of the RpoB protein in MDR-TB strains which leads to variations in binding pattern of the anti-TB drugs to the protein and thus results in multiple drug resistance. 3D structure of the wild type *rpoB* gene product, which was not available previously (GenBank accession: NP_215181) was modeled through modeller 9.11. It was observed that the mutant amino acids vary in charge, size and hydrophobicity values. The position of mutation is different in all models and might be responsible for rifampin resistance. The wild type and mutated RpoB protein models were docked with rifampin through docking server to explore variations in their binding pattern. The mutant residues were not found in their preferred conformation, slightly destabilizing the local conformation and thus affecting the pattern of hydrogen bonding. Variations in hydrogen bonding distressed the rifampin binding that might cause the drug resistance in *Mycobacterium tuberculosis* clinical isolates. It is hoped that current study will provide significant information related to drug resistance and can be useful for the development of new potent drugs to inhibit the β-subunit of RNA polymerase.

Introduction

Tuberculosis is one of the major fatal diseases with global mortality rate higher than any other disease. During the last few years, prevalence of TB has been increased worldwide due to multiple factors including drug resistance and thus threatening more

lives [1]. According to an estimate, there were 9.6–13.3 million prevalent cases of TB in 2008-09 with more than 3.6% MDR-TB [2], a disease caused by *Mycobacterium tuberculosis* strains which are resistant to at least isoniazid and rifampin [3].

Modern medicines have been revolutionized by antibiotics and have saved millions of lives worldwide. Despite of this success, the growing problem of bacterial resistance is due to extensive use of antibiotics [4]. Rifampin is a potent inducer of both the hepatic and intestinal cytochrome P-450 (CYP) enzyme system and P-glycoprotein (P-gp) transport system that results in numerous clinically significant drug interactions [5]. The emergence of drug resistance in *Mycobacterium tuberculosis* is negatively affecting the treatment of TB and is also a threat to the progress made in curing and controlling TB worldwide [6]. It has become a considerable public health concern, as there are only few or even sometimes no effective antimicrobial agents available against such bacteria [7]. Mutations in hot spot region of *rpoB* gene encoding the β-subunit of RNA polymerase are considered to be responsible for rifampin resistance in *Mycobacterium tuberculosis.* It has been recently used as an alternative tool to identify MDR and non MDR Mycobacterium [8]. Comparative studies of *rpoB* from MDR-TB and wild type strains showed multiple mutations associated with rifampin resistance [9].

The application of bioinformatics tools have significant importance in managing large amount of biological data for better understanding and has enabled the researchers to extract more valuable information [10]. Bioinformatics tools are continuously increasing the knowledge at molecular level in different fields of biology. The *in silico* analysis includes database searching, quantitative structure-activity relationships, similarity searching, pharmacophore identification, computational modeling and docking [11, 12]. Such methods have been frequently used in the discovery and optimization of novel molecules with affinity to a target and physicochemical characterization [13].

Proteins are essential for both the structure and function of cells and viruses. They usually do not exist as linear polypeptides but as a compact and folded structure determining the protein functions. Structure prediction is therefore an important area of computational biology [14]. This study was carried out to explore how structural variations affect the binding pattern of RpoB protein in MDR-TB strains and will thus provide a better opportunity to enhance our perceptive about the antibiotic resistance conferred by different point mutations.

Material and Methods

Wet lab analysis

Clinical isolates of *Mycobacterium tuberculosis* were sequentially recovered from pulmonary tuberculosis patients from Ohja Institute of Chest Disease (OICD), Dow University of Health Sciences, Karachi. DNA was isolated from the MDR-TB strains by using the protocol described by Paolo et al (2006) [15]. A hot spot region of 157bp from *rpoB* gene was amplified by using synthetic oligonucleotide primers TR8 and TR9 [16-19]. Macrogen Company, Korea, sequenced PCR product of our amplified region. DNA sequence data was analyzed through applied bio system sequence Scanner v1.0 software, while mutations were observed by aligning all the sequences with the reference sequence (NP_215181) of reference strainH37Rv.

