Original Article

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TANAFFOS

Insights into Pyrazinamidase and DNA Gyrase Protein Structures in Resistant and Susceptible Clinical Isolates of *Mycobacterium tuberculosis*

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Background: Mutations in *pncA* and *gyrA* genes cause pyrazinamide (PZA) and fluroquinolone resistance in *Mycobacterium tuberculosis* (*MTB*). In the present study, structures of pyrazinamidase (PZase) and DNA gyrase proteins were studied in resistant and susceptible clinical isolates of *MTB*.

Materials and Methods: Sixty clinical isolates of *MTB* were used in this study. Polymerase chain reaction (PCR) amplification of *pncA* and *gyrA* genes was accomplished on purified DNA. Sequence of the fragments was determined by an Applied Biosystems™ apparatus. Bioinformatic analysis was performed by online software and three-dimensional (3D) structures of proteins was predicted using Molegro Virtual Docker (MVD) Modeler software.

Results: Amplified 744 and 194 bp fragments of *pncA* and *gyrA* genes, respectively were yielded suitable sequence results. Predicted 3D structures of proteins showed some differences between wild-type and mutant structures. Mutation in amino acid No.31 (T92C) caused an increase in distance from metal ion position to enzyme active site, but it was considered as a polymorphism. Docking results by MVD revealed a relationship in quinolone resistance-determining regions (QRDR) amino acids in interaction with antibiotic. T92C mutation in PZase from non-polar aliphatic amino acid Ile (ATC) to polar aliphatic amino acid threonine (ACC) was a polymorphism.

Conclusion: Structural changes in two important proteins related to drug resistance were proven in clinical isolates of *MTB*.

Key words: *Mycobacterium tuberculosis*, Pyrazinamidase, DNA Gyrase, Protein

INTRODUCTION

Pyrazinamide, a nicotinamide analog, is one of the most important first line drugs for treatment of *MTB* infections.

Pyrazinamide is bactericidal to *MTB* and reduces the tuberculosis treatment time (1), because PZA appears to kill at least 95% of the semi-dormant bacterial population, since its activity is present only in the acidic environment found in active inflammation (2). Pyrazinamide is a prodrug that must be activated by a bacterial PZase to the active form i.e. pyrazinoic acid, which is toxic for *MTB* (3,4).

Mutations in the *pncA* gene causing PZA resistance (5) occur along the entire *pncA* gene open reading frame and in its putative regulatory region (1). These mutations lead to loss of PZase activity and limit its effectiveness.

Any change in the *pncA* gene, may cause PZA resistance in these bacteria (6-11). Somoskovi et al. (6) in a study on six-resistant bacteria, found a mutation at nucleotide 11 -, pncA promoter. Their results was approved by Lee in 2001 (8). Somoskovi et al. (6) found a number of strains with mutations at nucleotides 70, 138, 139, 169 and 254. Perdigao et al. proved mutation at nucleotide 2 in 6 resistant samples in 2008 (5). They also found mutation at the nucleotide 374 in 11 samples. Sheen et al. in 2009 found mutation at nucleotides 143 on 3 resistant and one susceptible sample (10). They also found mutations at nucleotides 145, 152, 309 and 490 in pncA gene

Mutations in PncA are the main mechanism of PZA resistance in *MTB* isolates (8-11). The mechanism of PZA resistance in the *MTB* isolates from Brazil was shown by 22 changes in nucleotide sequence of *pncA* gene among the PZA-resistant isolates of *MTB* (12). On the other hand, in a study by Portugal et al. in 2004, strains that both nucleotides 359 and 374 contain mutations leading to resistance to Pyrazinamide greater than the other strains. They found that more than 42% of the mutations occurred exclusively in both nucleotides 359 and 374 (11).

During the process of replication and transcription, the opening DNA leads to the production of positive and negative super coils. Topoisomerase openes these supercoils that are created in front of replication forks.

Gyrase enzyme can be attached to a piece of DNA called G segment. ATP binding leads to a near two-domain enzyme and changes its three dimensional structure. Then, it cuts both strands of a DNA G segment and connects the other two strands of the T segment. ATP binds to the two ATPase domains of Topoisomerase II and dimerises them. After performing the reaction, ATP hydrolysis resets the topoisomerase for a new reaction cycle. The enzymatic cutting is done by the attack of a tyrosine to phosphate quinolones, which target the two bacterial type II topoisomerases and exert their antibacterial activity by interfering with the enzymatic reaction cycle. These drugs bind to a complex composed of topoisomerase and DNA and form a covalent bond (tyrosyl-DNA phosphate). The resulting ternary complexes block DNA replication and lead to cell death (12-17).

