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Der Pharmacia Lettre, 2016, 8 (14):85-91
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Study *in silico* and characterization of mutations in *rpoB* and *katG* genes in patients of tuberculosis in Jayapura, Papua province, Indonesia

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ABSTRACT

RIF resistance due to mutations in the *rpoB* gene, the gene that produces RNA polymerase β -subunit and INH resistance is largely due to mutations in the gene *katG*. With the increasing number of people with HIV/AIDS cause TB disease, WHO categorizes as a re-emerging disease. Objective of this study was to obtain information MDR-TB relations with the relevant genes, as well as information combined genotype of *M. tuberculosis*. Here, we reported that their C1363A nucleotide changes (Pro535His) in *M. tuberculosis* sensitive six antituberculosis drugs showed entire *rpoB* mutations causing resistance properties. On the basis of this phenomenon, it can be suggested that the formation mechanism of MDR-TB strains begins with *rpoB* mutations followed by mutation *katG*. This study shows that the mechanism of resistance to an anti-tuberculosis drug that only affects a single gene, such as rifampin affecting *rpoB*, will be more easily controlled than anti-tuberculosis drugs that affect multiple genes, for example isoniazid affecting other genes other than *katG*. The analysis showed a residue that forms a bond with a hydroxyl group of RIF will provide the most impact when there is a change of amino acid residues. Ser411 residue (531 homolog in *M. tuberculosis*), has the shortest distance to the RIF and form bonds. Changes in the amino acid residues have caused the greatest effect on the phenotype of *M. tuberculosis*. The change of leucine amino acid serine be causing changes in the bond and also the distance between the amino acid residues with the RIF. It is visible at the level of resistant isolates have mutations *rpoB*531 relatively higher compared to other mutations in codon *rpoB*.

Keywords: Characterization, *in silico*, *rpoB* and *katG*, resistance of β -subunit RNA polymerase, Jayapura-Papua Province

INTRODUCTION

Tuberculosis is an infectious disease in humans caused by the bacterium *Mycobacterium tuberculosis*. This chronic disease characterized by tissue death (necrosis) caused by delayed-type hypersensitivity, namely the phagocytosis and presentation of epitopes (antigen recognition) by macrophages in cell surface resulting in a series of processes that trigger the reaction of cells T lymphocytes main problem continued increases in the treatment and control of TB is the multidrug-resistant isolates of *M. tuberculosis* (MDR-TB), which is defined by the World Health Organization, WHO, as *M. tuberculosis* isolates were resistant to RIF and INH [1-3].

Treatment of TB patients are usually carried out by administering three types of anti-tuberculosis medicines with the main option is rifampin (RIF) and isoniazid (INH), then accompanied with streptomycin or pyrazinamide. RIF resistance due to mutations in the *rpoB* gene, the gene that produces RNA polymerase β -subunit and INH resistance is largely due to mutations in the gene *katG* [4-5]. With the increasing number of people with HIV/AIDS cause TB disease WHO categorizes as reemerging disease.

Research related to MDR-TB have been done. Due to a mutation of *rpoB*, particularly in the hotspot or RRDR (*rifampin resistance-determining region*), then RIF can not inhibit the action of RNA polymerase because it can not bind β -subunit, causing resistance to RIF. Meanwhile, INH requires activation process by the catalase-peroxidase enzyme produced by *M. tuberculosis*. Most of INH resistance occurs due to mutations in *katG* gene, the gene that produces the enzyme catalase-peroxidase, so that INH can not be converted into an active form. Until recently identified *katG* mutation causes resistance mutation at codon 315. just a small percentage of INH resistance can occur because of a gene mutation *inhA*, *ahpC*, and *kasA*, as well as other genes that correlated [6].

The data shows more than 95% of RIF-resistant *M. tuberculosis* is caused by mutations in *rpoB* and 60-70% INH resistant *M. tuberculosis* is caused by mutations in *katG*. Some publications also mentioned *M. tuberculosis* isolates that are resistant to RIF phenotype or genotype INH but there are mutations in the *rpoB* or *katG* gene [2, 7-10].

