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Analysis and Mutation of Codon in *rpoB* and *katG* Genes and Bioinformatics Study of RIF Binding Model by RNA β Polymerase Subunit: Study in Tuberculosis Patients at Merauke General Hospital-Indonesia

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Abstract

Treatment of TB patients is usually done by administering three types of antituberculosis drugs with the main options being Rifampin (RIF) and Isoniazid (INH), then accompanied by streptomycin or pyrazinamide. RIF resistance is attributable to the mutation of the *rpoB* gene, the gene that produces the RNA polymerase β-subunit, and the INH resistance is largely due to the mutation of the *katG* gene. The aim of this study was to obtain information on the association of MDR-TB with related genes, as well as information on the combination of *Mycobacterium tuberculosis* genotype in tuberculosis patients in Merauke. Here we reported that most of the MDR-TB isolates are resistant to other antituberculosis drugs, and the mutation frequency of *rpoB*526 and *rpoB*531 (mutations that occur on both sides/this place almost always occur together) is almost the same but the *katG*315 mutation is present in only 16 isolates (the number of mutations that occur in *katG*315 is less than in *rpoB*526 and *rpoB*531). The presence of C1363A nucleotide changes in sensitive *Mycobacterium tuberculosis* of six antituberculosis drugs showed that not all *rpoB* mutations caused resistance. On the basis of this phenomenon, it can be proposed that the mechanism of formation of MDR-TB strains begins with a *rpoB* mutation followed by a mutation of *katG*. This study demonstrates that the mechanism of resistance to a drug that affects only one gene, such as rifampin that affects *rpoB*, is more easily controlled than antituberculous drugs affecting several genes, such as isoniazid which affects other genes besides *katG*.

Keywords: Characterization; *rpoB and katG*; Resistance RNA polymerase β-subunit; *katG* genes; Merauke district; Papua province-Indonesia

Introduction

The increasing problem of TB treatment and control is the Multidrug-Resistant Tuberculosis (MDR-TB) isolate, defined by the World Health Agency WHO as a RIF and INH-resistant tuberculosis isolate. Treatment of TB patients is usually done by administering three types of antituberculosis drugs with the main options being Rifampin (RIF) and Isoniazid (INH), then accompanied by streptomycin or pyrazinamide. RIF resistance is attributable to the mutation of the *rpoB* gene, the gene that produces the RNA polymerase subunit-β, and the INH resistance is largely due to the mutation of the *katG* gene [1].

Research related to MDR-TB has been widely practiced. As a result of *rpoB* mutations, especially in hotspots or RRDR (Rifampin Resistance-Determining Region), RIF can not inhibit RNA polymerase because it can not bind to the β-subunit, causing RIF resistance. Meanwhile, INH requires an activation process by the enzyme catalase-peroxidase produced by *Mycobacterium tuberculosis*. Most of the INH resistance is due to the *katG* gene mutation, the gene that produces the catalase-peroxidase enzyme, so that INH can not be converted into an active form. Until now the identified *katG* mutations cause mutation resistance only in codon 315. A small proportion of INH resistance can occur due to the mutations of the *inhA*, *ahpC*, and *kasA* genes, and other correlated genes. The data shows that more than 95% of *Mycobacterium tuberculosis* RIF-resistant is caused by the mutation of *rpoB* and 60%-70% of *Mycobacterium tuberculosis* that is resistant to INH caused by the *katG* mutation. Some publications also mention the presence of *Mycobacterium tuberculosis* isolates that are phenotypically resistant to RIF or INH but genotypically there are no mutations in the *rpoB* or *katG* gene [2,3].

Materials and Methods

Isolation, identification and genotype characterization

Mycobacterium tuberculosis isolates were obtained from sputum, pulmonary fluid, or other body fluids from tuberculosis patients obtained from Merauke Regency General Hospital, Papua Province-Indonesia. Clinical specimen collection followed by isolation and identification was done during January-May 2018. Isolation of *Mycobacterium tuberculosis* was done by culture method using Löwenstein-Jensen media. The inoculated medium is then incubated at 37°C for 4-6 weeks or until colony growth develops.

