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Mutant vary region of *pncA* gene sequence of pyrazinamide resistance among multidrug resistant *Mycobacterium tuberculosis* isolates

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ABSTRACT

Introduction: Pyrazinamide (PZA) is one of the potent front-line drugs that act as antituberculosis (antiTB) for nonresistant or resistant *Mycobacterium tuberculosis*. Mutation of *pncA* gene is considered to be main target of PZA resistance mechanism. This study aims to determine the mutant gene sequences, location, and correlation of *pncA* gene mutations with PZA resistance in MDR *Mycobacterium tuberculosis* as a base for the rapid molecular examination.
Objective: This study aims to determine the mutant gene sequence and location of *pncA* gene with PZA resistance in multidrug resistant (MDR) *Mycobacterium tuberculosis* need a rapid molecular examination for consideration of MDR TB therapy management in Indonesia.
Methods: MDR *Mycobacterium tuberculosis* were identified and tested for PZA resistance with BACTEC MGIT 960 as a gold standard, followed by DNA extraction, PCR amplification and *pncA* gene sequencing.
Results: An analysis of 561 bp sequence of nucleotides was performed to determine type and location of mutations. A total of 35 isolates of this study showed 14 isolates of *pncA* gene mutation (40%), and revealed in 13 resistant and 1 sensitive isolate. The correlation analysis of *pncA* gene mutation to PZA resistance was significant (p = 0,003 and r = 0,452). Mutations in 3 (three) specific regions of *pncA* gene are 1 isolate at codons 51-76, 1 isolate at codons 130-142, and 3 isolates at codons 163-180.
Conclusion: Types of mutations in the *pncA* gene include substitution of 11 isolates, insertion of 2 isolates, and no deletion. Insertion of 178 CGCGCTGGAGGAGATGCGCACCGCC and multiple mutations in one isolate.

Keywords: mutant region vary of pncA gene, PZA resistance, MDR-TB
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INTRODUCTION

Tuberculosis (TB) is caused by acid fast bacilli, *Mycobacterium tuberculosis* that still remains a major problem globally and has been associated with significant morbidity and mortality. Global TB report estimated that one million people are new TB cases yearly and 1,5 million people are death because of TB infections.[1] The emergence of Multiple Drug Resistant (MDR) TB is a challenge to solve currently.[2,3]

Pyrazinamide (PZA) is still used in Indonesia as a first line key agent in the effective treatment of TB, including MDR TB cases[3]. Good bactericidal activities especially for intra-celluler organisms are unique characteristic of PZA. It is able to kill semi-dormant bacteria that are in an acidic environment during acute inflammation.[4-7] Several countries showed a higher prevalence of PZA resistance among MDR TB patients i.e. Papua New Guinea 10%, Turkey 25%, Thailand 49%, Central Africa 50%, South Africa 52%, Japan 53%, Taiwan 55%, Pakistan 77%, and South Korea 85%.[8] The PZA susceptibility test is phenotypically difficult because it requires an acidic environment for the activation of PZA, while acidic conditions can kill *Mycobacterium tuberculosiss.*[9]

PZA is a prodrug that enters *Mycobacterium tuberculosis* cell in a passive diffusion and converted into active form of pyrazinoic acid (POA) by the intra cellular bacterial enzyme pyrazinamidase (PZase). The POAs will be excreted by defective efflux and protonated at an acidic environment. The protonated POA is reabsorbed and accumulated into the cell. It makes cytoplasmic acidification and affects membrane transport. Effective pumping functions become inefficient and bacterial cells break down.[10]

The PZase enzymes are present in the cytoplasm and encoded by the *pncA* genes. The absence of PZase activities are common in clinically isolates with PZA resistant. Mutation of the *pncA* gene is considered a major cause of PZA resistance in *Mycobacterium tuberculosis.*[11] Several studies have reported that the correlation between resistant PZA and *pncA* gene mutations varies considerably around 41-80%.[12-15]

This study aims to determine the mutant gene sequences, location, and correlation of *pncA* gene mutations with PZA resistance in MDR *Mycobacterium tuberculosis* as a base for the rapid molecular examination. Early detection

of PZA resistance in *Mycobacterium tuberculosis* isolates is crucial for appropriate treatment to prevent the development of further resistance and the spread of resistant strains.

METHODS

A total of 35 *Mycobacterium tuberculosis* clinical isolates were recovered from MDR TB patients that stored in Surabaya Health Laboratory Center, Indonesia as National Referral Laboratory of *Mycobacterium tuberculosis* culture and drug susceptibility testing phenotypically.