In *MTB*, subunit A of topoisomerase II is encoded by gyrA gene. Effective mutations in gyrA gene cause resistance to quinolones. These powerful antibiotics interfere with enzymatic reactions of topoisomerases. The resulting ternary complexes block DNA replication and lead to cell death.

This study aimed to assess the PZA and DNA gyrase structures in resistant and susceptible isolates of *MTB*. The main implication of this study was to comprehensively understand the actual mechanism of resistance to PZA and quinolon antibiotics in clinical isolates of *MTB* for effective treatment of patients.

MATERIALS AND METHODS

Bacterial Samples

A total of 60 clinical strains of *MTB* were isolated from sputum samples of patients with tuberculosis. All specimens were processed for drug susceptibility test-proportion method. DNA Extraction was carried out by Chelex 100 (Sigma, USA) method.

PCR Amplification of *pncA* and *gyrA*

The extracted DNA was amplified using *pncA* primers (Pnc-8 :5'-GGTTGGGTGGCCGCCGGTCAG-3', and Pnc-11: 5'-GCTTTGCGGCGAGCGCTCCA-3') in an authorized thermal cycler (Eppendorf) (12).

A 194 bp DNA fragment of *gyrA* gene of *MTB* isolates was obtained in order to perform subsequent sequencing method. The oligonucleotide primers were as gyrA-F: 5'-CGATTCCGGCTTCCGCCCGG-3', gyrA-R: 5'-CCGGTGGGTCATTGCCTGGCG-3' that produced a product size of 194 bp in annealing temperature of 68°C (18).

The PCR reaction in a total volume of 50 µL of a reaction mixture, containing 10 µL purified DNA, 5 µL 10x Taq Buffer (contained $(NH_4)_2SO_4$ and 20 mM $MgCl_2$), 1 µL of deoxynucleoside tri-phosphates mix (dNTPs), 10mM each (Fermentas R0192), 1 U *Taq* polymerase (Cinagene) and 25 pmols of each set of primers (Cinaclone) was accomplished. Forty cycles started at 94°C for 40 seconds,

68°C for 1 minute and 72°C for 20 seconds and a final cycle at 72°C for 10 minutes were selected to complete the elongation of the PCR intermediate products.

Gel electrophoresis method was accomplished for the detection of gene bands on agarose gel (Merck Darmstadt, Germany) for 1.5 hours through 1.5% agarose gel in 1x TAE buffer with addition of ethidium bromide and polyacrylamide gel (Merck Darmstadt, Germany). In this manner, standard DNA marker of GeneRuler (50bp-Fermentas) was used for measuring the size of the bands and was observed in a UV Trans illuminator and photographed using a Geldoc.

DNA Sequencing

The purified PCR product was sequenced in an automated DNA Sequencer ABI PRISM® 310 Genetic Analyzer. The nucleotide sequences obtained were aligned and analyzed by Chromas, BLASTx and MEGA software to identify the amino acid changes in comparison with the wild type *MTB* (H37Rv).

Bioinformatic analysis

Nucleotide sequence alignments were carried out using the Mega4 and Chromas software programs. Dimensional structures of mutant proteins were predicted by MVD Modeler v9.13 software as comparative modeling; and in this case we used the crystal structure of wild type proteins as template (PDB=3PL1 for pncA, & PDB=3ILW for gyrA) and after that the energy of predicted models were minimized and optimized by YASARA server. In the next phase, the differences between wild and mutant type proteins and proteins with different point mutation evaluated by Molegro Virtual Docker (MVD) software. Furthermore, we used this software to calculate the binding affinities between proteins and some specific antibiotics such as Moxifloxacin (CID_15294) and Ofloxacin (CID_4583).

RESULTS

Sixty clinical isolates of *MTB* were studied to detect any mutations in the *pncA* gene. *In vitro* susceptibility testing showed that 39 of these isolates were resistant to PZA. The PCR product band of 744 base pairs (bp) was observed on a 2% agarose gel and confirmed the suitable amplification of the *pncA* gene of *MTB*. Furthermore, 194 bp band of a *gyrA* fragment containing quinolone resistance-determining regions (QRDRs) was identified by electrophoresis.