Genotype characterization was performed on the basis of an analysis of four genes of *Mycobacterium tuberculosis*, two genes producing membrane proteins and the other two being the *rpoB* and *katG* genes causing *Mycobacterium tuberculosis*-resistant properties to

RIF and INH. Characterization of the *rpoB* and *katG* genotypes is preferred in codon *rpoB*526, *rpoB*531, and *katG*315 using multiplex PCR and nucleotide sequencing methods, whereas the analysis on *efpA* and *Rv1877* uses only the nucleotide sequencing method. The primers we use in this study are as follows (Table 1).

Primer Name	Nucleotide Sequence	Temperature (°C)
Forward primer *rpoB*	5'-GTCGCCGCGATCAAGGA-3'	56
Reverse primer *rpoB*	5'-TGACCCGCGCGTACAC-3'	56
Inner primer *rpoB531*	5'-ACAAGCGCCGACTGT C-3'	48
Inner primer *rpoB526*	5'-GTCGGGGTTGACCCA-3'	50
Forward primer *katG*	5'-GCAGATGGGGCTGATCTACG-3'	64
Reverse primer *katG*	5'-AACGGGTCCGGGATGGTG-3'	60
Inner primer *katG315*	5'-ATACGACCTCGATGCCGC-3'	62

Table 1: The primar nucleotide sequence used in multiplex PCR *rpo*B526, *rpo*B531, and *katG*315.

Reactions of PCR rpoB531 and rpoB526 under initial denaturation conditions 96°C for 3 min; 5 cycles 95°C for 45 sec, 60°C for 1 min, and 72°C for 30 sec; 5 cycles 95°C for 40 sec, 59°C for 50 sec, and 72°C for 30 sec; 22 cycles 94°C for 50 sec, 55°C for 40 sec, and 70°C for 30 sec; final elongation of 72°C for 3 min.

Determination of the nucleotide sequences of the *efpA* and *Rv1877* genes was performed to see the association of these genes in MDR-TB resistant properties. Analysis of these two genes was only performed on 12 MDR-TB isolates representing high levels of resistance and low resistance levels. Meanwhile, the determination of the *rpoB* and *katG* nucleotide sequences is only partially performed, i.e. in segments flanked by forward and reverse primers used in multiplex PCRs. and the determination of this nucleotide sequence using the services of Macrogen Inc., Seoul of South Korea. All data on the results of this nucleotide sequence were analyzed using the Seqmen star DNA program.

Results and Discussion

Characterization of *rpoB* and *katG* genotypes

The resistance to RIF occurs because of the mutation of the *rpoB* gene, the gene that produces the RNA polymerase subunit-β. Approximately 95% of RIF-resistant isolates are caused by the mutation of the *rpoB* gene in the 80-bp region flanked by codons 507 and 533. This region is known as RRDR. These results are consistent with those reported by Hirano et al. [4-9] that over 70% of RIF-resistant properties in *Mycobacterium tuberculosis* in the Asian region are caused by the mutation of *rpoB*526 and *rpoB*531. The results of the analysis of the nucleotide sequence data and the mutations that we obtained also showed the same thing, which is an indication that the two mutations in the *rpoB* region are related to the nature of bacterial resistance. The number of isolates we analyzed was multiplexed PCR by 42, the number of patients with RIF resistance was 40, while those who experienced resistance to INH were 16 based on allegations of mutations in the area.

The determination of the *rpoB* segment nucleotide sequence shows highly variable results in a sample that amplifies two DNA segment bands for multiplex PCR. Meanwhile, almost all PCR multiplex analysis results for *rpoB*526 and *rpoB*531 showed similar results with nucleotide sequence determination. Different results were obtained from Papuan isolate samples, i.e., no mutation with PCP multiplex *rpoB*526 but the electroferogram result indicated the existence of A1576G mutation (codon *rpoB*526). Undetectable *rpoB*526 mutation with multiplex PCR is probably caused by amplification of other segments resulting from the inadequacy of the primary attachment. RIF is the main anti-tuberculosis drug used for the treatment of TB patients. A total of 10 amino acid residues in RNA polymerase subunit-β are involved in direct interaction with RIF.

