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Mutation in *pncA* and distortion in PZase model structure as a basis of pyrazinamide resistance in *Mycobacterium tuberculosis*

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ABSTRACT

Basis of pyrazinamide (PZA) resistance in M. tuberculosis is commonly associated with mutations in the pncA gene encoding pyrazinamidase (PZase) enzyme. The complete pncA open reading frame of 0,6 kb from R2 and R6 clinical isolate of PZA-resistant Mycobacterium tuberculosis has been characterized. A group mutations of T40C, G419A and A535G which link to amino acid replacements of Cis14Arg, Arg140His, Ser179Gly was identifed in pncA gene from the R2 PZA-resistant Mycobacterium tuberculosis, meanwhile a G511A mutation which replace amino acid of Ala171Thr was found in pncA gene from the R6 PZA-resistant strain. The structure modelling study to determine the effect of amino acid substitutions in the R2 and R6 PZase was carried out. Superposition of the generated model for R2 and R6 PZase with the wild-type PZase structure gave root mean square deviations (RMSD) of 0.24 and 0.23˚A respectively, suggesting highly similar structures. However, the mutant structures of R2 and R6 PZase amended several interactions in its wild-type structure, expecially in the regions that contain the catalytic residues. The physical-chemical changes around the active site may be unfavorable for R2 and R6 PZase activities, and in turn create PZA resistance in both M. bacterium strains.

Key words: *pncA* gene, PZA resistance, PZase, *Mycobacterium tuberculosis*

INTRODUCTION

Tuberculosis (TB) is a bacterial infectious disease, caused by *Mycobacterium tuberculosis*, which primarily affects the lungs. TB is the second leading killer in adults and classified as the most deadly of all infectious diseases. The disease has a serious health problem in the world especially in developing countries. The World Health Organization (WHO) has recorded Indonesia as a "high burden country" for tuberculosis, occupied the highest fifth rank of the 22 countries considered to be high burden. There are about 500,000 new cases of TB annually and 175,000 of them are deaths in Indonesia [1]. Of the TB cases in Indonesia, about 2 percent of new cases and 12 percent of the recurring cases are the multidrug-resistant strain [2]. The high incidence of multidrug resistant TB pose a serious health problem, because patients have lower cure rates and higher mortality than patients with drug-susceptible TB. Mortality rates in these outbreaks of multidrug-resistant tuberculosis has been generally recorded > 70%, with most fatalities occurring among patients infected with human immunodeficiency virus [3].

Pyrazinamide (PZA) is a mainstay drug used to treat TB, due to its ability to kill a population of semidormant tubercle bacilli that reside in an acidic environment without being influenced by other TB drugs. Along with isoniazid (PZA), rifampin (RMP), and ethambutol (EMB), PZA is part of the currently used shortcourse treatment regimen [4]. PZA, an analog of nicotinamide, is a prodrug that transformed by bacterial pyrazinamidase (PZase) to active form, pyrazinoic acid (POA) in order to affect the tubercle bacilli [4,5]. *Mycobacterium tuberculosis* strains that have no PZase activity, are resistant to PZA [6]. The correlation of loss PZAase with PZA resistance remains unclear, because the *pncA* deficient in PZA-resistant *M. tuberculosis* strains which remove completely the PZase activity are rare. Although insertion, deletion, and frameshifts in *pncA* gene can induce the complete loss of the

functional gene product, then assosiated with high levels of PZA resistance, but the majority of the mutations identified in clinical isolates are single point mutations that result PZA resistance in intermediate levels [5,7].

The profile mutations in the *pncA* gene is unique in PZA-resistant mycobacterial isolates from different geographical regions [8]. Determination of the frequency of mutations underlying drug resistance in isolates from different geographical areas is important for the basis to make genetic marker for rapid means of detecting drug resistance in clinical isolates and also to study resistance mechanism. This paper illustrates the moleculer of *pncA* gene in PZA-resitant *M. tuberculosis* from two Indonesia clinical isolates, the correlation between the *pncA* mutation with PZA resistance level, and the link of model stucture of PZA mutant with PZA resistance.