The *pncA* and *gyrA* fragments was sequenced and mutations were detected (Figure 1). The *pncA* protein encoded by these genes in both the presence and absence of mutations was evaluated. Analysis of the all protein's 3D structures by MVD software showed differences of wild-type and mutant structures of two proteins. This software predicted these structures with a high assurance (70%).

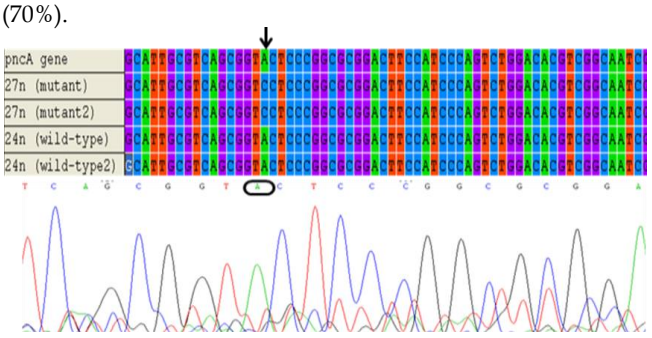

Figure 1: Sequence of *pncA* gene in wild-type and mutant strains of *MTB*

According to Figure 2, the change in amino acid No. 31 (related to T92C nucleotide change) compared to wild-type condition increased the distance from metal ion position to enzyme active site.

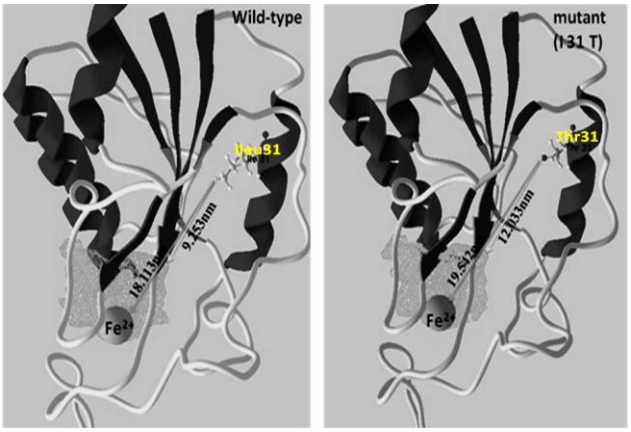

Figure 2. Comparison of wild type and polymorphism at position 31.Substitution of amino acid 31 of PZase enzyme, increases distance from active site and metal ion position of the enzyme. However, any change in I31T would be polymorphism.

In accordance with the distance and position of active protein, and no correlation between amino acid 31 and other amino acids in the active site position, structural

stability of mutation is acceptable; therefore it can be considered as a polymorphism. Comparison of these distances is shown in Table 1.

Identification of the secondary structures and amino acids involved in the activation of gyrase protein was performed by MVD (Figure 3). Alfa-helix and beta-sheet were shown in amino acid sequencing (Figure 3) as bold lines and the arrows, respectively.

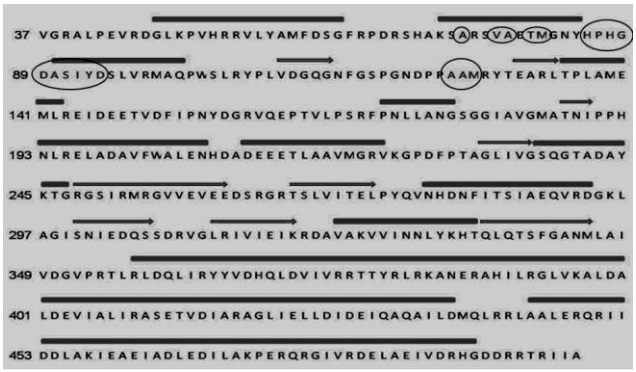

Figure 3. A part of amino acid sequence of QRDR of gyrase protein. The bold lines are alfa-helix, the arrows are beta-sheet and circles are amino acids involved in creating the hydrophobic holes and bond to drugs.

The circles in Figure 3 are the amino acids involved in creating the hydrophobic holes and bond to the medicine.

