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Nucleotide Sequences and Mutations in *Katg* Gene in Clinical Isolates of *Mycobacterium tuberculosis* Isolates Resistant to Isoniazid in Papua-Indonesia

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Abstract: Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. Resistance to RIF is caused by mutations in the *rpoB* gene encoding the β subunit of RNA polymerase, with the highest frequency at codon 526 and 531. While Isoniazid is a *prodrug*, must be activated by the enzyme catalase-peroxidase encoded by the gene katG of M. tuberculosis, this gene mutation resulting in INH resistant. The purpose of this research is to obtain information on the cause of the genotype level resistance to INH in clinical isolates of the MDR-TB. Stages of research conducted here is Polymerase Chain Reaction (PCR) allele-specific multiplex *katG*, agarose gel electrophoresis, determining the nucleotide sequence, and *in silico* analysis. Results PCR and agarose gel electrophoresis for all isolates showed two DNA bands measuring 0.4 kb and 0.3 kb. Homology analysis to compare the results of sequencing electropherogram 0.4 kb fragment *katG* gene of the isolates with the same fragment of *M. tuberculosis* strain H37Rv standard. PyMOL modeling results describe the position of each amino acid as a result of mutations in the DNA level in the three-dimensional structure of protein molecules M. tuberculosis catalase-peroxidase. Analysis of data obtained showed that the mutation G946T three isolates located at codon 316, GGC into TGC, resulting in the amino acid glycine is mutated to cysteine. Simulation of the spatial structure of catalase peroxidase with PyMOL program showed 316 amino acid residues near the active site binding INH. Catalase-peroxidase simulation with PyMOL program showed 290 amino acid residues located in the N terminus loop area and relatively far from the active site, the effect of these mutations and their relationship in the nature of resistance to INH unknown. Other isolates G795A mutated nucleotide located at codon 265, TTG into TTA, but did not cause amino acid changes that can be ascertained that the mutation is not the cause of the nature of the resistance. While isolate mutated at codon 315, which has been proven as a cause of INH resistance. The results of this study are expected to provide new information on the position of the mutation in the gene katG of M. tuberculosis that is resistant to INH therefore G946T mutation (Gly316Cys) and C896T (Ala290Val) to isolate not been previously reported.
Keywords : Isoniazid, *katG* gene, MDR-TB, PCR, Isolate Papua.

Introduction

Tuberculosis (TB) is one health problem that has always been a concern of society and the government of Papua province, Indonesia.[1] TB is caused by an organism called *Mycobacterium tuberculosis*, and can spread

from person to person through the air. TB disease usually occurs in the lungs, but can occur elsewhere in the body called Miliary TB if the tubercle bacillus enters the bloodstream and carried throughout the body, they grow and cause disease in many places. TB disease must be treated for at least 6 months, in some cases even longer. The initial treatment of TB disease include four drugs: isoniazid, rifampin, pyrazinamide, and ethambutol. TB disease should be treated with at least two drugs which are prone basil. When using only one drug to treat TB, will lead to the growth of the population of TB bacilli resistant to the drug. Drug-resistant can also be caused if the patient does not take the medicine according to the recommended dosage.

MDR TB is a condition in which TB bacteria are resistant to at least two drugs are rifampicin (RIF) and isoniazid (INH). MDR-TB is a disease that continues to grow and some studies suggest a link between the epidemic of HIV/AIDS by increasing the rate of TB. Therefore, when the attention is reduced then there will be the possibility of TB cases to rise again. Research into the causes of MDR-TB is very important in order to know how best prevention and treatment.[2]

Research has been conducted on MDR-TB showed that RIF resistance occurs due to mutations in the gene *rpoB* and resistance to INH is caused by mutations in several genes that *katG*, *inhA*, *kasA* and *ahpC*. However, mutations that occur most often in the *katG* gene. KatG protein serves as a peroxidase enzyme catalase that degrades H_2O_2 and organic peroxides, the only one who has the activity of catalase enzyme in *M. tuberculosis*. *katG* prodrug activating INH become reactive species to inhibit the formation of cell walls. In a previous study found that *M. tuberculosis* resistant to INH and RIF, it is known that there is a mutation in the gene rpoB but no mutations in codon that usually occurs in the gene mutation katG315. This study aims to analyze the gene katG and determine whether there is a mutation at codon 315 in addition to contributing to the nature of resistance of *M. tuberculosis* to INH.