EXPERIMENTAL SECTION

Bacteria and plasmid

Two isolates of PZA-resistant *M. tuberculosis* (R2 and R6) and an PZA-sensitive isolate named as H37Rv strain were obtained from Health Research Center, Bandung, Indonesia. The TOP10 *Escherichia coli* strain (Invitrogen, Maryland, USA) was employed for cloning host of the *pncA* gene. The pGEM-T plasmid (Invitrogen, Maryland, USA) was used as a vector for cloning of *pncA* gene.

Bacterial growth conditions

M. tuberculosis was grown in a solid medium, named as Lowenstein-Jensen (LJ) at 37ºC for 2 weeks until be found single colony. The LJ medium is composed by egg suspension 60% (v/v) ; malachite green 1% (w/v); glycerol 0,8% (v/v); KH2PO4 0,2% (w/v); $MgSO_4$.7H20 0,02% (w/v); and citrate magnesium 0,04% (w/v). While the *E. coli* was grown in Luria broth (Difco) at 37°C. Wherever necessary, 100 µg of amphicilin/ml was added to the medium.

Chromosomal DNA isolation

Chromosomal DNA of PZA resistant *M.tuberculosis* and H37Rv strain were isolated with alkali lyses method using *wizard genomic DNA purification kit (Promega)*. According to the kit protocol, the *M.tuberculosis* cells were suspended in 480µl 50mM EDTA, then added 120µl lysozyme and incubated at 37ºC for 1 hour. After that, it was centrifuged for 2 minutes at 13.000g and the supernatant was discharged. Concerning to cell pellet was added by *Nuclei lysis Solution* and *Protein Precipitation solution* solvent, incubated for 5 minutes at 80oC, then cooled to room temperature. The mixture then was added by 3µl Rnase, incubated at 37ºC for 15 minutes. Furthermore, it was done adding of 200µl of protein precipitation solution to the mixture, inubated on ice for minutes, then centrifuged for 2 minutes at 13.000g. The supernatant was transfered to a clean tube containing 600µl isopropanol, then centrifuged for 2 minutes at 13.000g to precipitate the cromosomal DNA. After the DNA was washed by 600µl of 70% ethanol, it was solved in 100µl of rehydration solution. The chromosal DNA then was used as template for PCR.

DNA Amplification

The amplification of a full length of *pncA* gene was performed by using *pncA*F and *pncA*R primers that have sequences: 5'-GAG *CAT* <u>*ATG* CGG GCG TTG ATC ATC</u>-'3; and 5'-GA*A GAT* <u>*CT*G GAG CTG CAA ACC AAC</u> TC –'3 respectively. The primers were constructed having nucleotides adapter for an NdeI restriction sites in *pncA*F and BglII restriction sites in *pncA*R. The restriction nucleotides are marked by italic fonts. The reaction mixture for PCR was created in 25 µL volume which consists of 2,5 pmol of each primer, 50 ng of template DNA, 1,25 unit *Taq*-DNA polymerase, 1x PCR buffer (20 mM Tris-Cl, pH 8.4, 50 mM KCl), and 0,2 mM dNTPs. PCR process was runned by *DNA-thermal Cycler* machine, consisting of an initial 10 min denaturation and enzyme activation at 94°C followed by 25 cycles of 94°C denaturation for 1 min, 54ºC 1 min, and 72°C elongation for 2 min and a final 7-min elongation. The PCR products were analyzed in agarose gel electrophoresis and purified by GFX purification kit (Amersham, New Jersey, USA).

Gene Cloning

The PCR-produced *pncA* gene was ligated to a pGEM-T vector in the reaction mixture that consisted of 1µl *pncA* gene, 1 µl pGEM-T, 5µl of 2x ligase buffer, 1µl T4 DNA ligase and adjusted with ddH2O to a final volume of 10 µl. The ligation reaction was conducted at 4ºC for 16 hours. The product of this reaction then was transformed into *E. coli* TOP10 using the CaCl2 method [9]. Transformant cells were screened at agar plate containing Luria-Bertani (LB) medium, 100 µg/mL ampicillin, and 5-bromo-4-chloro-3-indolyl-*β*-D-galacto-pyranoside. Some white colonies of transformants that grown in the medium were selected as bacteria cell for isolation of a pGEM-T-*pncA* recombinant. Furthermore, the DNA recombinant was characterized by a resctriction analysis and PCR.