Some of the *rpoB* mutations detected between nucleotides 1521 and 1730 are new mutations that have never been published. This study obtained several isolates containing *rpoB* mutations outside and inside the RRDR region flanked by the codon *rpoB*507 and *rpoB*533. *rpoB* mutations present in the RRDR region other than nucleotides C1592T (*rpoB*531), C1576G and C1576T (*rpoB*526), among them are the mutation of nucleotide sequences A1538T, A1534T and C1536G (R5) and C1548T and C1654T. Previous bioinformatics research suggests that changes in *rpoB* gene residues at RRDR at positions 511, 512, 515, 521, and 529 did not significantly affect the RIF's minimum inhibitory concentration MIC, but this study showed that *rpoB*512 mutations can also cause high levels of resistance. Meanwhile, Zenkin et al. [10], suggesting a change in the 12 amino acids in the protein (pocket) area resulted in the area being out of direct contact with RIF.

In other studies, most of the mutations occurring in the β-subunit RNA polymerase-β were found in region I (amino acid residue position 505 to 537) and region II (amino acid position 562 to 572). The results of the structural model analysis of RNA polymerase subunit-β proteins that bind RIF to *T. aquaticus*, show only a few amino acid residues that bind directly to RIF because they have the same polarity and can form bonds between nitrogen or oxygen, with RIF hydroxyl groups as seen in the following picture (Figure 1). The amino acid residues are Glu393 (homologous 513 to *Mycobacterium tuberculosis*), Phe394 (homolog 514), His406 (homolog 526), Arg409 (homologous 529), and Ser411 (homolog 531).

The picture below shows that the residue that forms the bond with the RIF hydroxyl group will give the greatest impact if there is a change of amino acid residues. Ser411 residue (homologous 531 on *Mycobacterium tuberculosis*) as seen in the drawing, has the closest distance to the RIF and forms a bond. Amino acid changes in this residue cause the greatest effect on the *Mycobacterium tuberculosis* phenotype. The presence of serine amino acid changes into leucine causes changes in the bond and also the distance between the amino acid residues and RIF. This is seen in the isolation level of isolates experiencing *rpoB*531 mutations relatively higher compared with other *rpoB* codon mutations.

Based on multiplex PCR analysis and nucleotide sequencing, some *rpoB* mutations, either amino acid or silent mutations, are obtained. The determination of the nucleotide sequence correctly identifies the amino acid changes occurring in the *rpoB* segments analyzed to obtain several previously unknown mutations. The *rpoB* mutation data

detected by determining the nucleotide sequence can be seen in Table 2.

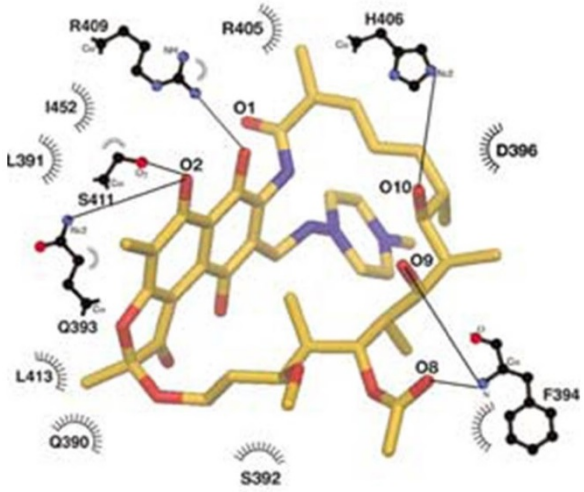

Figure 1: RIF binding model by RNA polymerase subunit-β on *T. aquaticus*. Rifampin in the form of a yellow structure, surrounded by residual RNA polymerase subunit-β. Five residues form the strongest bond with RIF so that the mutations in these five residues carry the greatest influence on resistance. Residues H406≈526 and S411≈531 on *Mycobacterium tuberculosis*. The thin black line shows the bond between the RNA subunit-β RNA polymerase residue and RIF; red color is a group containing oxygen while blue is a nitrogen group [2].