3D structure analysis

The 3D structure of RpoB reference sequence from wild type *Mycobacterium tuberculosis* with Gene Bank accession number: NP_215181 was modeled through comparative homology modeling by using Modeller 9.11 [20], which was not available previously and is a prerequisite for comparative structure analysis. All the sequenced mutations of *rpoB*, were substituted in the predicted 3D model of the reference sequence through MUTATE_MODEL [21]. All the predicted models improved through energy minimization and were refined through FG-MD (http://zhanglab.ccmb.med.umich.edu/FG-MD/) for quality enhancement. After fulfilling the basic criteria of energy minimization and refinement, the models were validated for quality assurance through ProSA-web [22], PROCHECK [23], ERRAT [24], WHATIF [25] and RAMPAGE 9 [26, 27]. The 3D structure of rifampin was retrieved from PubChem, a database maintained in NCBI [28]. The energy minimization of rifampin was conceded by MMFF94 force field and Gasteiger partial charges were added to the ligand atoms [29]. Non-polar hydrogen atoms were merged and rotatable bonds were defined.

Docking

The wild type H37Rv and nine mutant RpoB protein models were docked with the ligand rifampin to investigate the change in binding pattern responsible for drug resistance through AutoDock4.0. Essential hydrogen atoms, Kollman united atom type charges and solvation parameters were added to improve the AutoDock tools. Affinity (grid) maps of 20×20×20 Å grid points and 0.375 Å spacing were generated, using the appropriate Autogrid program [30]. AutoDock parameter set-and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were done through

Lamarckian Genetic Algorithm (LGA) and the Solis & Wets local search method [31]. Original position, orientation and torsions of the ligand molecules were randomly set. Every docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250,000 energy evaluations. The population size was set 150 during the search and a translational step of 0.2 Å quaternion and torsion steps of 5 were applied.

Results

Nucleotide sequences accession numbers: The new alleles found in this study have been submitted in DDBJ (DNA data bank of Japan) under accession no: AB711167, AB711168, AB711169, AB711170, AB711171, AB711173, AB711174, AB711175, and AB711178.

Structure analysis

For 3D structure, a high level of sequence identity usually ensures accurate alignment between the target sequence and template structure. Chain C of crystal structure of RNA polymerase holoenzyme from *T. thermophilus* at 2.6 Å resolution (PDB: 1IW7) [32] was selected as template from the BLAST search against PDB, having 50% identity and 94% query coverage. The modeled structure was subjected to molecular dynamics using CHARMM force field after energy minimization (Figure 3a). All the sequenced mutations were stepwise substituted in the native model through MUTATE_MODEL to get the mutant structures of RpoB protein. Graphical comparison of mutant model showed the variations as presented in figure 1. Mutations of RpoB were systematically analyzed to investigate the impact of structural variations on binding pattern with the rifampin inducing multiple drug resistance in the Mycobacterium strains. The position of each mutation in 3D structure is different while all mutations were detected in rifampin resistant clinical isolates, which might be responsible for drug resistance.

Docking studies with rifampin drug

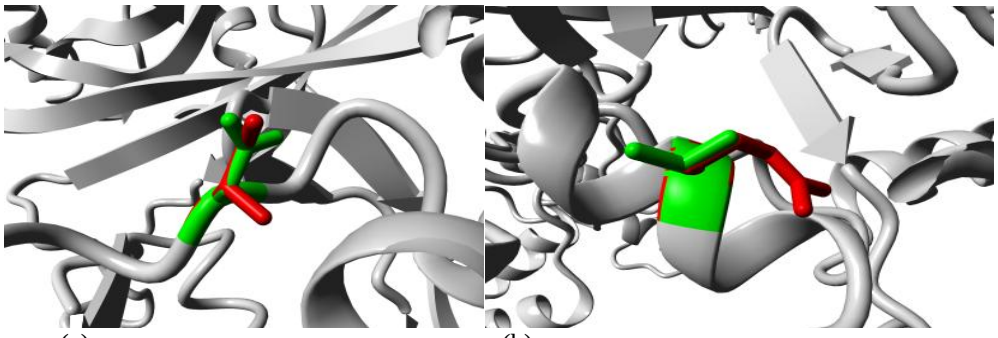
Computational investigation of docking score and interaction energy between ligand rifampin and *Mycobacterium tuberculosis* RpoB modeled proteins were carried out through AutoDock4.0 (Figure 3c) Docking simulations produced nine conformers for each docked complex out of ten docking runs using 2.0 Å RMSD tolerances. Docked complexes were carefully analyzed and the interactions were visualized through PyMOL and UCSF Chimera 1.6.1 software [33]. Hydrogen bond interactions along with binding distances were measured for the best conformers showed in Table. The negative value of electrostatic energy means enhanced interaction and vice-versa. Variations were also observed in the bonding patterns among the key binding residues of mutant and wild type residues (Table). Imprecise bonding pattern of mutated RpoB with rifampin, may lead to resistant in *Mycobacterium tuberculosis* against rifampin.