Restriction analysis

Luria-Bertani broth (50 ml) containing amphicilin (100µg/ml) was inoculated with a fresh colony of *E. coli* TOP10 containing pGEM-T-*pncA* recombinant and grown at 37°C overnight. The *E. coli* cells were collected by cold centrifugation at 12,000×g for 10 min. The recombinant plasmid was extracted from the cells using QIAgen Spin Plasmid Miniprep Test Kit (Qiagen, Santa Clarita, USA), and digested with the restriction enzyme, NdeI.

DNA sequencing and analysis

Automated DNA sequencing was performed by dideoxy-Sanger method at First BASE Laboratories Sdn Bhd, Selangor, Malaysia. All of *pncA* genes were sequenced with the primers not only used for amplification, *pncA*F and pncR, and but also used M-13 and T7-promoter primers that reside in part of pGEM-T vector flanking of *pncA* gene. Sequence analyses were conducted with DNA Star Software using programs of SeqManTMII for DNA sequence and MegAlignTM for the amino acid sequence [10]. All sequencing runs included the PZA-susceptible strain *M. tuberculosis* H37Rv (ATCC 27294) as a wild-type control. Each sequence was compared with that of both the control strain and the appropriate published sequence.

Modeling of the three-dimensional structure of PZase

A model of the PZase mutant of *M. tuberculosis clinical isolate* was constructed by SWISS-MODEL [11] on the basis of the known three-dimensional structure of PZase from PZA-sensitive *M. tuberculosis H37Rv*, which has a number access "3PL1.pdb" at Protein DataBank. The superposition of the generated and 3PL1 structures was done using SuperPose version 10 [12]. The model structures of mutant PZase was visualized in PyMOL 1.3 [13].

RESULTS AND DISCUSSION

The chromosomal DNA of two PZA-resistant (R2 and R6) and a PZA- sensitive *M. tuberculosis* (H37Rv) were isolated by Wizard genomic DNA purification kit (Fig 1), then each of the chromosomal DNA was used as template DNA for *pncA* gene amplification. The coding region of *pncA* gene from each of *M. tuberculosis* strain was amplified by PCR using *pncA*F and *pncA*R primers, generated a DNA fragment at 0.6 kb in agarose gel electrophoresis (Fig 2). The PCR-producted DNA fragment showed a similiar size with the *pncA* of *M. tuberculosis* H37Rv that deposited in GenBank (ID number: 888260), which is 561 bp. Futhermore, the 0,6 kb PCR fragment of each *M. tuberculosis* strains was cloned into pGEM-T plasmid, a linearized vector with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly made a compatible overhang for PCR product that runned by Taq polymerase. Characterization of pGEM-T containing *pncA* gene was performed by single digestion with NdeI restriction enzyme to give a fragment of 3.6 kb (Fig 3, lanes 3–4), which corresponds to the size of the plasmid harbouring the *pncA* gene. The present of *pncA* in pGEM-T plasmid was also confirmed by PCR using *pncA*F and *pncA*R primers to generated a DNA fragment, 0,6 kb (Fig 4, lanes 1–2). The results showed that each *pncA* gene from two PZA-resistant *M. tuberculosis* strains (R2 & R6) and a PZA-sensitive *M. tuberculosis* strain (H37Rv) have successfully been inserted into pGEM-T vector.

Table 1. Characteristics of PZA-resistant *M. tuberculosis*

Gene	Clinical *M. tuberculosis*	Mutation				Level of PZA Resistance [µg/mL]
		Σ	Nucleotide	Σ	Amino acid	
pncA	R2	3	T40C G419A A535G	3	Cys14Arg[a] Arg140His[a] Ser179Gly	40
	R6	1	G511A	1	Ala171Thr[a]	10

[a] new mutations have not found in publications

Fig.1. Electropherogram of crhomosomal DNA of *M. Tuberculosis*
Lane 1: PZA sensitive M. tuberculosis strain (H37Rv); lanes 2-3: PZA-resistant M. tuberculosis strains (R2 & R6)

