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# DISCOVERING OF PYRAZINAMIDE RESISTANCE IN LOCAL STRAIN OF *Mycobacterium tuberculosis* CLINICAL ISOLATES BY MOLECULAR DETECTION OF *pncA* GENE ENCODING PZase

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**ABSTRACT**

Pyrazinamide (PZA) is one of the anti-tuberculosis (TB) drugs that have an action on the *pncA* gene encoding the PZase enzyme. *Mycobacterium tuberculosis* clinical isolates R14 and R7 showed PZA resistance up to doses 40 and 30 µg/mL, however, the basis of their resistance was unknown. The pncA gene of them was cloned into a pGemT vector, then characterized by sequencing. The *pncA* measuring 0.6 kb has been inserted into pGemT to form recombinant DNA at 3,6 kb. The *pncA* nucleotides of R14 and R7 showed four and two mutations toward the PZA-sensitive isolate H37RV. The mutations of G76T; G112C; A403C; G426A changed amino acids A26S; A38P; T135P were found in R14, while mutations G115A and T506C which changed A39T and V169A amino acids found in R7. The mutation triggered the decrease of two hydrophobic amino acids in R14 PZase, but one in R7. An additional polar amino acid was also found in R7 PZase. The PZase structure of both mutants changed conformation from the native structure H37RV with RSMD as 0.5 and 0.3 Å. The *pncA* mutations that followed by the change in protein properties and structures might induce the emergence of PZA resistance in the isolates.

**Keywords:** *pncA*, Pyrazinamide, Mutation, *Mycobacterium Tuberculosis.*

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## INTRODUCTION

One of the deadly infectious diseases is tuberculosis (TB), caused by *Mycobacterium tuberculosis*. These rod-shaped bacteria are acid-fast, non-spore-forming, and encapsulated. The wall of *M. tuberculosis* is very complex, consisting of a fairly high-fat layer (60%). The main components of the *M. tuberculosis* cell wall are mycolic acid, complex waxes, trehalose dimycolate called cord factor, and mycobacterial sulfolipids which play a role in virulence. TB disease is transmitted through air contaminated with *M. tuberculosis* which is released when a TB patient coughs, while in children the source of infection generally comes from adult TB patients. These bacteria often enter and collect in the lungs and multiply (especially in people with low immune systems), and can spread through blood vessels or lymph nodes.[1,2,3] In the lungs, *M. tuberculosis* is captured by macrophages. The interaction of TB with macrophage cells involves mycobacterial cell surface components with lipoarabinomannan (LAM). Porin protein OmpA and hemagglutinin HbhA with macrophage receptors such as mannose receptors, surfactant protein SP-A, and CD14. During contact with these receptors, *M. tuberculosis* secretes several virulent compounds into macrophage cells. Experimental studies show that *M. tuberculosis* ESAT-6 and CFP-10 proteins function as intracellular pathogenic compounds.[3,4,5,14] TB therapy is generally carried out by administering anti-TB drugs, one of which is pyrazinamide (PZA). The PZA is a prodrug that must be activated into pyrazinoic acid (POA) by the PZase enzyme, a protein of the pncA gene. In its active form, POA is able to fight *M. tuberculosis*. When used concurrently with isoniazid and rifampin, PZA was much more effective in accelerating healing from 9 months to 6 months of treatment. This is because PZA is able to kill semi-dormant bacteria.[6,7]

Although PZA is a recommended anti-tuberculosis drug, the mechanism of action of PZA has not been fully described because of its unique and paradoxical properties. The mechanism of action of PZA in vitro


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on *M. tuberculosis* isolates was initiated by the absorption of PZA through a passive diffusion process by *M. tuberculosis*.[7,8] In bacterial cells, PZA will be converted into POA which has a weak lipophilic group (Fig.-1), then in turn, it diffuses freely in bacterial cells. POA was initially formed into its anionic form ($POA^-$) at a neutral pH environment and had no antibacterial activity. $POA^-$ exists in bacteria via passive diffusion or an incomplete efflux pump. In acidic media $POA^-$ is found in the protonated form (HPOA). HPOA penetrates the membrane until there is a balance of concentrations inside and outside the membrane. Under acidic conditions, it is easier for POA to enter the cell (influx) than to leave the cell (efflux), so not only the formation of HPOA but also the accumulation of POA- and protons in the cell. In bacterial cells, the conversion of HPOA to its anion is preferred at neutral pH. Therefore, HPOA that enters the cell carries protons to the cytoplasm which in turn triggers the acidification process, interferes with proton strength, reduces energy so that it inhibits membrane transport functions, and prevents the formation of important enzymes.[6,8] This explains the higher PZA activity in old cells than in young cells and explains PZA activity under microaerophilic or anaerobic conditions. In this condition, the bacterial cell only has membrane potential as an energy source but will be lost quickly as a result of the acidic environment. Briefly, the change from PZA to POA can be explained by the mechanism in Fig.-1.