Table. Hydrogen bonding pattern of interacting residues in active site of rpoB proteins along with their distances and binding affinity of the best docked conformations

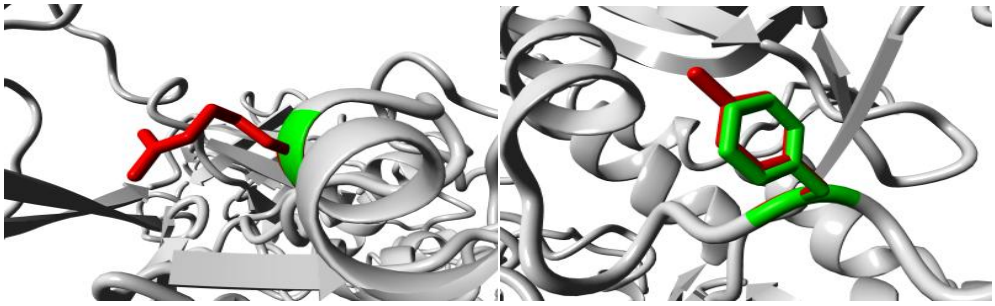
Type of structure	NHB	IR	LHB	BA
H37Rv-Model-Reference Strain	3	NE2 GLN 432 NE2 GLN 432 NH1 ARG 448	3.48 3.10 3.79	-8.4
AB711167 (N437I), (H445R)	4	NE2 GLN 432 OD2 ASP 435 NH1 ARG 448 O ASN 487	3.20 3.50 2.97 3.54	-8.7
AB711168 (S428R), (N437I), (H445R)	4	CB GLN 432 OD2 ASP 435 NH1 ARG 448 O ASN 487	3.12 3.49 2.92 3.86	-8.8
AB711169 (D435Y), (N437I)	4	NH1 ARG 448 NH2 ARG 448 ND2 ASN 487 NH1 ARG 487	2.95 3.27 3.19 2.85	-8.7
AB711170 (N437I), (N438D)	4	OD2 ASP 435 NH1 ARG 448 NH1 ARG 448 OD1 ASN 604	3.42 3.31 3.73 3.06	-8.1

AB711171-(N437I)	5	CB ASN 673 NH1 PHE 433 OD2 ASP 435 NH1 ARG 448 NH1 ARG 448	3.80 3.67 3.60 3.31 3.54	-9.3
AB711173-(H445L)	3	NE2 GLN 432 NE2 GLN 432 NH1 ARG 448	3.10 3.27 3.23	-8.3
AB711174 **(N437I), (L440M)**	3	CB GLN 432 OD2 ASP 435 NH1 ARG 448	3.28 3.40 2.89	-9.6
AB711175 **(M434I), (D435Y)**	5	OD1 ASN 604 O GLN 429 NH1 ARG 448 NH2 ARG 448 CB ASN 487	2.88 3.42 3.18 3.66 3.28	-9.4
AB711178-(H445R)	3	NE2 HIS 674 OD1 ASP 435 OD2 ASP 435	3.28 3.07 3.19	-9.6

NHB = No. of hydrogen bonds IR = Interacting residues
LHB = length of hydrogen bond (A^0) BA = Binding affinity (Kcal/mol)



(a) (b)



(c.) (d)

