

**DETECTION OF ISONIAZID RESISTANCE
Mycobacterium tuberculosis STRAINS BY
MULTIPLEX ALLELE-SPECIFIC PCR FROM INDONESIA**

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ABSTRAK

Tuberculosis (TB) is an infected disease caused by *Mycobacterium tuberculosis*, and treatment with anti-tuberculosis drugs could cure the disease. Multi-drug resistant TB (MDR-TB) has been defined by World Health Organization as strains of tuberculosis that are resistant to at least the two main first-line drugs, namely isoniazid (INH) and rifampicin (RIF). One particular substitution reported to be the most frequent that confers resistance to INH is G944C, in codon 315 which replaced, AGC to ACC, hence Ser mutated to Thr. Our research group has six clinical isolates MDR-TB, which genotypically showed mutation in *rpoB* gene codon 526 and 531, but no mutation found in *katG*315 based on PCR experiment assays. The aim of our research is to find the genotype information of the above resistance INH in six clinical isolates. Our research methods consist of PCR multiplex specific alleles *katG* assays, gel agarose electrophoresis, nucleotide sequencing, and *in silico* analysis. The PCR multiplex assay employed three primers, two outer primers KF and KR; and one inner reverse primer K315. Here we showed that multiplex PCR of all isolates showed DNA fragment of 0.43kb and 0.29kb bands. Electrophoregram of 0.43kb *katG* gene fragment were compared to the same fragment of *M. tuberculosis* wild type. Homology analysis showed three isolates have mutation in nucleotide 946, G to T; and an isolate (R2) has mutation in nucleotide 869, C to T. Two other isolates, L4 has mutation in nucleotide 795, G to A; and L7 isolate have two mutations in nucleotides 944 and 946 which change G to C and G to T respectively. Pymol modeling showed each amino acid position in three dimensions structure protein as the effect of mutation in the DNA level. Data analysis obtained here showed that G946T mutation in three isolate located in codon 316, GGC change to TGC, with the consequence of replacing amino acid glycine to cysteine. Pymol program showed the three dimensional structure of catalase-peroxidase enzyme, and the residue 316 was located close to the active site of the enzyme. The recent report have proved that mutation in the residue 315 confers INH resistance. The fact that three isolates have no mutation in the residue 315, we suggest that mutation in

residue 316 was responsible for the resistance phenotype. The R2 isolate has mutation in nucleotide C869T located in codon 290, GCT change to GTT, hence amino acid alanine replaced by valine. Pymol program showed amino acid residue 290 located in the loop region of N terminal and was located far from the active site. The effect of this mutation and the relation in resistance INH has not been yet known. L4 isolate has mutation in nucleotide G795A located in codon 265, TTG change to TTA, which is a synonym substitution. Therefore, the cause of resistance of INH in the L4 isolate has not been yet known. While L7 isolate has mutation in codon 315, which confers INH resistance. The implication of our results is to give the new information about mutation position in *katG* gene *M. tuberculosis* which is resistance INH because of mutation G946T (glycine to cysteine) in isolate L10, L18, L19; and C869T (alanine to valine) in isolate R2 has not been published before.

Key words: Isoniazid, gene *katG*, MDR-TB, PCR.

INTRODUCTION

Multidrug resistant (MDR) TB is a condition in which TB bacteria resistant to at least the two drugs namely rifampicin (RIF) and isoniazid (INH). MDR-TB is a disease that continues to grow and some studies suggest a relationship between the epidemic of HIV / AIDS by increasing the rate of TB. Therefore, if attention is reduced, it would be possible TB cases will increase again. Research on causes of MDR-TB is very important to note the way the best prevention and treatment.

Research has been done to the MDR-TB showed that RIF resistance is due to mutations in the *rpoB* gene, and resistance to INH is caused by mutations in several genes that *katG*, *inhA*, and *ahpC* gene. However, the most common mutation in the gene is *katG*. *KatG* protein functions as a catalase peroxidase enzyme which degrades H₂O₂ and organic peroxides, the only enzyme which has catalase activity in *M. Tuberculosis*. FUDR activate *katG* of INH into a reactive species to inhibit the formation of cell walls. In previous studies showed *M. tuberculosis* resistant to INH and RIF, we know that there are mutations in the *rpoB* gene mutation but not in codon that normally occurs in the gene mutation *katG*315. This study aims to analyze the genes *katG* and determine whether there is a mutation at codon 315 in addition to contributing to the resistance of *M. tuberculosis* in some samples to INH resistant *M. tuberculosis* from Indonesia.