MATERIALS AND METHODS

M. tuberculosis isolates obtained, tested six kinds of resistance to anti-tuberculosis drugs. This test is performed by proportional method using Löwenstein-Jensen medium. The suspension isolates are made by inserting a colony of *M. tuberculosis* into a sterile tube containing saline and Glassbead thus achieving McFarland turbidity. The suspension was then diluted to 10x and 100x. 0.5 ml bacterial suspension from 10x dilution is taken and put in a test medium, namely media Löwenstein-Jensen slant which already contains anti-tuberculosis drugs are different in every tabunganya. Control is made by inserting a bacterial suspension of 10x and 100x dilution of 0.5 ml into the media control (not containing anti-tuberculosis). The cultures were incubated at 37 °C for 4-6 weeks [11].

Genotype characterization and *in silico* analysis

Characterization of genotypes is based on an analysis of four genes of *M. tuberculosis*, the two genes that produce membrane protein and the other two are the *rpoB* and *katG* genes that cause the nature of *M. tuberculosis* resistant to INH and RIF. Characterization of *rpoB* and *katG* preferred genotype at codon *rpoB*526, *rpoB*531, and *katG*315 using multiplex PCR and determining the nucleotide sequence, while analysis on *efpA* and Rv1877 only use the method of determining the nucleotide sequence. Analysis *efpA* gene, Rv1877, as well as segments of *rpoB* and *katG* do with the method of determining the nucleotide sequence based on the amplification primer pair. In addition, analysis of *rpoB* and *katG* performed by multiplex PCR method that uses three primary, the forward primer, reverse primer, and inner primer. Primer is used to detect mutations in codon *katG*315, *rpoB*531, and *rpoB*526 thus generated by the primary segments within segments of DNA called a variable. If there is a mutation, then the 3'-end of the primer can not be paired with its complement and variable segments of DNA amplification process can not occur. Forward and reverse primer *rpoB* and *katG* gene will amplify segments each measuring 0.25 kb and 0.44 kb [12-14]. The results of amplification primer pairs are called non-variable band because there must always be to mark the passage of multiplex PCR process.

Determining the nucleotide sequence of the gene *efpA* and Rv1877 done to see how the genes in the MDR-TB resistant properties. Analysis of both genes are only done on 12 isolates were MDR-TB which represented the high levels of resistance and resistance levels are low. Meanwhile, the determination of the nucleotide sequence of the *rpoB* and *katG* only done partially, namely in the segment flanked by the forward and reverse primer used in multiplex PCR. Cell lysis method for isolating DNA and determination of these nucleotide sequences using the services of Macrogen Inc., Seoul, South Korea. All data is the result of the determination of the nucleotide sequence is analyzed *in silico* using DNASTAR program with a variety of applications such as *Seqman* and *MegAlign* [15-17].

RESULTS AND DISCUSSION

Characterization of *rpoB* and *katG* based on the determination of the nucleotide sequence on Papuan isolates

Analysis of determining the nucleotide sequence of DNA segments performed using forward primer amplification product and turning on multiplex PCR. The primer pair amplifying the band length of approximately 0.25 kb and 0.44 kb, respectively for the *rpoB* and *katG*. Primer *rpoB* nucleotide flanking region between 1521 and 1730, or between codons 507 and 576. Thus, a hotspot for nature resistant to rifampin or also called RRDR located between codons 507 and 533 amplify entirely. Meanwhile, the determination of the nucleotide sequence of *katG* segment bounded by the nucleotide sequence of 675 and 1104. amplified *rpoB* and *katG* segments can be seen in Fig. 1 below.