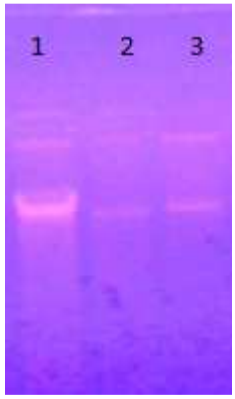


Fig.2. Amplification products of the *pncA* gene using PCR
Lane 1, 100 bp DNA marker; lane 2, negative control of PCR; lane 3-4, fragment DNA (0.6 kb) of PZA-sensitive M. tuberculosis H37Rv; lane 5-6, fragment DNA (0.6 kb) of PZA-resistant M. tuberculosis R2 & R6

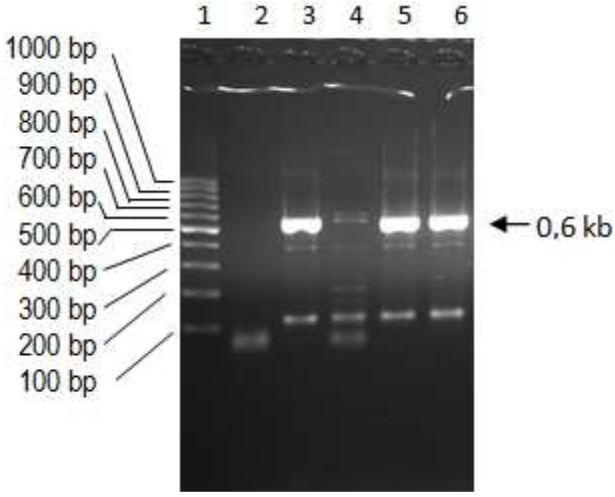


Fig. 3. The profile of restriction analysis of recombinant pGEMT-*pncA*
Lane M, marker DNA λ/HindIII; Lane 1-3, pGEM-T-pncA (H37Rv, R2 and R6 respectively) was digested by NdeI enzymes, resulting a DNA fragment of 3,6 kb that exhibite the combinantion of pGEM-T 3.0 kb and the pncA fragment 0.6 kb

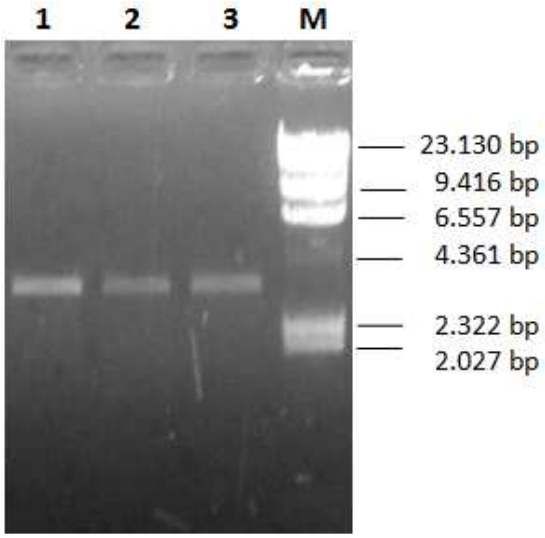


Fig. 4. Amplication products of *pncA* gene in recombinant pGEMT-*pncA* as template using PCR
Lane M, 1 kb DNA marker; lane 1-2, fragment DNA (0.6 kb) of H37Rv isolate; lane 3-4, fragment DNA (0.6 kb) of R2 isolate; lane 5-6, fragment DNA (0.6 kb) of R6 isolate