Material and Methods

Isolates were used obtained from this study that has been collecting clinical specimens such as sputum or lung fluid TB patients who come to the laboratory of Biochemistry, University of Cenderawasih, Jayapura, Indonesia.

Test of genotype

Tests conducted to determine the position of the mutation in the gene *katG* of the isolates were resistant to isoniazid and rifampin. The test is done using PCR methods, ie outside forward and reverse primer that will stick to the template DNA and primers specific to the *wild-type* allele. Outer primer pair will amplify band *invariable*. Primer in will stop at the end of 3' region of codon targets and a specific fragment of the *wild-type* allele. Changes bases are connected to the 3' end of the specific primer pairs causes the error between the DNA template and primer so that it will prevent the polymerase to extend the primer and no fragments are amplified.[1,3]

*katG*315 gene multiplex PCR with primers in the reverse *katG315* in the position 3' end of the pair with second base (G) of codon 315 *wild-type* allele (AGC). The absence of the mutation at position *katG315*, produce 0.29 kb fragment amplified with the outside primer KF and reverse primer in KR. If the mutation occurs, the results obtained are errors in pairs at the 3' primer in without any specific PCR products. Two outside primer KF and KR flanking the whole area *katG315* and fragment 0.43 kb. PCR quality controlled by 0.43 kb fragments resulting from the amplification primer KF and KR. Primer sequences used are: (Table 1).

Table 1. DNA primer used in this research

Name of primer	The nucleotide sequences (5' → 3'))
outside primer KF	GCA GAT GAT GGG GCT GAT CTA CG
outside primer KR	AAC GGG TCC GGG ATG GTG
reverse inner primer 315*katG*	ATA CGA CCT CGA TGC CGC

PCR reactions were performed with PCR machine Perkin-Elmer GeneAmp PCR System 2700 on the conditions: initial denaturation 96 °C for 3 min. Five cycles of 95 °C 1 min, 62 °C 1 min, 72˚C 30 sec and, five cycles of 95 °C 1 min, 60 °C for 40 sec, and 72 ˚C 30 sec. Twenty-two cycles of 94 °C 1 min, 58 °C for 40 sec, and 72 °C 30 seconds. Final elongation 72 °C for 3 min. A total of 5µL fragment amplification results in an agarose gel electrophoresis with a standard composition of 1.5%, using a marker pUC and visualized under UV light.[4]

Determination of nucleotide sequence and *in silico* analysis

katG gene amplification along the 0.43 kb without using a primer in, with the same PCR conditions PCR. Results amplification is used to determine the nucleotide sequence (sequencing). While *in silico* analysis using the nucleotide sequence of DNAstar programs. To determine the positions of the mutated residues in the three-dimensional structure of proteins *catalase-peroxidase* using PyMOL program.

Results and Discussion

Several MDR-TB isolates were amplified by multiplex PCR method for determining the presence of mutations in nucleotides 944, codon 315, AGC became ACC. The mutation causes the inner-primer, K315 could not be able to stick to the second base codon 315 as a result there will be no amplification of nucleotides along 0.29 kb.[5] If there is no mutation there will be amplification of nucleotides along 0.43 kb and 0.29 kb. PCR results can be viewed by using agarose gel electrophoresis (Figure 1).