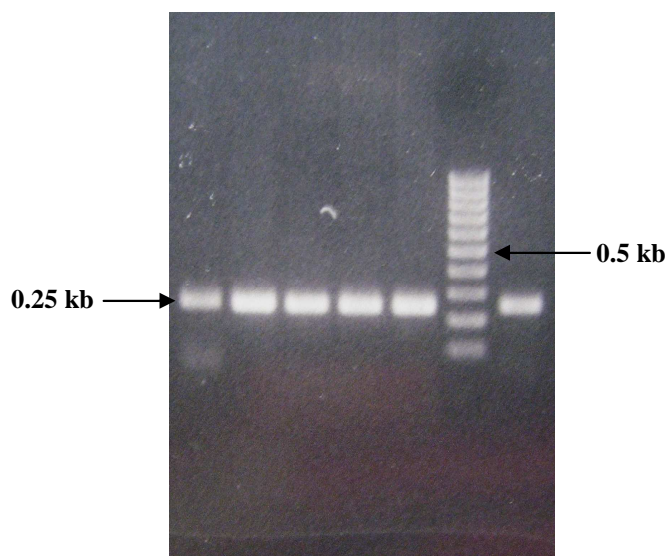


Fig 1. Amplified *rpoB* fragment of 0.25 kb in size. Multiplex PCR results are dubious in some isolates, such as P1-P5, and other isolates will be confirmed by determining the nucleotide sequence. After reaching a sufficient concentration, along the 0.25 kb fragment is then used to determine the nucleotide sequence; (M) band marker of 100 bp