MATERIALS AND METHODS

Isolates were derived from our research group has collected clinical specimens of sputum or fluid pulmonary tuberculosis patients who came into the Biochemistry Laboratory, Dok V Hospital, Jayapura, Papuan province. Testing conducted to determine the position of the *katG* gene mutations in isolates resistant to isoniazid and rifampin. This test was carried out using multiplex PCR method, which is outside the forward and reverse primer that will stick to the DNA template and allele-specific primers in the wild-type. Outer primer pairs will amplify invariable bands. Primary in will stop at the end of the 3' region and amplify the target codon-specific alleles are wild type. Changes in bases that are connected at the 3' end specific primers in causing errors in pairs between template DNA and primers that will prevent the polymerase extending the primer and no amplified fragment (Mokrousov et al., 2003, Mokrousov et al., 2002).

KatG 315 gene PCR with primers in the reverse *katG*315 tip positioned 3' paired with a second base (G) of codon 315 wild-type allele (AGC). The absence of mutations in *katG*315 position, yielding 0.29 kb fragment amplified with outside primers KF and KR primers in reverse. If there is no mutation, the results obtained are errors in pairs at the 3' primer in no specific PCR product. Two out of KF and KR primers flanking the entire region *katG*315 and amplify 0.43 kb. PCR quality controlled by the 0.43 kb fragment generated from the amplification primers KF and KR. Primer sequences used were:

Table.DNA primers used in this study

<i>Primer</i>	<i>Nucleotide sequence</i>
outer primer KF	5'-gCA gAT gAT ggg gCT gAT CTA Cg-3'
outer primer KR	5'-AAC ggg TCC ggg ATg gTg-3'
Inner primers the reverse <i>katG</i> 315	5'-ATA CgA CCT CgA TgC CgC-3'

PCR reactions performed with Perkin-Elmer PCR machine GeneAmp PCR System 2700 under conditions: initial denaturation 96 °C for 3 minutes. Five

cycles of 95 °C 1 minute, 62 °C 1 minutes, and 72 °C 30 seconds, five cycles of 95 °C 1 minute, 60 °C 40 seconds, and 72 °C, 30 seconds. Twenty-two cycles of 94 °C 1 min, 58 °C 40 seconds, and 72 °C, 30 seconds. Final elongation 72 °C for 3 minutes. A total 5µL amplified fragments electrophoresed on agarose gel 1.5% with a standard composition, using marker pUC and visualized under UV light.

KatG gene amplification along the 0.43 kb without using the primers, with PCR conditions similar to PCR. Amplification is used to determine the nucleotide sequence. While *in silico* analysis of nucleotide sequences using DNASTAR program. To determine the positions of mutated residues in three-dimensional structure of proteins using the program PyMOL Peroxidase catalase.

RESULTS AND DISCUSSION

Multiplex PCR

Several isolates of MDR-TB in the amplification with the PCR multiplex to see a mutation at codon 944 nucleotides 315, AGC into ACC. These mutations lead to primary-in K315 would not be able to stick to the second base codon 315 as a result there will be no amplification of nucleotides along the 0.29 kb (Mokrousov et al., 2002). If the mutation does not happen then there will be amplification of nucleotides along the 0.43 kb and 0.29 kb. PCR results can be viewed by using agarose gel electrophoresis.

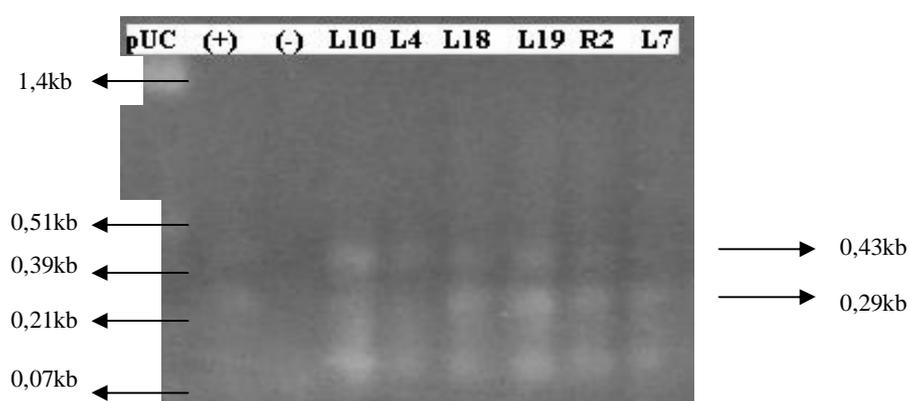


Figure 1. Agarose gel electrophoresis of PCR product. KatG315 six gene MDR-TB isolates (L10, L4, L18, L19, R2, L7), control (+): the normal strain H37Rv isolate, control (-): water. Six isolates and control (+) gave two bands at 0.43 kb and 0.29 kb. 0.29 kb bands shows that there is no mutation in the katG gene codon 315.