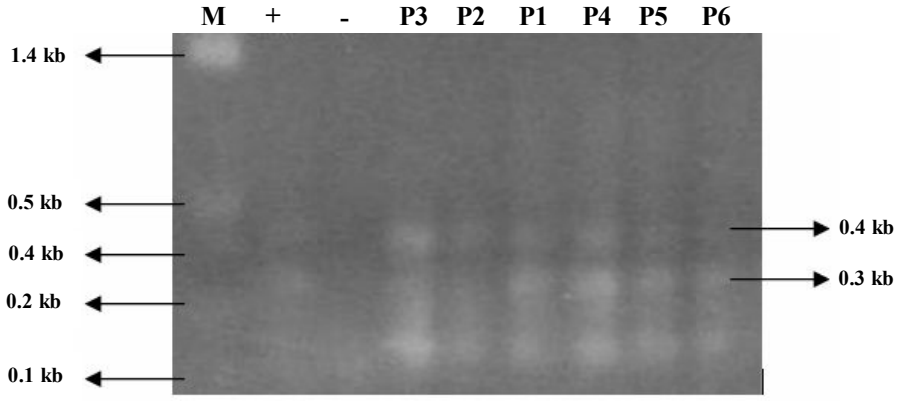

Fig 1. Agarose gel electrophoresis results of PCR multiplex. 315*katG* gene of the isolates MDR-TB from Papua, control (+): isolates normal strains H37Rv, control (-): water. Six isolates and controls (+) gives two bands at 0.4 kb and 0.3 kb. Pita 0.3 kb indicates that there is no mutation in the gene *katG* codon 315.

In Fig 1 shows the results of multiplex PCR six isolates were MDR-TB is not mutated in *katG*315. Based on this test it is known that the six isolates that do not have mutations in *katG*315. Isolates DNA fragments are amplified along 0,43kb, and to confirm the results of PCR conducted to determine the nucleotide sequence by dideoxy method of Sanger.

The results of the determination of the nucleotide sequence

Determination of nucleotide sequence using the Sanger dideoxy method produces electropherogram and the nucleotide sequences of the isolates MDR-TB compared with normal strains H37Rv. The following are the nucleotide sequences of isolates Papua sequencing results.

The nucleotide sequences of isolates Papua sequencing results:

TTTTTAAGCC GGCGCTCGGA GTACGACTGC CTCCTTCGGA TTGGTCTTCG 50
GTCGCGAAAG CTGAATGGAA AGGCCCGCGT GCAAAAATCA GCCCCGTCTG 100
CAGGGGGTGT TCGTCCATCC GACCCCTATG CAGCTGGTGA TCGCGTCCTT 150
ACCGGTTCCG GTGCCATACG AGCTCTTCCA GCCCAAGCCC ATCTGCTCCA 200
GCGGAGCAGC CTCGGGTTCG GGGCCGACCA GATCGGCCGG GCCGGCGCCA 250

TGGGTCTTAC CGAAAGTGTG ACCGCCGACG ATCAGCGCCG CTGTTTCGAC 300
GTCGTTCATG GCCATGCGCC GAAACGTCTC GCGAATGTCG ACCGCCGCGG 350
CCATGGGGTC CGGGTTGCCG TTCGGCCCCT CCGGGTTCAC GTAGCATCAG 400
CCCCATCTGC AAA 413

Analysis of homology

Homology analysis using DNAstar Seqman program compares the six isolates with normal strains of *M. tuberculosis* H37Rv. *katG* gene of three isolates, which are shown only isolates P1, compared with natural strains H37Rv katG gene at nucleotide 944 and 946. In addition, compared also with P2 isolates that are MDR-TB but without the mutation at nucleotide 944 and 946; and with MDR-TB isolates P3 that have mutations in the two nucleotides. The results of homology analysis showed that three isolates (P4, P5, P6) had a mutation at nucleotide 946, changes the base G into T. (Figure 2)