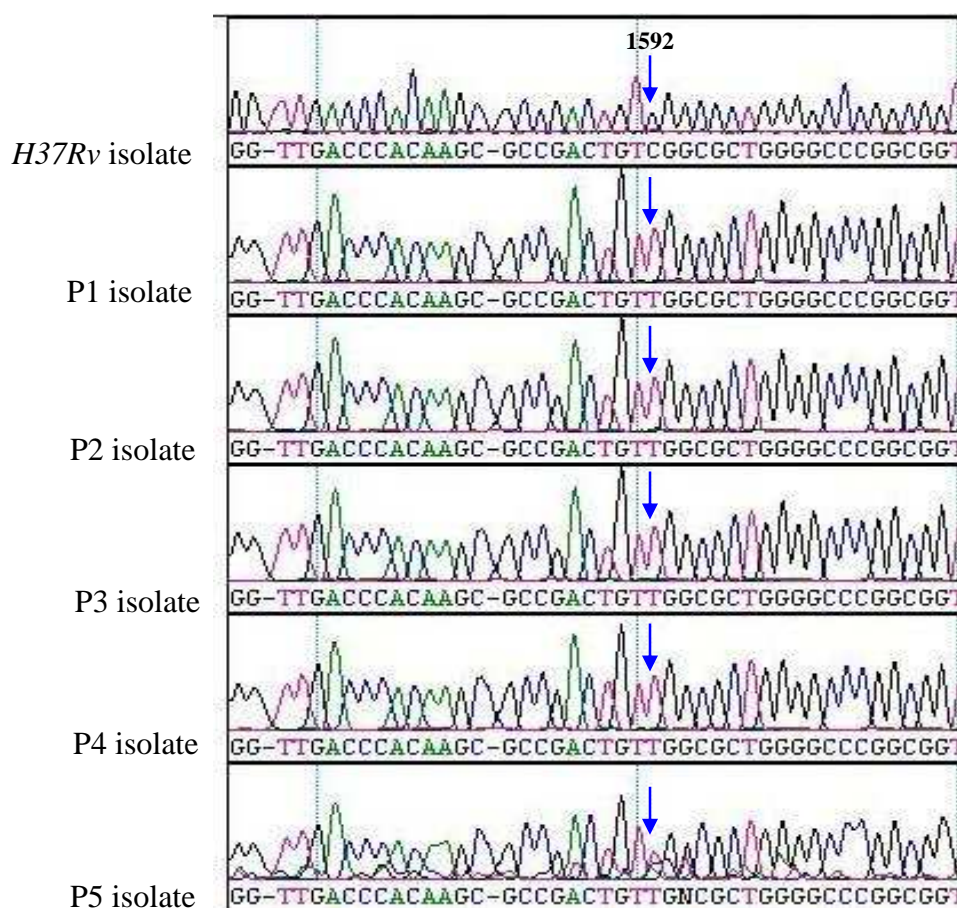


Fig 2. Elektroferogram of *rpoB* gene on some isolates of MDR-TB. On the graph shows the change in the nucleotide isolates P1, P2, P3, P4 and P5 compared with standard isolates of *M. tuberculosis* H37Rv. Mutations are C1592t, analysis using DNASTar with Seqman application

Electropherogram analysis results starting with the nucleotide sequence determination focused on nucleotides 1578-1580 (*rpoB*526) and nucleotides 1593-1595 (*rpoB*531). Some isolates detected a mutation in codons with multiplex PCR method, also appear to provide the same results in the electropherogram.

Elektroferogram result of the determination of the nucleotide sequence of *katG* segment on MDR-TB isolates showed different results with multiplex PCR. Isolates amplify two DNA segments ribbon on multiplex PCR showed no mutations in the nucleotide sequence *katG315* or 944. Similarly, isolates the non-variable amplifying the band alone, showed mutations in the nucleotide sequence of 944, ie codon *katG315*. One of the wild-type isolates *katG315*, namely P3 carrying mutations in the nucleotide silent T882C.

Gene of *rpoB* and *katG* each having a length of 4810 bp and 3516 bp while the segment analysis of the nucleotide sequence of the *rpoB* and *katG* carried out successively in order 1521-1730 and 675-1104 nucleotides for *katG* segment. On the results of multiplex PCR *rpoB526*, *rpoB531*, and *katG315*, in addition to the ribbon non-variable and variable, are also found non-specific band segments of DNA in some samples. It is likely that the number template DNA is added to the PCR mixture too much so that the PCR reaction showed specific results. The use of three primer on multiplex PCR primer causing the temperature to well more than one type.

Non-specific band of the multiplex PCR results do not affect the analysis of genotypes for *rpoB531*, *rpoB526*, and *katG315* because this method is only used to detect the presence of DNA fragment bands variable size 0.18 kb and 0.17 kb for the codon *rpoB526* and *rpoB531*, and tape measure 0.25 kb for the detection of codon *katG315*. DNA samples used in multiplex PCR is the result of cell lysis is not measured concentrations of DNA, causing the DNA concentration in each sample in a multiplex PCR reaction is different.

The nature resistant to RIF occurs because of the *rpoB* gene mutation, the gene that produces β -subunit RNA polymerase. Approximately 95% of RIF resistant isolates is caused due to the *rpoB* gene mutations in the region of 81 bp flanked by codons 507 and 533. The area is known by the name RRDR [8, 18-19]. Mutations that often happens is a change Ser531Leu (TCG \rightarrow TTG) and His526Asp (CAC \rightarrow GAC) [20-21]. *rpoB* analysis in this study focused on RRDR region, in particular codon *rpoB526* and *rpoB531*, to determine the cause of RIF-resistant properties of the MDR-TB.

The results showed about 81% of MDR-TB isolates were determined by multiplex PCR mutated, whereas 19% of MDR-TB isolates were not detected mutated *rpoB526* or *rpoB531*. These results are consistent with another study reported that over 70% of RIF-resistant properties of the *M. tuberculosis* in the region are caused by mutations *rpoB526* and *rpoB531* [15-16].

Determining the nucleotide sequence of the *rpoB* segment showed a very varied in the samples to amplify two DNA segments ribbon for multiplex PCR. Meanwhile, almost all the results of multiplex PCR analysis for *rpoB526* and *rpoB531* showed similar results with the determination of the nucleotide sequence. The different results obtained from the sample isolates P3, ie no mutations with multiplex PCR *rpoB526* but elektroferogram results showed A1576G mutation (codon *rpoB526*). *rpoB526* mutations that are not detected by multiplex PCR amplification may be caused by other segments as a result of inaccuracies annealing. This imprecision generate DNA bands other but with the same size.