Figure 1 shows the PCR product were six isolates of MDR-TB which is mutated in katG315 not. Multiplex PCR, this isolate isolates retested previous research. Based on this test found that six isolates namely L4, L7, L10, L18, and R2 L19 does not have mutations in katG315. Isolate DNA fragments, all isolates were amplified 0.43 kb, and to confirm the PCR product were performed to determine the nucleotide sequence with the Sanger dideoxy method.

Results Determination of Nucleotide Sequences

Nucleotide sequencing using Sanger dideoxy method produces electropherogram nucleotide sequences of six isolates of MDR-TB (L4, L7, L10, L18, L19, R 2) compared with the normal strain H37Rv. One of the electropherogram (isolate L18) is shown in Figure 2. Here is the nucleotide sequence of isolate L18 sequencing results.

Nucleotide sequence of sequencing results isolates L18:

TTTTTAAGCC	GGCGCTCGGA	GTACGACTGC	CTCCTCGGA
TTGGTCTTCG	50		
GTCGCGAAAG	CTGAATGGAA	AGGCCCGCGT	GCAAAAATCA
GCCCCGTCTG	100		
CAGGGGGTGT	TCGTCCATCC	GACCCCATG	CAGCTGGTGA
TCGCGTCCTT	150		
ACCGGTCCG	GTGCCATACG	AGCTCTCCA	GCCCAAGCCC
ATCTGCTCCA	200		
GCGGAGCAGC	CTCGGGTTCG	GGGCCGACCA	GATCGCCGG
GCCGGCGCCA	250		
TGGGTCTTAC	CGAAAGTGTG	ACCGCGACG	ATCAGCGCCG
CTGTTTCGAC	300		
GTCGTTTCATG	GCCATGCGCC	GAAACGCTC	GCGAATGTCG
ACCGCCGCGG	350		
CCATGGGGTC	CGGGTTGCCG	TTCGGCCCT	CCGGGTTCAC
GTAGCATCAG	400		
CCCCATCTGC	AAA		413

Homology analysis and isolates L10, L18, L19

Homology analysis using DNASTAR Seqman comparing the six isolates with a normal strain of *M. tuberculosis* H37Rv. KatG gene of three isolates (L10, L18, L19), which is shown only isolates L18, compared with natural strains H37Rv katG gene at nucleotide 944 and 946 (indicated by arrows). In addition, compared with isolates L4 MDR-TB nature but do not have mutations in the nucleotides 944 and 946; and L7 with MDR-TB isolates that have mutations in these two nucleotides. The results of homology analysis showed that three isolates (L10, L18, L19) mutation at nucleotide 946, G to T base change