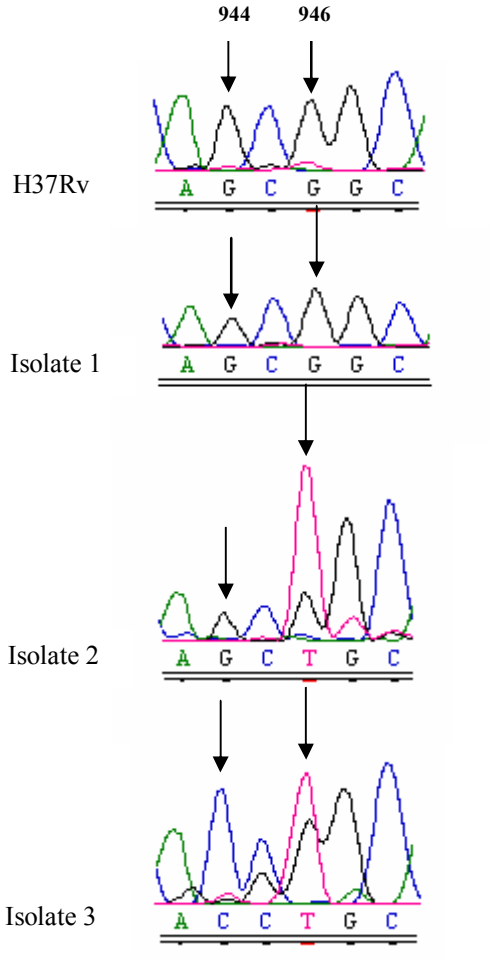

Fig 2. Homology analysis isolates Papua 2. Isolate 2 mutation in nucleotide 946, C to T, codon 316, GGC into TGC; and without the mutation at nucleotide 944, codon 315 AGC into ACC. Compared with H37Rv and other isolates of MDR-TB is not mutated in two positions. Benchmarking was also carried out with a 3 isolates had mutations at positions 946 and 944. The line amber indicates a base at codon 315, the green line shows the base at codon 316. The arrows indicate the nucleotides at positions 944 and 946 bases.

Analysis of amino acid homology H37Rv compared with three other isolates:

H37Rv:

301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 **316** 317 318 319 320
901 aag agc tcg tat ggc acc gga acc ggt aag gac gcg atc acc agc **ggc** atc gag gtc gta
Lys Ser Ser Tyr Gly Thr Gly Thr Gly Lys Asp Ala Ile Thr Ser **Gly** Ile Glu Val Val
K S S Y G T G T G K D A I T S **G** I E V V

Isolat P1, P2, P3:

301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 **316** 317 318 319 320
901 aag agc tcg tat ggc acc gga acc ggt aag gac gcg atc acc agc **tgc** atc gag gtc gta
Lys Ser Ser Tyr Gly Thr Gly Thr Gly Lys Asp Ala Ile Thr Ser **Cys** Ile Glu Val Val
K S S Y G T G T G K D A I T S **C** I E V V

Analysis of the data showed that the nucleotide 946 located at codon 316 first bases, GGC into TGC, resulting in the amino acid glycine is mutated to cysteine. This isolates all three did not have mutations in the gene *katG* codon 315. Previous studies have reported that mutations in the gene katG serin315threonin is the most frequent mutation.[7] Mutation at codon 315 resulting in reduced affinity peroxidase enzyme catalase to INH and can turn hydrogen bonds.[7-9] Effect of mutations in codon Gly316Cys against binding INH in the *catalase peroxidase* enzyme unknown but is thought to be the cause resistance properties of three isolates (P1, P2, and P3) to INH.

Homology analysis of isolates P3

P3 isolates homology analysis showed that the *katG* gene mutation in its nucleotide 944, base G to C; and nucleotide 946 bases G into T. G944C mutation at codon 315 that converts the amino acid serine to threonine has been shown to cause resistance to INH. However, the results showed PCR amplification on 0.43 kb and 0.29 kb. This could happen because of the condition of PCR is so sensitive that there is an error in pairs on the inner-primer (Figure 3).[3]

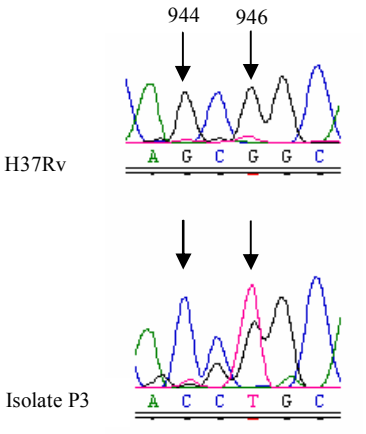

Fig 3. Homology analysis isolates P3. P3 isolates have mutations at nucleotide 944, G to C, codon 315, AGC became ACC; and nucleotides 946, G to T, codon 316 GGC into TGC.