Nucleotide Position	Codon	Change of codon	Amino acid Changes
1532, 1534	512	AGC→TGG	Ser→Trp
1536	513	CAA→CTA	Gln→Leu
1546	516	GAC→GAT	Asp→Gly
1574	526	CAC→GAC	His→Asp
1574	526	CAC→TAC	His→Tyr
1575	526	CAC→CGC	His→Arg
1590	531	TCG→TTG	Ser→Leu
1602	535	CCC→CAC	Pro→His
1615	539	TCA→TCC	Ser→Ser
1630	544	GGG→GGC	Gly→Gly
1646	550	GTG→ATG	Val→Met
1652	552	CCG→TCG	Pro→Ser
1666	556	GGC→GGT	Gly→Gly
1669	557	CGG→CGT	Arg→Arg
1693	565	GAG→GAA	Glu→Glu

Table 2: *rpoB* mutations in MDR-TB isolates were detected by analysis of nucleotide sequencing.

katG analysis is only directed to (*katG*315) as a cause of resistance to INH because no other catgod mutations have been found yet. According to van Doorn et al. [8], 60%-70% of the INH-resistant properties of *Mycobacterium tuberculosis* isolates are due to the presence of the *katG*315 mutation and as much as 10%-15% of the resistance to INH is caused by mutations in the inhaler gene promoter region. The multiplex PCR to detect the *katG*315 mutation showed very low results when compared with published research results. Based on the PCR multiplex results of *katG*315 in 42 MDR-TB isolates, only 16 isolates (38.1%) were detected (*katG*315). Ramaswamy et al. [6] detected (*katG*315) in 25 isolates of 37 (67.6%) isolates of INH resistance analyzed. Mokrousov et al. obtained 93.6% of *Mycobacterium tuberculosis* isolates (*katG*315) in Russia. Previously, [3] obtained 22 of 24 (91.7%) isolates of *Mycobacterium tuberculosis* resistant INH were (*katG*315) in Russia.

Multiplex PCR results obtained by 16 MDR-TB isolates (*katG*315) increased with nucleotide sequencing results. The only change of nucleotide in the *katG* segment detected in this study was a mute mutation of T884C codon *katG*294 on isolate of Papuan 1. This study shows that the magnitude of (*katG*315) prevalence-occurring in the isolated *Mycobacterium tuberculosis* origin of Merauke is only part of the prevalence occurring in the world.

Some of the mutations occurring in the *katG* gene, especially the partial deletions of these genes, can cause *Mycobacterium tuberculosis* to be unable to cope with the oxidative stress derived from peroxides produced by macrophage cells. *katG*315 mutations are the most minimal mutations affecting the ability of *Mycobacterium tuberculosis* cells to face oxidative stress. Until now no other mutations of *katG* have been found that can cause resistance but do not affect the ability of *Mycobacterium tuberculosis* to face oxidative stress. Meanwhile, Mn^{2+} metal ions are required to solidify the protein structure of catalase-peroxidase.

The low frequency of the *katG*315 mutations occurring in the MDR-TB isolates originating from Merauke, Papua-Indonesia may be caused by other mechanisms. So far there has been no research that mentions the mutation data in *Mycobacterium tuberculosis* that give rise to resistance to INH in Indonesia, especially in Bandung. The resistance to INH is a very complex process. INH affects several genes so that the high genotype $(katG)^{+}$ associated with INH-resistant traits suggests that antituberculosis drugs mechanisms affecting some genes will be more difficult to observe than antituberculosis drugs that affect only one gene, as does RIF. INH resistant properties can be caused by mutations of genes other than $(katG315)^{-}$, but usually occur at low frequencies. The existence of time and cost constraints, does not allow analysis of other genes. In addition, the limitation also caused not all MDR-TB isolates were analyzed by determining the nucleotide sequence. Well-detected MDR-TB isolates undergoing mutations by multiplex PCR analysis (a very clear amplified band) are excluded from the next method of determining the nucleotide sequence.

Conclusion

Some of the detectable *rpoB* mutations between nucleotides 1521 and 1730 are newly unpublished mutations in tuberculosis patients in Merauke district, Papua province-Indonesia. This study obtained several isolates containing *rpoB* mutations outside and inside the RRDR region flanked by the codon *rpoB*507 and *rpoB*533. The results of the analysis showed that changes in 12 amino acids in the area (*pocket*) proteins resulted in the area being not in direct contact with

RIF. Most of the mutations occurring in the β-subunit RNA polymerase β are found in region I (the position of amino acid residues 505 to 537) and region II (the position of amino acids 562 to 572). Some of the mutations occurring in the *katG* gene, especially the partial deletions of these genes, can cause *Mycobacterium tuberculosis* to be unable to cope with the oxidative stress derived from peroxides produced by macrophage cells.

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