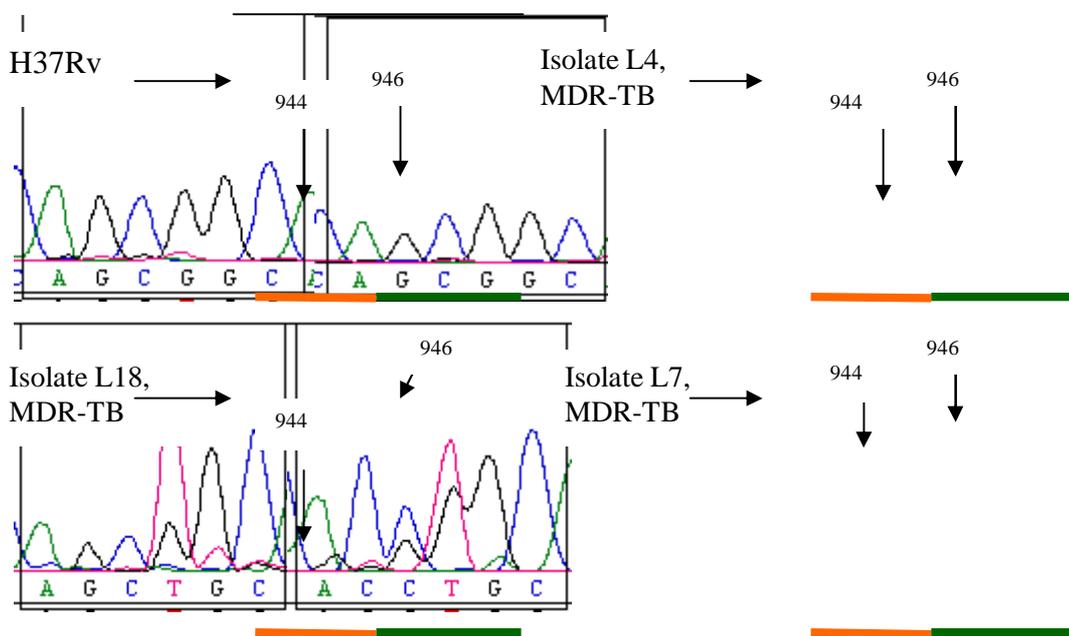


Figure 2. Homology analysis of isolates L18. Isolate L18 mutation at nucleotide 946, C to T, codon 316, GGC to TGC; and have not undergone mutation at nucleotide 944, codon 315 AGC to ACC. Compared with H37Rv and MDR-TB isolates R2 that do not have mutations in two positions. Benchmarking is also done with isolate L7 which has mutations in positions 946 and 944. The orange line shows the bases at codon 315, the green line shows the bases at codon 316. The arrows indicate nucleotide positions at 944 and 946 bases.

Analysis of amino acid homology H37Rv compared with three isolates (L10, L18, L19):

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

Isolates L10, L18, L19:

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 these data shows that 946 nucleotides located on the first base codon 316, GGC to TGC, resulting in the amino acid glycine is mutated to cysteine. These three isolates had no mutations in all genes katG codon 315. A previous study has reported that the katG gene mutations in serin315threonin is the most common mutations (Mokrousov et al., 2002). Mutation at codon 315 result in reduced affinity of the enzymes catalase peroxidase to INH (Wengenack et al., 1998) and can alter the hydrogen bond (Bertrand et al., 2004). Effect of mutation at codon glisin316sistein against the binding of INH in the catalase peroxidase enzyme is unknown but is estimated to be the cause of the resistance properties of the three isolates (L10, L18, L19) to INH

Isolate R2 Homology Analysis

Homology analysis of isolates R2 compared with a natural strain H37Rv and the isolates L4. The third alignment results *katG* gene of *M. tuberculosis* showed that the isolates R2 mutation at nucleotide 869, cytosine bases changed to thymine. Once analyzed, it turns out to be 869 nucleotides at codon 290, GCT to GTT, and the base changes resulted in amino acid alanine changed to valine. Ever reported mutations are at codon 291, alanine to proline amino acid (Fang et al., 1998). Homologinya analysis is shown in Figure 3.

Amino acid homology analysis of MDR-TB isolates R2 compared with the amino acids of normal strain H37Rv:

H37RV:

281 282 283 284 285 286 287 288 289 **290** 291 292 293 294 295
296 297 298 299 300

841 gcc gat ctg gtc ggc ccc gaa ccc gag **gct** gct ccg ctg gag cag
atg ggc ttg ggc tgg

Ala Asp Leu Val Gly Pro Glu Pro Glu **Ala** Ala Pro Leu Glu
Gln Met Gly Leu GLy Trp

A D L V G P E P E A A P L E Q M
G L G W

Isolate R2:

281 282 283 284 285 286 287 288 289 **290** 291 292 293 294 295
296 297 298 299 300

841 gcc gat ctg gtc ggc ccc gaa ccc gag **ggt** gct ccg ctg gag cag
atg ggc ttg ggc tgg

Ala Asp Leu Val Gly Pro Glu Pro Glu **Val** Ala Pro Leu Glu
Gln Met Gly Leu GLy Trp