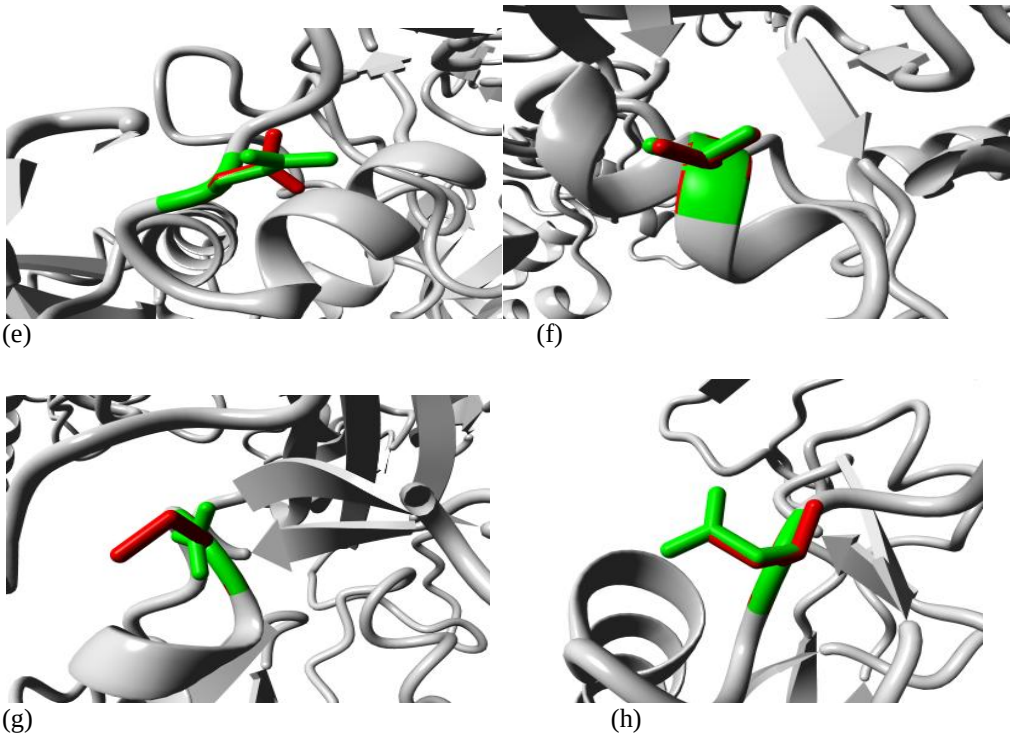


Figure 1. Close-up of the mutation (seen from a slightly different angle). The protein is colored grey, the side chains of both the wild-type and the mutant residue are shown and colored green and red respectively
(a) Mutation of Asparagine into Isoleucine(N to I) at position 437 (518).
(b) Mutation of Histidine into Arginine (H to R) at position 445 (526).
(c) Mutation of Serine into Arginine (S to R) at position 428 (509).
(d) Mutation of Aspartic acid into Tyrosine(D to Y) at position 435 (516).
(e) Mutation of Asparagine into Aspartic acid (N to D) at position 438 (519).
(f) Mutation of Histidine into Leucine(H to L) at position 445 (526).
(g) Mutation of Leucine into Methionine (L to M) at position 440 (521).
(h) Mutation of Methionine into Isoleucine (M to I) at position 434 (515).

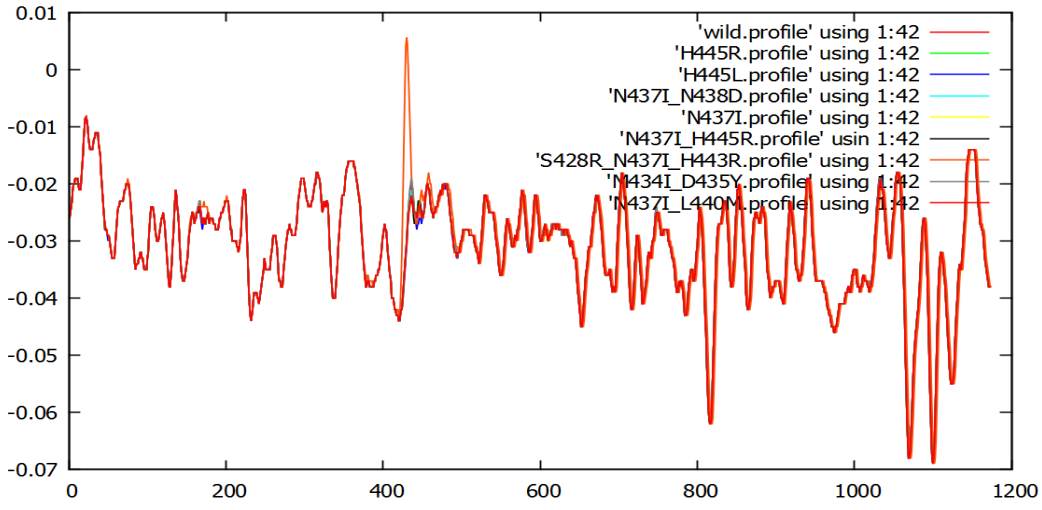


Figure. 2. Graphical comparison among the wild type (H37RV) and mutated RpoB 3D models, developed through gnuplot 4.6. Green circles are highlighting the variable regions