Study of data docking results using MVD, indicated a relationship between the main amino acids in the QRDR area of gyrase protein such as H85, P86, H87, G88, D89, A90, S91, I92 and D94 in the process of connecting to the antibiotics (Figure 4).

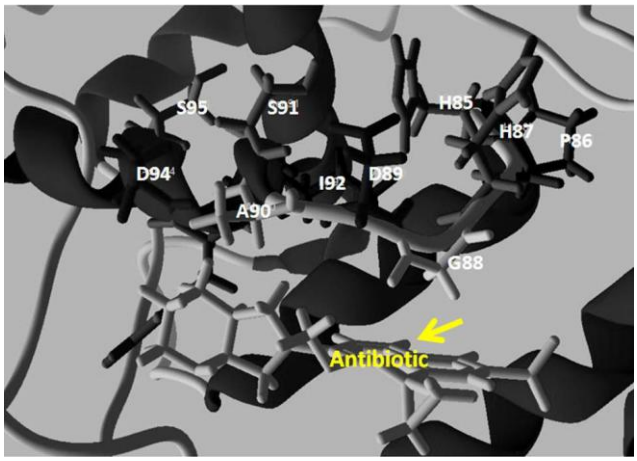

Figure 4. Amino acids involved in interaction with antibiotics in QRDR area of gyrase protein

Gyrase enzyme has two different alpha subunits, which orientates side-chain amino acids such as Y93 yielding a site suitable for binding of substrate. Antibiotics would occupy this position (Figure 5).

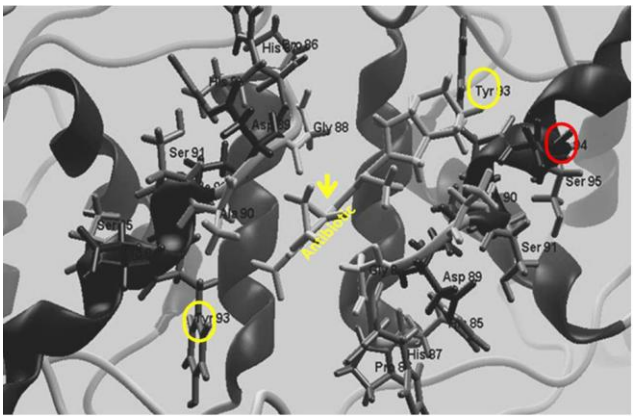

Figure 5. The amino acids involved in the process of connecting two alpha subunits of gyrase to antibiotic.

Distance and orientation of the amino acids can be considered as criteria for their importance in activity of the enzyme (Figures 6, 7).

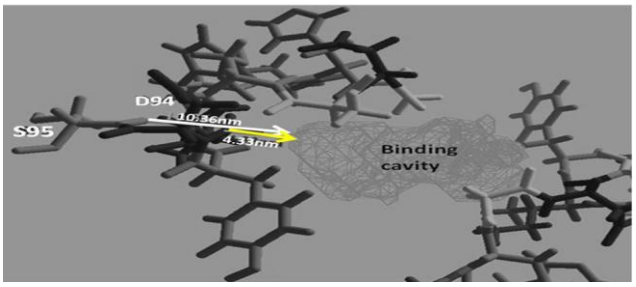

Figure 6. The distances of D94 and S95 from active site of gyrase

Figure 7. Global view of gyrase and its interaction with drug. Quinolones occupy binding sites of DNA (G-Segment) and inhibit enzyme activity

Table 1. Data from bioinformatic analysis using MVD software

Effect on protein activity	Distance to cavity		Distance to the metal ion		
	Mutant	WILD-TYPE	Mutant	WILD-TYPE	
Mutation (to inhibit enzyme activity)	23.0768nm	19.9544nm	26.6282nm	24.1225nm	Mutant (V125 G)
Polymorphism (no effect on enzyme activity)	12.033nm	9.253nm	19.542nm	18.111nm	Mutant (I 31 T)

As shown in Figure 6, due to long distance and specific orientation of amino acid 95 compared to amino acid 94, any change at 95 would be polymorphism and not related to resistance.

DISCUSSION

In this study, amplification of the two *pncA* and *gyrA* gene fragments, was sequenced and the detection of mutations in the strains was examined, then the protein encoded by these genes in both the presence and absence of mutations was evaluated. Careful study of the amino acids involved in the position of mutations in the gene encoding Pzase suggests that the changes in this gene as a T92C mutation, non-polar aliphatic amino acid Ile (ATC) is converted to polar aliphatic amino acid threonine (ACC) (Figure 8).