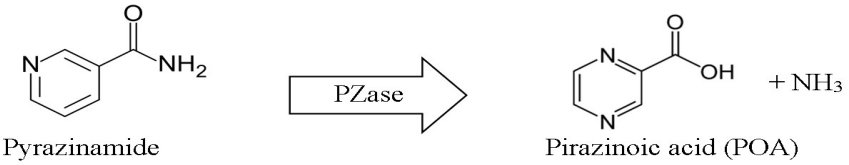


Fig-1. Conversion of Pyrazinamide to POA by PZase[7,8]

The resistance of *M. tuberculosis* to PZA was caused by a decrease in PZase activity in converting PZA into its active form, POA. 70%-90% of *M. tuberculosis* isolates that are resistant to PZA have mutations in the *pncA* gene encoding the pyrazinamide enzyme.[7,8,9] Research on the analysis of *pncA* gene mutations against PZA resistance in local isolates of *M. tuberculosis* is very important to produce genetic biomarkers. Two local clinical isolates R7 and R14 mycobacterium tuberculosis were PZA-resistant at doses of 40 and 30 g/mL, but the cause was unknown. This paper explores the *pncA* gene from the two isolates as the molecular explanation for PZA resistance.

## EXPERIMENTAL

### Sample

Isolate of PZA-sensitive *M. tuberculosis* H37RV as well as PZA-resistant strains carried from Bandung of the health research center, Indonesia.

### Isolation of the DNA Genome and Plasmid

The DNA genome of *M. tuberculosis* sensitive and PZA-resistant strains was extracted by genome preparation kit from Promega. Meanwhile, the isolation of DNA plasmid was carried out by the plasmid extraction kit from Qiagen, Santa Clarita, USA). The isolated DNA was then detected in agarose gel electrophoresis.[9,10,11,12]

### Cloning of *pncA* Gene

The pncA gene was replicated from the DNA genome by PCR using primer pairs 5'-gagcatatgcgggcgttgatcatc-'3 and 5'-gaagatctggagctgcaaaccaactc-'3. The PCR process was carried out for 25 cycles with a DNA-thermal Cycler machine, where each cycle was regulated with the following conditions: denaturation temperature of 94ºC 1 minute; annealing temperature of 50ºC for 1 minute, and polymerization at 72ºC for 2 minutes. Predenaturation and post-extension conditions were carried out at 94ºC and 72ºC for 4 and 5 minutes, respectively. The DNA fragment of the *pncA* gene resulting from the PCR was then purified with the GFX purification kit from PROMEGA and then inserted into the T plasmid following the pGEM®-T Easy Vector kit protocol, then transformed into E coli TOP10 with the help of cold CaCl2 and heat shock at 42ºC. The selection of clones carrying pGemT-*pncA* recombinant DNA was performed on ampicillin-solid LB media. The *pncA* gene was then subcloned into the pET30a vector using the T4 DNA ligase enzyme. Characterization of recombinant DNA in each plasmid vector was carried out by cutting with restriction enzymes.[9-12]

**Restriction and Sequencing of *pncA* Gene**

The presence of *pnc*A in the recombinant plasmid DNA was identified by the digestion of the recombinant with the *Nde*I enzyme. The digestion was performed according to the protocol in the restriction enzyme manual. Meanwhile, the nucleotide sequence of *pncA* was determined through analysis services at Macrogen Inc. using T7 promoter and SP6 primers. The sequencing results were processed using the DNASTAR program Seq Man TMII and Meg Align TM software.[11,12]

**Structural Modeling of Mutant PZase**

Construction of the PZase structural model was done by SWISS-MODEL Server using a template of 3D structure for PZase of *M. tuberculosis* H37Rv named 3PL1.pdb. Checking Root mean square deviation of the protein model was calculated based on Superpose 1.0 online server.[13]

## RESULTS AND DISCUSSION

The target of PZA as an anti-TB drug is the *pncA* gene. Based on this, the observation of PZA resistance cases in a clinical isolate of *M. tuberculosis* (R14 and R7) was searched by profiling the gene. For this purpose, the *pnc*A was amplified by PCR from the genome DNA of the mycobacterial samples. The electropherogram profile of the genome DNA from *M. tuberculosis* sensitive and PZA resistant is shown in Fig.-2. Meanwhile, the *pncA* gene PCR results are listed in the electropherogram in Fig.-3, showing a DNA band of 0.6 kb. The *pncA* gene was cloned in Escherichia coli cells using the vector plasmids pGemT and pET30a, resulting in multiple transformant cells (Fig.-4). Screening of transformant cells on LB media containing ampicillin could produce positive clones.