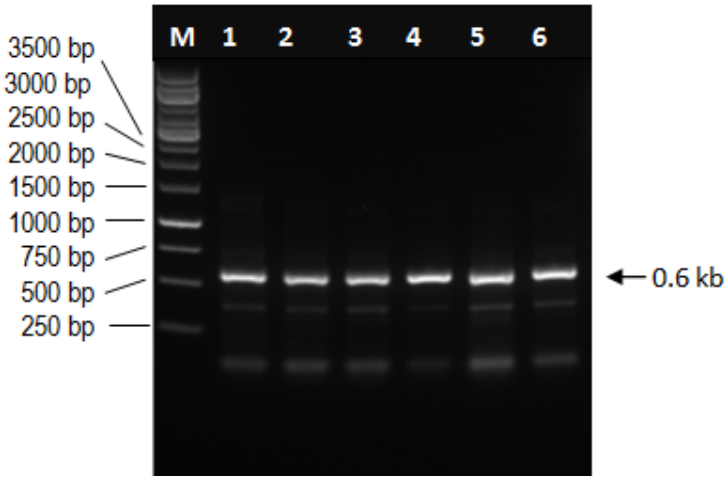
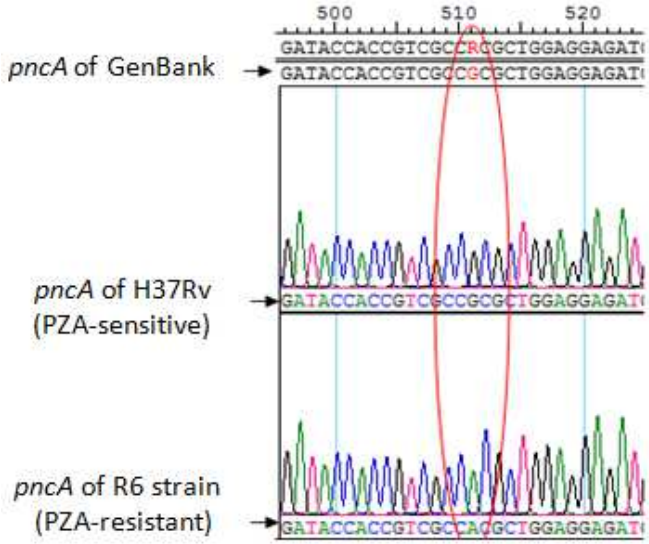


Fig.5.The alignment of *pncA* of PZA-resistant *M. tuberculosis* (R6) with *pncA* Genbank and *pncA*- sensitive *M. tuberculosis* (H37Rv)
Compared with the GenBank and H37Rv pncA, the pncA of clinical R6 showed a nucleotide difference (red underline), consisting of G511A, corresponding to amino acid substitution Ala171Thr.



The corresponding 0,6 fragment was completely sequenced. The nucleotide sequence analysis of the 0,6 fragment of PZA-sensitive *M. tuberculosis* strain (H37Rv) showed 100% identity with genbank *pncA* (ID number: 888260). Three nucleotide variations were found in *pncA* gene of R2 PZA-resistant *M. tuberculosis* strain toward the H37Rv strain and genbank *pncA* genes. The mutations were put at 40, 419 and 535 position, altering the T, G and A nucleotides into C, A, and G sequentially, then followed by amino acids replacement in PZase protein at 14, 140 and 179 position from Cys, Arg and Ser residues into Arg, His and Gly respectively (Table 1). A varian was found in *pncA* gene of R6 PZA-resistant *M. tuberculosis* strain, which was G instead to A at 511 position (Fig 5), and substitute the Ala amino acid at 171 position to Thr (Table 1).