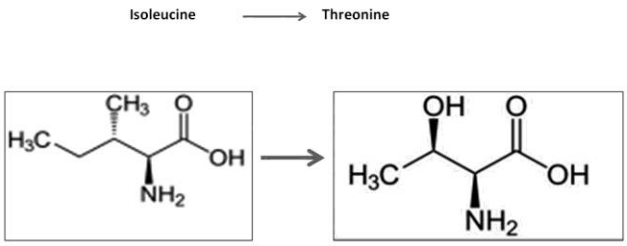

Figure 8. The differences between side-chain of isoleucine and threonine

In the gene encoding PZase (pnc-A), many changes occurred but not all of them were mutations. Mutation of amino acid 92 from Isoleucine, a non-polar amino acid, to a polar aliphatic amino acid threonine as T92C was a polymorphism. In aqueous environments, threonine has a hydroxyl group (OH) capable of establishing hydrogen bonds with water molecules, but isoleucine does not have this capability. In comparison with T92C, mutations in the positions of 359 and 374 that change the hydrophobic amino acids leucine and valine, into proline and glycine had higher effects on protein structure. Despite these amino acids that lead to creation of loops or turns in the secondary structure of a polypeptide, threonine and isoleucine are different only in their polarity and the reaction with their surrounding environment. Ultimately T92C is a polymorphism and does not cause structural changes or resistance to PZA.

The type of amino acid involved in the creation of the position polymorphisms in gyrase proteins in sensitive samples shows that at codon 95, serine changed to threonine. Both of these amino acids have hydroxyl group (-OH) at their side-chain and both are in a family of amino acids with aliphatic and polar side chains. Considering the same structural features, conversion of serine to threonine does not change the three dimensional structure of protein and these changes can be considered as polymorphisms.

According to bioinformatics analysis, the change in amino acid No. 31 (related to T92C nucleotide change) compared to wild-type condition increased the distance from metal ion position to enzyme active site. These parameters are shown in Table 1.

Figure 5 shows the interaction between antibiotic and two connection holes, which were created by two alpha subunits getting closer. Drug has occupied the position for connecting to DNA and prevents the enzyme activity for opening the DNA super coils. Consequently, the bacterium will die. Amine acids involved to create the active site are D94, G88, Y93, D89, A90, M127 and M81. Amino acids involved to create the holes show that 94 is a key amino acid to allow the interaction due to its close distance to the position. Any changes in 94 cause resistance and prevent

the bond. On the other hand, amino acid 95 has no role in this bond (due to its far distance from the position); therefore, changes in that will not be considered as mutation, and will not create drug resistance.

According to Figure 6, the distance of the amino acid serine 95 from cavity is approximately 2.5 times the amino acid 94 and its orientation is such that it cannot play a role in drug binding. Also, it is direct interaction with 95 codon of cavity. Mutations in this area will result in polymorphisms and do not affect the enzyme activity. Gyrase is a multi-subunit protein. Active site of enzyme is created by two subunits together. One of the important amino acids in the junction of DNA at the active site is amino acid tyrosine 93. The result of the creation of this junction is that both the tyrosines are located in front of each other at the position of active site. Quinolones target and bind to topoisomerases and interfere with their action (18). The resulting ternary complexes block DNA replication and lead to cell death (7, 14). As it can be illustrated, distance and direction of amino acid 95 is in a way that it cannot be involved in drug bonding, and the mutation here will be polymorphism; in contrast, amino acid 94 is one important involved amino acid in drug bonding with DNA.

Considering the occurrence of peptide bonds between theY93 and D94, distances and directions in formation of these bonds, it was suggested that these two amino acids were placed in enzyme active site. Furthermore, any changes in codon 94 would be changed the distances of peptide bonds (θ, ψ) and orientation of tyrosine 93 within the enzyme active site. Validation of detected changes with sequencing method in the isolates with mutated phenotypes can be showed accurate of these predictions.

CONCLUSION

In this study, alterations in structural proteins of Pzase and quinolone were found to be related to drug resistance in clinical isolates of *MTB*. Any changes leading to polymorphism or drug resistance validated by sequencing were predicted in the proteins by the bioinformatics tools. This finding are important for more effective diagnosis and treatment of tuberculosis.

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