A D L V G P E P E **V** A P L E
Q M G L G W

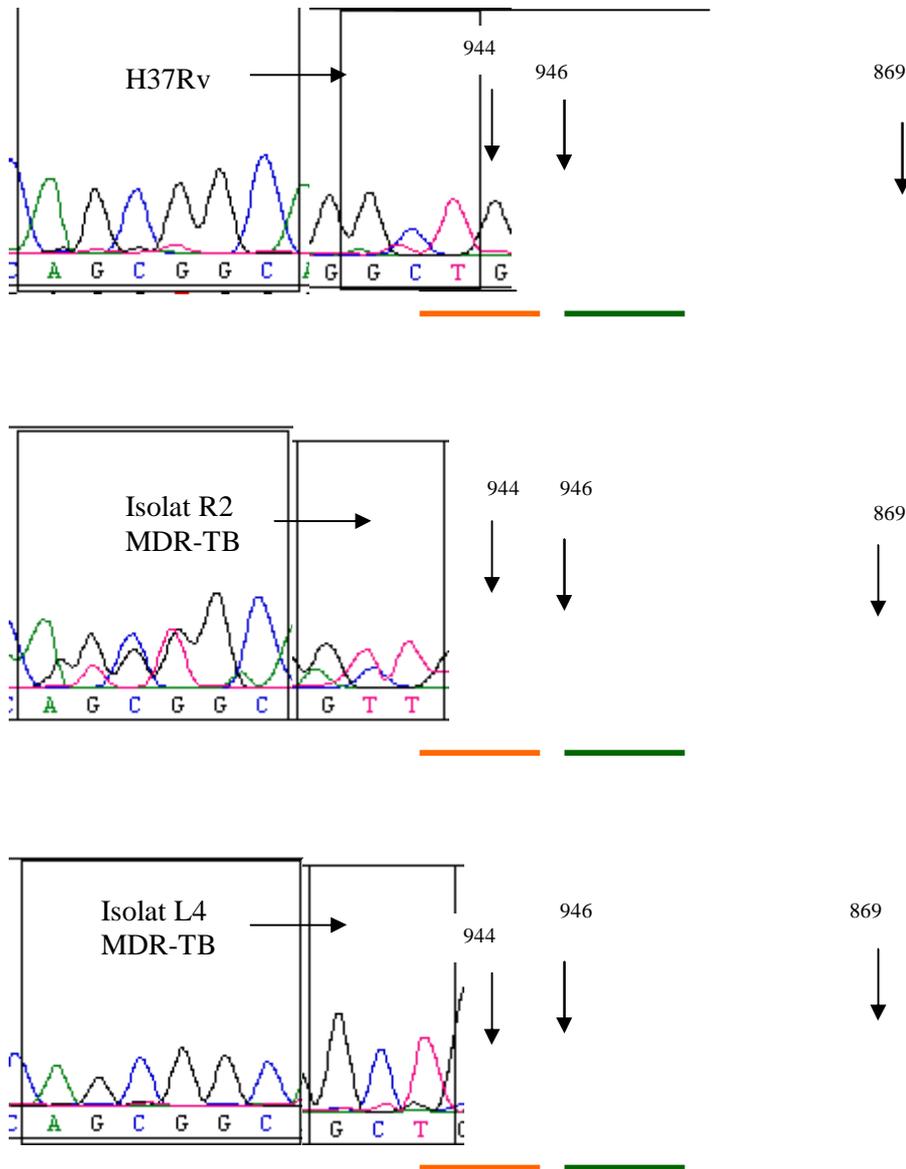


Figure 3. Homology analysis of isolates R2. R2 isolates mutated at nucleotides 869, C to T, second base of codon 290 GCT to GTT. R2 isolates no mutation at nucleotide 944, codon 315 (orange line) and nucleotide 946, codon 316 (green line). Compared with H37Rv and the isolates are MDR-TB L4.

Homology analysis of isolates L4

L4 compared with H37Rv isolates and isolates L10. Isolates L4 mutation at nucleotide 795, G to A, which is located at codon 265, TTG to TTA, but did not cause amino acid changes that can be ascertained that these mutations did not cause resistance to INH. Thus the cause of INH resistance isolates L4 is unknown.