Analysis of amino acid homology isolates P3 with natural strains H37Rv:

H37Rv:

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      301 302 303 304 305 306 307 308 309 310  311 312 313 314 315 316 317  318  319  320
901   aag agc tcg  tat ggc acc gga acc ggt aag  gac gcg atc acc agc ggc atc  gag  gtc  gta
      Lys Ser Ser Tyr Gly Thr Gly Thr Gly     Lys Asp  Ala Ile Thr Ser Cys Ile  Glu  Val
Val
       K   S   S   Y   G   T   G   T   G   K   D   A   I   T   S   G   I   E   V   V
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Isolat P3:

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      301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320
901   aag agc tcg tat ggc acc gga acc ggt aag gac gcg atc acc acc tgc atc gag gtc gta
      Lys Ser Ser Tyr Gly Thr Gly Thr Gly     Lys Asp  Ala Ile Thr Thr Cys Ile Glu
Val   Val
       K   S   S   Y   G   T   G   T   G   K   D   A   I   T   S   G   I   E   V   V
```

Other mutations in isolates P3 is at nucleotide 946, changing the base G to T, codon 316 GGC into TGC. Mutations in the same position with three other isolates. It is estimated that the mutation at codon 316 has become one of the causes of the nature of INH resistance in isolates P3, but the exact effect is not yet known. Another frequent mutation is at codon Arg463Leu, but this mutation has proven not associated with resistance to INH. Several mutations have been reported at codon Arg128Gln, Ala291Pro, and Thr275Pro.[10] His-108, which is one INH-binding residues have been reported mutated in INH resistant isolates, into acids glutamate and glutamine.[11-12]

Mutations in the active site Asp-137 is an important role in binding INH has never been reported to have mutations, but mutations are the residues surrounding that N138S, A139P, S140N, or D142A. Mutants are given effect by changing the local conformation so that it can change the orientation of the side groups Asp-137 consequently can not bind INH.[13-14]

Catalase-peroxidase protein visualization using the program PyMOL

Previous research has crystallize *M. tuberculosis* catalase-peroxidase and have determined the three-dimensional structure of the protein. This crystal structure data can be seen on public sites www.ncbi.nlm.nih.gov, with names 1SJ2.[6] Position residues that have mutations in the gene *katG* 1SJ2 can be seen on structure by using PyMOL program. Catalase-peroxidase structure visualization space with PyMOL program showed amino acid residues 316 near the active site binding INH. The results of further analysis showed the change of surface residue 316 due to mutations glycine to cysteine. Glycine is an amino acid cysteine simplest whereas larger and can form a disulfide bond with another cysteine. But the effect of this glisin316sistein mutations in resistance to INH unknown.

Previous research has shown that mutations at residue 315 lead to resistance to INH as result in changes in hydrogen bonds between heme and serin315.[1,6] Three isolates did not have mutations at residue 315 so that the mutation at residue 316 is strongly suspected to be the cause resistance properties.

Residues 278 to 312 on *catalase-peroxidase* enzyme in *M. tuberculosis* is local loop, conformation is similar in two other peroxidase catalase structure that is at *Haloarcula marismortui* and *Burkholderia pseudomallei*. In *Burkholderia pseudomallei* catalase-peroxidase, loop area is estimated to be the substrate binding INH place to interact with enzymes. But another researchers stated that the *M. tuberculosis* catalase-peroxidase, the local loop is not the most important binding sites INH.[14-18] Mutations at amino acid residue 290 is located relatively far from the active site and its influence on the nature of INH resistance is unknown.

Conclusion

The analysis of six isolates were MDR-TB, which is mutated in a multiplex PCR rpoB gene but this study did not have mutations in the gene katG codon 315. The analysis showed that the five isolates katG gene, by determining the nucleotide sequence, not mutated at codon position 315 and one strain mutated in these

positions. Three of the five isolates above have the same type of mutation that is Gly316Cys. Two other isolates each have mutations in different positions, namely the position of mutations that alter amino acids Ala290Val and other positions do not change the amino acid. Simulation of the structure using PyMOL program shows that residue 316 is close to the active site binding INH, while residue 290 in the region of the loop ends of N relatively far from the active site. Mutations in the local loop is unknown influence in INH resistant properties. Three isolates did not have mutations in codon 315, which has been shown to cause resistance to INH, but occurs at codon 316. The mutation causes resistance to INH in three isolates were allegedly due to mutation at codon position 316. The flow of further research is needed to confirm whether these mutations are the only cause of resistance to INH in Papua isolates in other regions and compared to the eastern part of Indonesia.

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