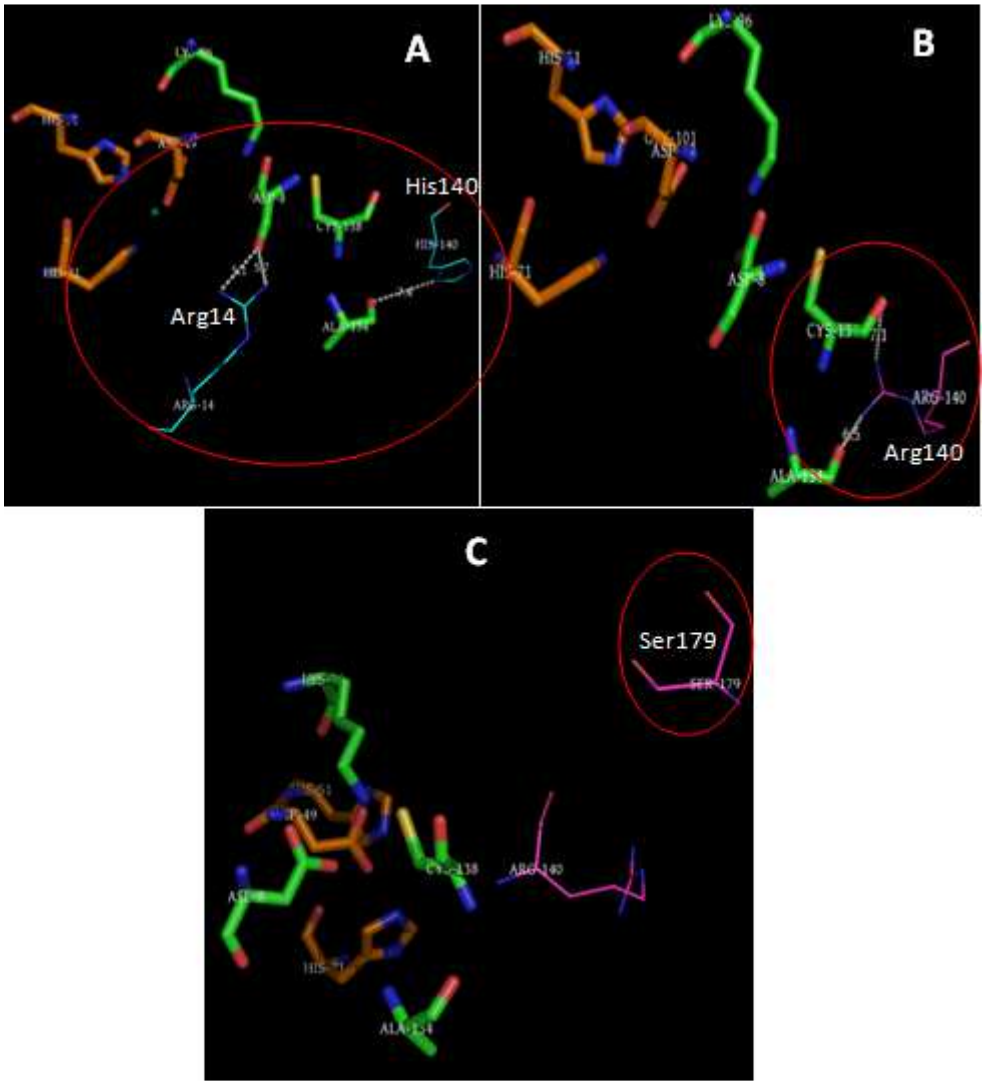
Single mutation in the R6 M. tuberulosis strain for Ala171Thr replacement connected to level of PZA resistance at PZA at 10 μg/ mL. Meanwhile the R2 strain that exhibited resistance to PZA at 40 μg/ mL connected to three mutations for Cys14Arg, Arg140His, Ser179Gly replacement. We suggested that the number of mutations in *pncA* gene correlated with the level of PZA resistance in clinical isolates of *M. tuberculosis*. The number of mutations may be necessary to bring the higher level of resistance or to maintain the virulence in a human host.

PZA-resistant strains showed a very diverse in the type and position of mutations in *pncA* gene, and no a special mutation dominantes [5,8]. All type and diverse mutations in the R2 and R6 PZase are classified as new mutations,

that have not been previously reported, except for Arg140Ser substitution that has been found from in others *pncA* of many PZA-monoresistant strains from many regions of Quebec [5].

Fig.6.Illustration of the effect of Cys14Arg, Arg140His and Ser179Gly substitutions in R2 PZase model structure

Residues of Asp8, Lys96, Ala8 and Cys138 in green ribbon structures are active residues of PZase catalitic site, while the Asp49, His51 and His71, the magenta ribbon structures are metal-binding residues. (A) The Arg14 in the R2 PZase put out a new interaction in the catalytic site environment, namely Van Der Waals interaction with Asp8. This interaction has not found for Cys14 in wild-type PZase. (B) the Arg140 in wild type PZase create Van Der Waals interaction with two amino acid residues, Ala134 and Cys138. These interactions are absent for His140 in R2 PZase. (C) The Ser179Gly doesn't affect significantly, because the residue at 179 position is far away from the active site, and also reside on the surface of the PZase structure.