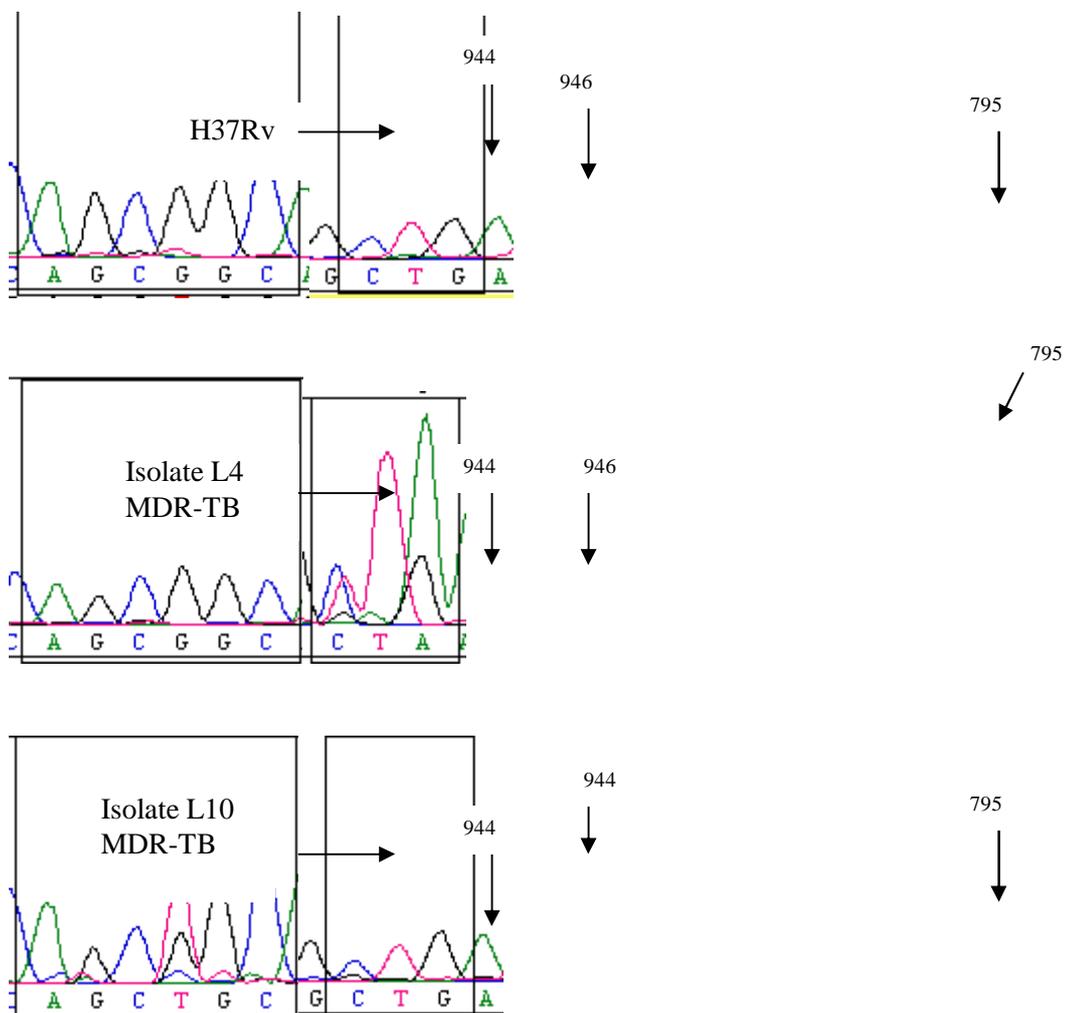


Figure 4. Homology analysis of isolates L4. Isolates L4 mutation at nucleotide 795, G to A base codon 265, CTG to CTA did not cause amino acid changes. H37RV and isolate compared with the MDR-TB L10. L4 isolates no mutation at nucleotide 944 and 946.

Analysis of amino acid homology of isolate L4 MDR-TB strains compared with natural amino acids H37RV:

H37RV:

261 262 263 264 **265** 266 267 268 269 270 271 272 273 274
 275 276 277 278 279 280
 781 gaa aca gcg gcg **ctg** atc gtc ggc ggt cac act ttc ggt aag
 acc cat ggc gcc ggc ccg

Glu Thr Ala Ala **Leu** Ile Val Gly Gly His Thr Phe Gly Lys Thr
 His Gly Ala Gly Pro
 E T A A **L** I V G G H T F G K
 T H G A G P

Isolate L4 MDR-TB:

261 262 263 264 **265** 266 267 268 269 270 271 272 273 274
 275 276 277 278 279 280
 781 gaa aca gcg gcg **cta** atc gtc ggc ggt cac act ttc ggt aag acc
 cat ggc gcc gcc ccg
 Glu Thr Ala Ala **Leu** Ile Val Gly Gly His Thr Phe Gly Lys
 Thr His Gly Ala Gly Pro
 E T A A **L** I V G G H T F G K
 T H G A G P

Homology Analysis of Isolates L7

Homology analysis showed that the isolate L7 katG gene was mutated at nucleotide 944, a base G into C; and 946 nucleotide base G into T. G944C mutation at codon 315 that converts the amino acid serine into threonin has been shown to cause resistance to INH. However, multiplex PCR results indicated that amplification of 0.43 kb and 0.29 kb. These things can happen because the highly sensitive PCR conditions so that there is an error on primer pairs, in (Mokrousov et al., 2002).