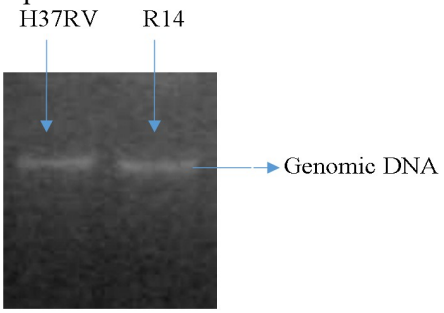


Fig.-2: Electropherogram of Genomic DNA from *M. tuberculosis* Sensitive and Resistant to PZA.

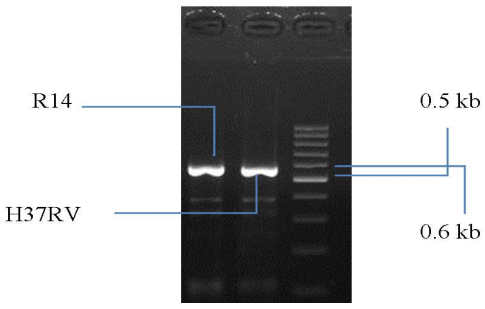


Fig.-3: Electropherogram of PCR Product for Amplification of *pncA* gene. Lane 1, 100 bp DNA Marker. Lane1 & 2 Fragment DNA of *pncA* from *M. tuberculosis* H37RV and Clinical Isolate R14

The characterization of recombinant DNA from the *pncA* gene cloned in the pGemT vector was able to confirm the successful formation of the correct pGemT-*pncA* recombinant plasmid. The results of cutting the pGemT-*pncA* recombinant plasmid with the *NdeI* restriction enzyme showed a single band measuring 3.6 kb in the agarose gel electropherogram. This site represents the combination of the 3.0 kb pGemT plasmid with the 0.6 kb *pncA* gene (Fig.-5).

**Correlation between PZA Resistance with Gene Mutation of *pncA* and Distortion of its Protein Structure**

The nucleotide base sequence of the *pncA* gene of *M. tuberculosis* sensitive and pyrazinamide-resistant isolates present in the pGemT-*pncA* recombinant plasmid was determined by the dideoxy Sanger method

using the T7 promoter and SP6 primers. The *pncA* genes of *M. tuberculosis* isolates that were clinically resistant to PZA R14 and R7 had different nucleotide sequences with the *pncA* isolates sensitive to PZA, with 4 and 2 nucleotides, respectively (Fig.-6).

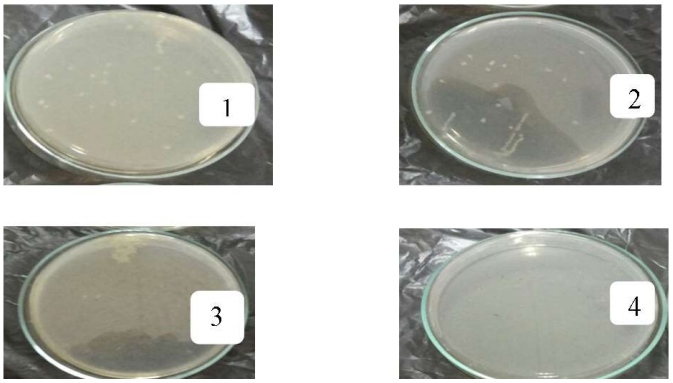


Fig.-4: Transformant Selection in Media LB-amphicilin. 1) pGemT-*pncA* H37RV, 2) pGemT-*pncA* R14, 3) Positive Control, 4) Negative Control

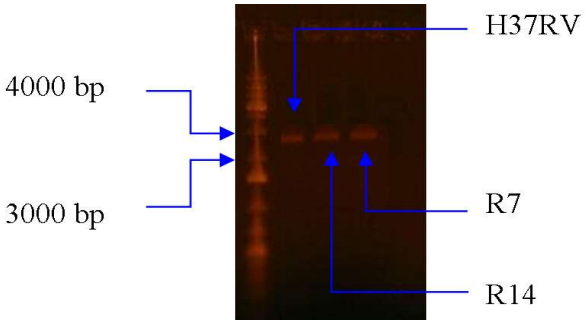


Fig.-5: Elecrophorogram Restriction of pGemT-*pncA* Recombinant with *NdeI*. Recombinant Plasmid of pGemT-*pncA* from Isolate H37RV, R7, and R14 Resulted in a Single Band After Cutted with *NdeI* enzyme. The Band Has a Size of 3.6 kb That Represents a Joining of DNA from pGemT (3.0 kb) and *pncA* (0.6 kb)