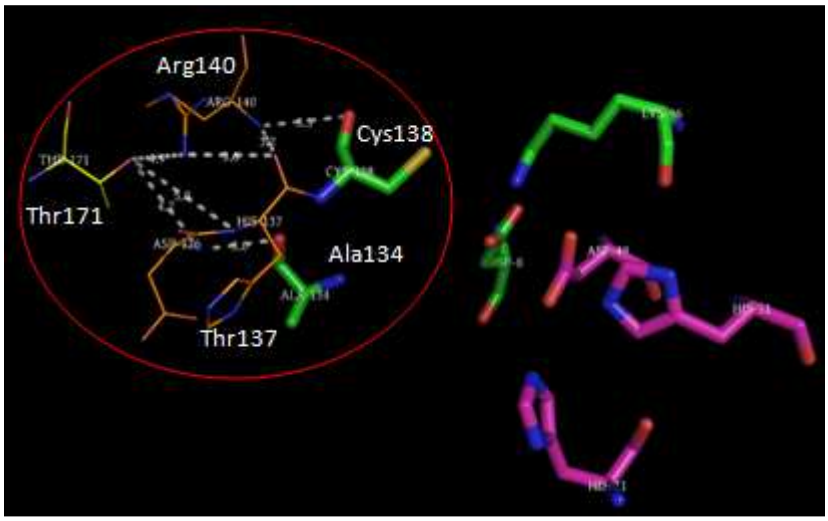


To determine the effect of amino acid substitutions in the R2 and R6 PZase, a structural modeling study has been carried out. The R2 and R6 PZase exhibited 98.4% and 99,5% respectively amino acid sequences to the wild-type PZase. The Cα-backbone superposition of the generated model for R2 and R6 PZase with the wild-type PZase structure gave root mean square deviations (RMSD) of 0.24 and 0.23˚A respectively, suggesting highly similar structures. However, the mutant structures of R2 and R6 PZase amended several interactions in its wild-type structure, expecially in the regions that contain the catalytic residues. PZase contain some catalytic residues, ie Asp8, Lys96, Ala134 and Cys138 in the active site, furthermore Asp49, His51 and His71 in the metal-binding site [14,15]. Substitution of Cys residue at 14 position with Arg in the R2 PZase put out a new interaction in the catalytic site environment, namely Van Der Waals interaction between the Arg14 residue with Asp8. This interaction has not found in WT PZase (Fig 6A). Meanwhile, the presence of His residue instead of Arg140 in R2 PZase reveals loss of Van Der Waals interactions between the amine groups in side chain of the Arg140 residue with two amino acid residues, Ala134 and Cys138 (Fig 6B). In addition to this, there is no significant effect due to the replacement of Ser179Gly, because the amino acid residue at 179 position is far away from the active site, and also reside on the surface of the PZase structure (Fig 6C). However, all amino acid substitutions might involve together in reducing of the R2 PZase activity.

Based on the model structure of R6 PZase, three new interactions are formed in the vicinity of the active site as a result of Ala171Thr substitution. The polar residue of Thr instead to Ala171 create a hydrogen interaction with Arg140 and two Van Der Waals interactions with Asp176 and His137 (Fig 7). The new interactions may destabilize the active site environment, because the three residues of Arg 140, Asp176 and His137 are located very close to the active site residues, and interact directly with two active residues Ala134 and Cys138 (Fig 7). It is proposed that the results of the physical-chemical changes around the active site may be unfavorable for R2 and R6 PZase activities, and in turn create PZA resistance in both M. bacterium strains. In the future, site-directed mutagenesis and enzyme activity studies should be accomplished to specify the role of each amino acid in PZase activity and also the structure determination of protein crystal to describe the molecular interactions exactly.

Fig.7. Illustration of the effect of Ala171Thr substitution in the R6 PZase model structure

The polar residue of Thr171 in R2 PZase create a hydrogen interaction with Arg140 and two Van Der Waals interactions with Asp176 and His137. The three residues in the end reside very close to the active site residues, and interact directly with two active residues Ala134 and Cys138, so the new interactions may induce to destabilize the active site environment.



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