First-line drugs (rifampicin, isoniazid, ethambutol, streptomycin, and pirazinamid) susceptibility tests were determined in a *BACTEC MGIT 960* system *(Becton Dickinson Microbiology Systems, Sparks, Md.)* in accordance with the manufacturer's instructions.[16] Identification of *Mycobacterium tuberculosis* isolates was confirmed by serpentine cord microscopically with *Ziehl Neelsen* stain and MPT64 rapid test (*SD Bioline Kit, Standard Diagnostics, Inc., Korea*).

Mycobacterial DNAs were extracted from *Lowenstein Jensen* medium by standardized boiling method. One loopful of *Mycobacterium tuberculosis* colonies were suspended in 250 µl of TE buffer (10mM Tris HCl, 1 mM EDTA, pH 8,0) and boiled in 100°C for 10 minutes. The suspension was centrifuged at 13000 rpm for 2 min. The supernatant was taken as 100 µl and stored at -20 °C until used as DNA template for amplification.[17]

For analysis of the presence of the *pncA* gene mutation, a 561-bp fragment was amplified using forward primer (5' GTCGGTCATGTTCGCGATCG 3') was located 105 bp upstream of the start codon and and reverse primer (5'GCTTTGCGGCGAGCGCTCCA 3') was located 60 bp downstream of the stop codon as recently described.[18-21]

The PCR mix composition consisted of Taq DNA polymerase, dNTPs, MgCl2, and buffer reaction (pH 8.5) (Promega, USA). PCR was performed in a total volume of 50 µl consisted of 25 µl of master mix, 2,5 ml of each primer (10µM forward and 10µM reverse primers), 2 µl of crude DNA and 18 µl of ddH_2O.

The PCR reactions were performed under the following conditions: initial denaturation at 95°C for 2 min; denaturation at 95°C for 40 sec; annealing at 58,6°C for 30 sec; 35 cycles were performed and followed by final elongation at 72°C for 3 min. The expected size of the pncA PCR products were 726 bp. PCR products were analysed by 2% agarose gel (**Figure 1**) and subsequently they were purified for sequencing with forward or reverse primer.

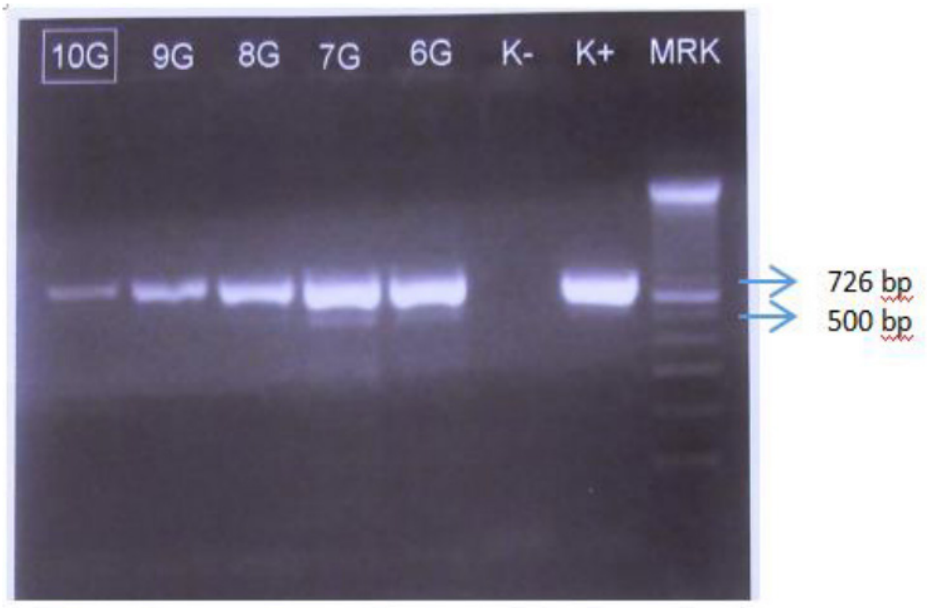

Figure 1. Visualization of *pncA* gene on 2% agarose gel. MRK, 100 bp DNA marker, K+, positive control of PCR (*Mycobacterium tuberculosis* H37Rv, K-, negative control of PCR (mixed PCR reagent without DNA), 6G-10G, sample isolates with pncA gene positive.