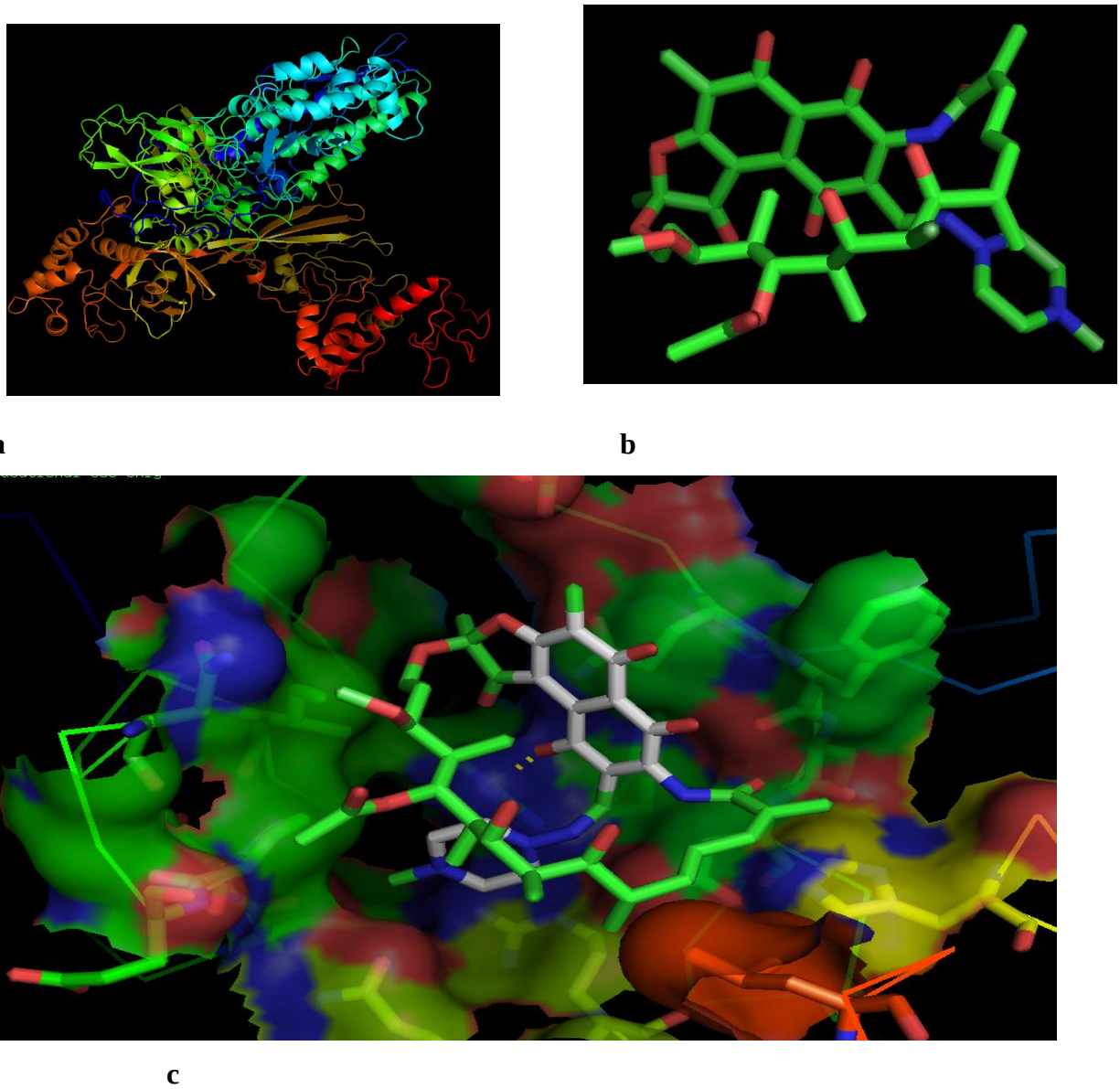


Figure. 2: Structural representation of rpoB enzyme and its inhibitor rifampin.
(a) 3D structure if rpoB enzyme in ribbon view.
(b) 3D structure of inhibitor rifampin.
(c) Binding of inhibitor to rpoB in the active pocket.