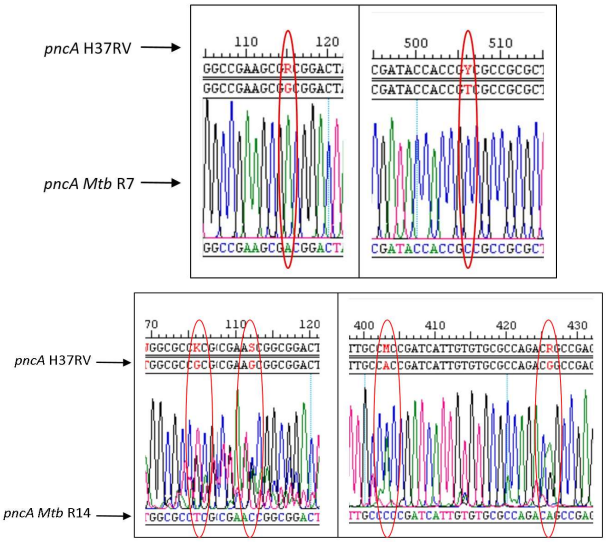


Fig.-6: The Alignment of Nucleotide from *pncA* of Clinical Isolate R14 and R7 toward *pncA M. tuberculosis* (H37RV)

G76T mutation; G112C; A403C; G426A in the gene *pncA* isolate R14 changes the 3 amino acids of the protein PZase it encodes, namely A26S; A38P; T135P (Table- 1), while the rest are classified as silent mutations. This *pncA* R14 gene mutation was associated with PZA resistance at a dose of 40 g/mL. Meanwhile, the *pncA* gene of clinical isolate R7 which is associated with PZA resistance at a dose of 30

ug/mL has a mutation of 2 nucleotide bases, G115A; T506C (Fig.-6) which converts 2 amino acids, A39T; V169A (Table-1).

Table-1: The Profile of Mutation in *pncA* Gene from R7 and R14

| Gene | Clinical *M. tuberculosis* | Mutations | | | | Level of PZA Resistance [µg/mL] |
|---|---|---|---|---|---|---|
| | | Σ | Nucleotide | Σ | Amino acid | |
| *pncA* | R14 | 4 | G76T; G112C; A403C; G426A | 3 | A26S; A38P; T135P; T142T | 40 |
| | R7 | 2 | G115A; T506C | 2 | A39T; V169A | 30 |

In silico translation of protein encoded by *pncA* R14 and R7 showed the change in some protein properties in PZase R14 and R7. The protein PZase of R14 has a decrease of two hydrophobic amino acids, while the R7 decreases one amino acid compared with the sensitive strain H37RV. The polar amino acid in PZase R7 increases one than the sensitive PZase (Table-2). The properties of two PZase might trigger to change protein structure of the PZase mutant from pyrazinamide resistant strain. The observing of model structure for PZase R14 and R7 compared with PZase H37RV showed the distortion structure that stated as Root mean square deviation (RSMD). The RMSD scores showed 0.5 and 0.3 respectively toward H37RV PZase (Table-3).

Table-2: Properties of PZase Encoded by *pncA* S (H37RV), R14 and R7

| Properties | Protein PZase encoded by *pncA* | | |
|---|---|---|---|
| | H37RV | R14 | R7 |
| Molecular Weight (kDa) | 19,6 | 19,6 | 19,6 |
| Amount of amino acids | 186 | 186 | 186 |
| Strongly Basic(+) Amino Acids | 10 | 10 | 10 |
| Strongly Acidic(-) Amino Acids | 27 | 27 | 27 |
| Hydrophobic Amino Acids | 71 | 69 | 70 |
| Polar Amino Acids | 45 | 45 | 46 |
| Isoelectric Point | 4.27 | 4.27 | 4.27 |

Table-3: Root Mean Square Deviation (RMSD) of PZase Structure R14 and R7 Toward H37RV

| PZase encoded by the *pncA* gene | RSMD* related to structure of *pncA* H37RV (Å) |
|---|---|
| R14 | 0..5 |
| R7 | 0.3 |

*The RSMD was calculated based on a protein superposition server (http://superpose.wishartlab.com)

The mutation of the *pncA* gene from PZA-resistant strains of R14 and R7 followed by the change of their protein properties as well as their structure might induce drug resistance in *M. tuberculosis* R14 and R7.

## CONCLUSION

At the phenotypic level, clinical isolates of M. tuberculosis R14 and R7 showed pyrazinamide resistance. Identification of the pncA gene since a target of PZA in both isolates showed mutations of G76T; G112C; A403C; G426A in R14, then mutations G115A and T506C in R7. The mutations linked to the change of protein properties as well as protein structure of mutant PZase of R14 and R7. The PZase structure of R14 and R7 changed to 0.5 and 0.3 Å respectively compared with native PZase from the M. tuberculosis H37RV strain. The pncA mutations that followed by the change in protein properties and structures might induce the emergence of PZA resistance in both isolates.

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## CONFLICT OF INTERESTS

All authors declared that there were no conflict of interest in this study.

## AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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