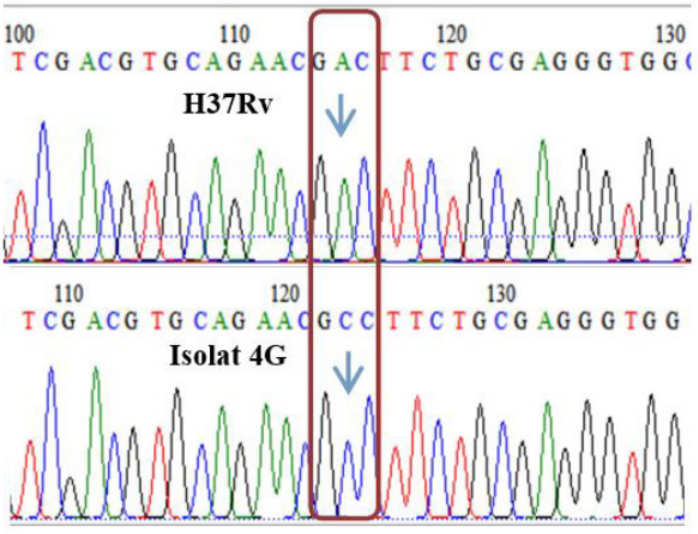

Figure 2. Electropherograms of *pncA* gene mutation. The 4G isolate showed substitution A become C.

The sequence analysing were compared with the sequence of Mycobacterium tuberculosis H37Rv pncA with *BioEdit sequence alignment editor* program (http://www.mbio.ncsu. edu/bioedit/bioedit.html) (**Figure 2**).

The correlation analysis of mutations in the *pncA* gene with PZA resistance in clinical isolates of *Mycobacterium*

tuberculosis MDR was determined by 2x2 chi square table analysis (contingency coefficient or Pearson's coefficient).

RESULTS

Of the 35 isolates studied, data on age and sex showed that 22 (62,86%) were males. The male:female ratio was 1,69:1. Most of the isolates (48,57%) were between 45-64 years old.

Of the 35 isolates tested, 22 isolates (62,86%) were PZA resistance by *BACTEC MGIT 960* as gold standard. The proportion of *pncA* gene mutations from a total of 35 *Mycobacterium tuberculosis* MDR isolates of this study based on PCR and sequencing was 40% (n = 14).

A total of 14 *pncA* gene mutation isolates of this study showed 13 resistant and 1 sensitive isolate. The correlation analysis of *pncA* gene mutation to PZA resistance was significant in **Table 1** (p = 0,003 and r = 0,452).

Mutations in 3 (three) specific regions of *pncA* gene are 1 isolate at codons 51-76, 1 isolate at codons 130-142, and 3 isolates at codons 163-180. Types of mutations in the *pncA* gene include substitution of 11 isolates, insertion of 2 isolates, and no deletion (**Table 2**).

Table 1. Correlation analysis of *pncA* gene mutations with PZA resistance in clinical isolates of Mycobacterium tuberculosis MDR.

Mutation		*pncA* gene		Correlation coefficient	P value
		No mutation			
PZA resistance	Resistant	13	9	0,452	0,003
	Sensitive	1	12		

Table 2. Characterization of *pncA* gene mutation.

No	No lab	Mutation type	Nucleotide changes	Frequency
1	2G	Insertion	CGCGCTGGAGGAGATGC GCACCGCC pada 535	1
2	4G	Substitution	A35C	1
3	9G	Substitution	A139C	1
4	14G	Substitution	C500T	1
		Substitution	C404G	
		Substitution	T408G	
		Substitution	G438T	
		Substitution	G443T	
		Substitution	C456G	
5	15G	Substitution	G461T	1
		Substitution	C509A	
		Substitution	C514A	
		Substitution	G519C	
		Substitution	C533A	
		Substitution	G541T	
6	17G	Substitution	C297G	1
7	18G	Insertion	T287	1
8	19G	Substitution	G485A	1
9	20G	Substitution	A29C	1
10	21G	Substitution	G71A	1
11	28G	Substitution	A226C	1
12	30G	Substitution	T545C	1
13	33G	Substitution	G41A	1
14	34G	Substitution	G41A	1

DISCUSSION

PZA was an important first line anti TB drug due to bactericidal activity especially in dormant bacilli.[4,7] Several studies showed that PZA can reduce duration of therapy and prevent relapse cases.[9] Emergence of MDR TB become complex issues due to ineffectiveness of treatment.[1] In high burden countries, PZA resistance have high prevalence especially in MDR TB cases (ranging between 10%-85%). The reason could be caused by widespread use of PZA with no drug susceptibility test or false-positive results. PZA susceptibility test should be performed but it has technical challenges phenotypically.[8] The difficulties are acidic condition to change PZA to active form, the other side show precisely the acidic atmosphere can kill bacteria.[9]