Discussion

The molecular basis and binding affinity of ligand molecule give us valuable information for the development of new potent drugs [31,34] against the rifampin resistant clinical isolates of *Mycobacterium tuberculosis.* We also studied the three dimensional spatial arrangement and conformational changes because of mutations at significant residues contributing in rifampin binding. We analyzed the 3D structure of eight different mutations positioned at different codon number of clinical isolates.

It was observed that the wild type residues were in their prefer conformation while the mutants preferred to be in other conformation, thus slightly destabilizing the local conformation [35]. Mutant residues are not of the same size with the wild type, which may affect hydrogen bonding between ligand and RpoB proteins (Figure 1). Green circles of

graphical presentation of 3D structure comparison showed the variations due to mutations in mutant models (Figure 2). Hydrophobicity of amino acids is another important factor that distresses hydrogen bonding. Mutations cause loss of hydrophobic interactions in the core of the protein-ligand complex. Binding residues are part of an interpro domain "RNA polymerase Rpb2", annotated with Gene-Ontology (GO) term GO: 0003677. The GO annotation indicates that the domain has a function in nucleic acid binding [36].

Structural arrangement and binding affinity of ligand and RpoB enzyme complexes were carefully and critically analyzed in docking calculations. Statistical data of mutant model is different as compared to wild type model that might be responsible for distressing the ligand binding affinity. Binding pattern of wild type RpoB indicates favorable and complimentary interactions for RpoB function. While the variable binding interaction of mutant residues, which are non- complimentary, might hampered enzyme function, causing drug resistance in Mycobacterium strains. In the docked complex, wild type RpoB protein (H37Rv) showed that the residues GLN432 and ARG448 made two and one single H-bond, with bond length of 3.48 Å, 3.10 Å and 3.79 Å respectively, which considered quite favorable bonding arrangement for drug function. In mutant models, we found different impact of two mutations at same codon 445, mutation H445L in AB711174 model showed similar attraction to H37Rv except the H-bond length and binding affinity. While different interacting residues were observed due to point mutations H445R in modeled sequence AB711178. This shows the impact of amino acid changes; different amino acids have different impact on binding attraction as both are the sequences from MDR clinical isolates. Furthermore, modeled sequence AB711167 has three point mutations S428R, N437I and H445R. Four H-bonds were found in this mutated model with 8.8 Å binding affinity, two new residues ASP435 and ARG448 were detected as interactive residues. The numbers of H-bonds have been increased up to five in modeled sequences AB711171 and AB711175, due to point mutations M434I, D435Y and N437I respectively. Similarly, number of H-bonds increased up to four with change in interacting residues e.g., AB711167-AB711170 (Table).

In this study, all mutated modeled showed changes in bonding residues, H-bond attractions, binding residues. We observed that number of H-bond increased in most of mutated models with low binding affinity, which showed the strong bonding. This strong bonding of rifampin might be unable to inhibit the RpoB functions [37]. The most important factors responsible for inhibition of drug function is change in binding residues and difference in H-bond pattern, as observed in all mutated models (Table). Resistance mechanism against anti-tuberculosis drugs, still poorly understood. Different approaches have been used for the identification of mutations associated with MDR-TB [38-41].

Our systematic computational analysis revealed that bonding pattren of wild type H37Rv and mutant models of RpoB were different which conferred that these variations would distort the enzyme function [36]. Affinity and structural arrangement of binding residues were found affected due to difference in size and hydrophobic properties of the mutant amino acids. Variations in hydrogen bonding distressed the rifampin functions that might cause the drug resistance in *Mycobacterium tuberculosis* clinical isolates. It has been hoped, that the knowledge gained from the current study will provide significant information related to drug resistance, level of resistance and virulence of strains.

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