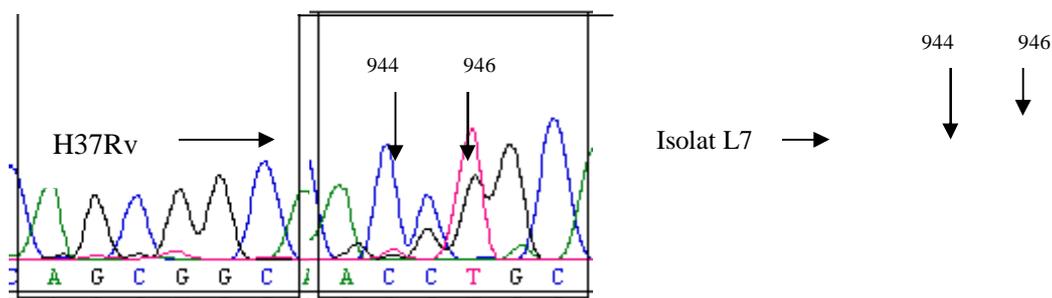


Figure 5. Isolates L7 Homology analysis. Isolates L7 mutation at nucleotide 944, G to C, codon 315, AGC to ACC, and nucleotide 946, G to T, codon 316 GGC to TGC.

Analysis of amino acid homology with isolate L7 natural strain H37Rv:

H37Rv:

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301 302 303 304 305 306 307 308 309 310 311 312 313 314
315316 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
```

Isolate L7:

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301 302 303 304 305 306 307 308 309 310 311 312 313 314
315316 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 of L7 is at nucleotide 946, changing the base G to T, codon 316 GGC to TGC. Mutations in the same position as three other isolates. It is estimated that this mutation at codon 316 is one reason the nature resistes isolates INH in L7, but the exact effect is unknown. Another frequent mutation occurred at codon Arg463Leu, but these mutations have been known not associated with resistance to INH (Shim et al., 1996). Several mutations have been reported to be at codon Arg128Gln, Ala291Pro (Fang et al., 1998), and Thr275Pro (Pym et al., 2002). His-108 which is one of the INH binding residues have been reported mutated in isoniazid-resistant isolates, the acid glutamate and glutamine (Rouse et al., 1995; Rouse & Morris, 1995).

Mutations in the active side of Asp-137 which plays an important role in the binding of INH has not been reported to have mutations, but mutations occurred at residues surrounding the N138S, A139P, S140N, or D142A (Zhang et al., 1992; Heym et al., 1995; Rouse et al., 1995; Cockerill et al., 1995; Musser et al., 1996). These mutants give effect by altering the local conformation so that it can change the orientation of Asp-137 side group is consequently unable to bind the INH (Jakopitsch et al., 2003).

Catalase Peroxidase Protein Visualization with PyMOL program

Research carried out Thomas Bertrand, et al., in the year 2004 has been crystallized catalase peroxidase *M. tuberculosis* and have determined the three-dimensional structure of the protein. The data of this crystal structure can be viewed on the public site www.ncbi.nlm.nih.gov with a 1SJ2. The position of residues that have mutations in the katG gene can be viewed by 1SJ2 structure using the program PyMOL. Fig 6 shows the position of residues 316 and 290 are mutated.

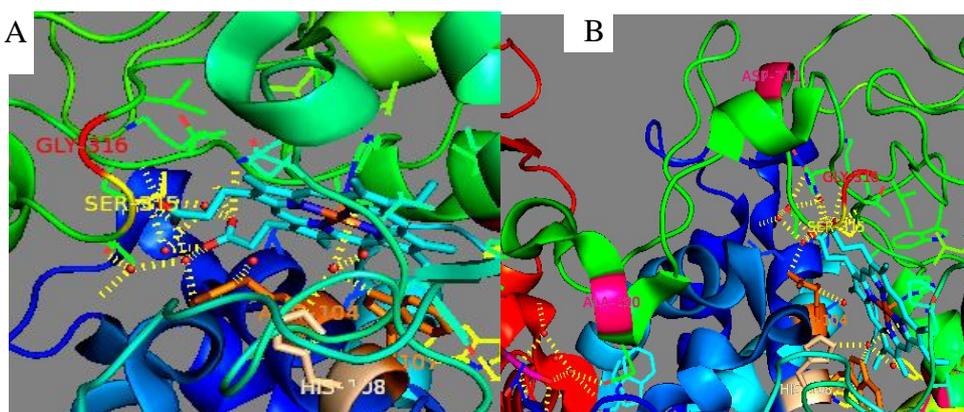


Figure 6. Residues are experiencing changes in position. Glycine residue position 316 mutated into cysteine (figure A) are indicated by red color. Alanine 290 residue positions that mutate into valine (figure B) is shown with a red label, alanine 290 in the loop regions (green).

Catalase peroxidase visualization space structure with the program PyMOL showed 316 amino acid residues located close to the active side of INH binding.

Figure 6 shows the surface changes due to mutation of glycine 316 residue to Aysteine. Glycine is the simplest amino acid, while cysteine, which is larger in size and can form a disulfide bond with another cysteine. But the effect of this mutation in the nature of resistance to INH is not known. Previous research has shown that mutations in residue 315 resulted in resistance to INH because the resulting changes in hydrogen bonding between the heme and serine315 (Bertrand et al., 2004; Yue et al., 2003). These three isolates had no mutation at residue 315 so that the mutation at residue 316 is strongly suspected to cause resistance

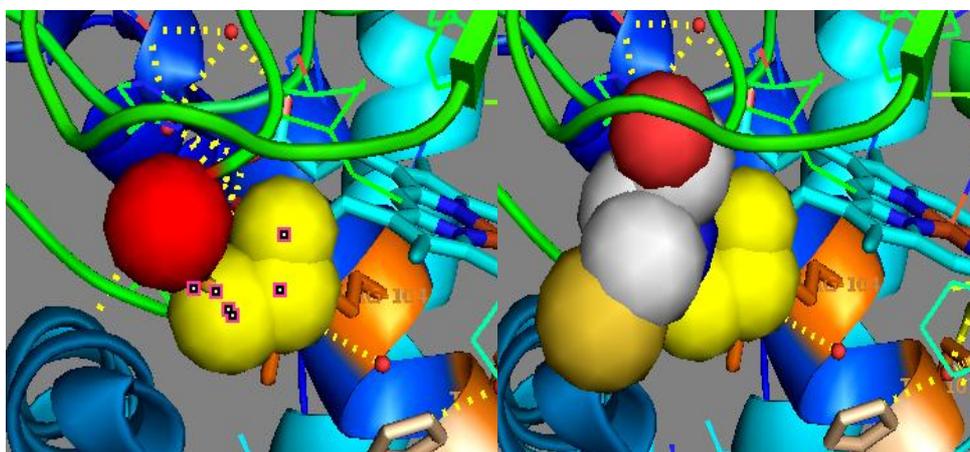


Figure 7. Visualization of changes of surface residue 316. (A) red: surface glycine 316, yellow: surface serine 315. (B) simulated changes in glycine 316 into cysteine, the larger the surface cysteine 316 are indicated by the color pink, gray, and dark yellow. Yellow: surface serine 315.

Residues 278 to 312 on the enzyme catalase peroxidase *M. tuberculosis* is in the loop region, the conformation is similar in two other peroxidase catalase structure that is at *Haloarcula marismortui* and *Burkholderia pseudomallei*. *Burkholderia pseudomallei* in the catalase peroxidase, loop area is estimated to be INH side of the substrate binding site interacts with the enzyme (Carpena et al., 2003). But Bertrand et al., and Pieratelli et al., Stating that in the catalase peroxidase *M. tuberculosis*, local loop is not the most important side of the binding of INH. Mutation at amino acid residue 290 is relatively distant from the active side and its influence on the nature of INH resistance is unknown.

CONCLUSION

Six isolates of MDR-TB that comes from Dok V Hospital, Jayapura, Papua province, a multiplex PCR in *rpoB* gene is mutated but not undergone mutation in the *katG* gene codon 315 results of this study, it showed that five isolates *katG* gene, based on nucleotide sequencing, not mutated at codon position 315 and one isolate mutated in these positions. Three out of five isolates of the above have the same type of mutation that is glisin316sistein. Two other isolates of each mutation in a different position, namely the position of mutations that alter amino acids alanin290valin; and other positions do not change the amino acid. Simulation of the structure using the PyMOL program showed that residue 316 is close to the active side of the binding of INH, whereas the 290 residues located at the tip of the N-loop regions that are relatively distant from the active side. Mutations in the loop region has not known the effect of INH-resistant properties. Three isolates had no mutation at codon 315, which had been shown to cause resistance to INH, but the mutation occurs at codon 316. The cause of resistance to INH in three isolates were allegedly due to mutation at codon position 316. However, further research is needed to confirm whether these mutations were the only cause of resistance to INH.

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