This study showed that the proportion of PZA resistance in MDR *Mycobacterium tuberculosis* was high enough (62,86%) by *BACTEC MGIT 960* as gold standard test. The results were correlated with those obtained from systematic review and meta-analysis by Whitfield *et al* in 2015 that showed PZA resistances were between 52.3%-68.6% among MDR TB cases.[22] Beside usage and technical issues, the other causes of increasing PZA resistance in MDR TB cases are higher frequency of spontaneous mutation rates ($1x10^{-5}$) than the other first line anti TB drug like rifampicin/RIF ($2,9 \times 10^{-9}$ - $2,4 \times 10^{-7}$) and isoniazid/INH ($2,56 \times 10^{-8}$).[23] The other theory by Stehr *et al* showed that MDR *Mycobacterium tuberculosis* may express extremely high NAD^+ levels for survival and inactivation of the drug. The accumulation of the nicotinamide produced may hamper PZA or POA.[24]

PZA must be changed by pyrazinamidase (PZase) enzymes that encoded by *pncA* gene to become active form, pyrazinoic acid (POA).[10] Several studies showed that *pncA* mutation has correlation as major mechanism of PZA resistance.[11] The correlation is ranging between 41-80%.[25] This study represents that the proportion of *pncA* mutations were 40% (14/35). Singh *et al* showed that the frequency of *pncA* mutation was varies (41%-97%).[26] The correlation between *pncA* mutation with PZA resistance in this present study are significant (p = 0,003 and r = 0,452).

The *rpsA* and *panD* gene have been found as other target of PZA resistance. Shi *et al* in 2011 suggested that *rpsA*

genes encode ribosomal protein S1 and can affect POA binding.[27] Zhang *et al* represent *panD* gene as a new target that have role in panthotenate and CoA biosynthesis for survival and pathogenesis of *Mycobacterium tuberculosis in vivo.*[11] This mechanism explained widely variety of correlation between *pncA* gene mutation with PZA resistance.

Du *et al* developed a model of theoretical homology structure based on nicotinamidase / PZAse from *Pyrococcus horikoshii* as template.[28] Zimic *et al* developed an enhanced theoretical model available in Protein Data Bank (GDP ID: 1X8A).

According to both models, the secondary structure of PZAse includes 4 alpha helix (α1-α4), 6 beta strands (β1-β6), and 10 loops (L1-L10).[29] This structure has catalytic center includes active site (residues D8, A134, and C138) and metal coordination site (residues D49, H51 and H71). Mutation at these positions are predicted to cause loss of enzyme activity. This region is found in codon 3-17, 61-85, and 132-142.[18] But, Li *et al* in 2016 studied that 3 specific region of *pncA* gene mutation (codon 51-76, 130-142, and 163-180) have been proven to be the part of active site and metal coordination site. The region has correlated with high level of PZA resistance.[30]

This study represented that *pncA* mutation have widely diverse and scattered distribution. The proportion of *pncA* mutation in 3 specific regions (codon 51-76, 130-142, and 163-180) was 18,2% and the others were in other codons. Mutations that do not occur in the active site, but close enough to them, the characteristics of the catalytic group will be disrupted. Mutations may not completely eradicate biochemical reactions, but alter reaction kinetics.[31]

The Thr47Ala (T47A) mutations in this study showed sensitive PZA results with *BACTEC MGIT 960*. The possibility of this clinical isolate has demonstrated low-level resistance, but not detectable phenotypically.[30]

Insertion of 178 CGCGCT GGAGGAGATGCGCACCGCC and multiple mutations in one isolate have never occurred before. This study requires further investigation to identify others PZA resistance mechanisms.

This study examined only a few isolates so information is still limited. Furthermore, this study can be continued with a number of samples that can represent the conclusions. These data also do not involve clinical and patient response to pyrazinamide therapy for resistance.

CONCLUSION

The proportion of PZA resistance in MDR *Mycobacterium tuberculosis* clinical isolates is high. There was moderate level of correlation between *pncA* gene mutations and PZA resistance from MDR *Mycobacterium tuberculosis* clinical isolates. Phenotype susceptibility test is required for the detection of PZA resistance. A rapid molecular examination might be used for consideration of MDR TB infection management therapy in Indonesia or the other high burden countries.

CONFLICTS OF INTEREST

The authors have none to declare.

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This study was self-funded without any contribution from third party.

AUTHOR CONTRIBUTION

All authors contributed equally in the writing of this article

ETHIC APPROVAL

This study had been ethically approved by ethical commission.

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