RIF is anti-tuberculosis drugs main options used for treatment of patients with TB. A total of 12 amino acid residues in the β -subunit RNA polymerase involved in direct interaction with the RIF. Substituted 11 of 12 amino acid residues will cause nature resistant to RIF [5, 12] mentions more than 90% of RIF resistance occurs because of the genetic changes in the 81 bp fragment RRDR *rpoB* gene which encodes the β -subunit of RNA polymerase. Meanwhile, several studies have shown *rpoB* gene mutations that are outside RRDR also can cause resistant properties.

Most of *rpoB* mutations were detected between 1521 and 1730 nucleotides is a new mutation that has never been published. This research obtains some isolates contained mutations in *rpoB* outside and inside the region flanked by the codon RRDR *rpoB507* and *rpoB533*. Mutations *rpoB* contained in the region RRDR besides nucleotide C1592T (*rpoB531*), C1576G and C1576T (*rpoB526*), among which are mutations in the nucleotide sequence A1538T (sample P1), A1534T and C1536G (sample P5), as well as C1548T and C1654T (Sample P4), G1389C silent mutations occur in isolates Papua 5, Papua 11, and papua 12 so that the alleged silent mutations are forms of *M. tuberculosis rpoB* gene polymorphism. Besides A1538T on P1 isolates, these mutations have not been published in research journals. Mutations A1538T (*rpoB513*) on Papua 1 isolates other investigators have been published [17].

Bioinformatics research conducted other researchers, suggests that changes residue *rpoB* gene in RRDR at positions 511, 512, 515, 521, and 529 did not significantly affect the MIC (*minimum inhibitory concentration*) RIF, but this study shows a mutation *rpoB512* can also lead to high levels of resistance. Meanwhile, research has shown a change in the amino acid 12 in the area (pocket) protein resulted in the area becoming no direct contact with the RIF. The other thing is that most mutations in β -subunit RNA polymerase discovered in the region I (the position of amino

acid residues 505 to 537) and region II (amino acid position 562 to 572). The results of the analysis of protein structure model- β subunit of RNA polymerase binding RIF on *T. aquaticus*, showed only a few amino acid residues that bind directly with RIF because it has the same polarity and can create a bond between the nitrogen or oxygen, the hydroxyl group of RIF. The amino acid residues are Glu393 (513 homologous to the *M. tuberculosis*), Phe394 (homologous 514), His406 (homologous 526), Arg409 (homologous 529), and Ser411 (homologous 531).

The figure below shows the residues which form a bond with a hydroxyl group of RIF will provide the most impact when there is a change of amino acid residues. Ser411 residue (531 homolog in *M. tuberculosis*) as shown in the picture, has the shortest distance to the RIF and form bonds. Changes in the amino acid residues have caused the greatest effect on the phenotype of *M. tuberculosis*. The change of leucine amino acid serine be causing changes in the bond and also the distance between the amino acid residues with the RIF. It is visible at the level of resistant isolates have mutations *rpoB531* relatively higher compared to other mutations in codon *rpoB*.

As shown in the figure above, the serine residue at *rpoB531*⁺ which forms a bond with RIF, changed into leucine distance was closer but the kind of bond that may occur with RIF weaker than before. The bond between hydrogen and oxygen on the interaction RIF and serine are polar covalent bond (*hydropathy index* (HI) -0.8). Meanwhile, leucine RIF form a bond with carbon and hydrogen which is a type of bond that is very non-polar (HI +3.8). *PyMOL* substitution performed by using the assumption that there is no change in the three-dimensional structure of another amino acid residue.

Mutations in *rpoB526* detected in this study will change the amino acid histidine into arginine or tyrosine. As shown in the figure below, the main consequence of the mutation at residue 526 is a distance change with RIF residue causing the bond between the residue with RIF weaker than the wild-type residue.

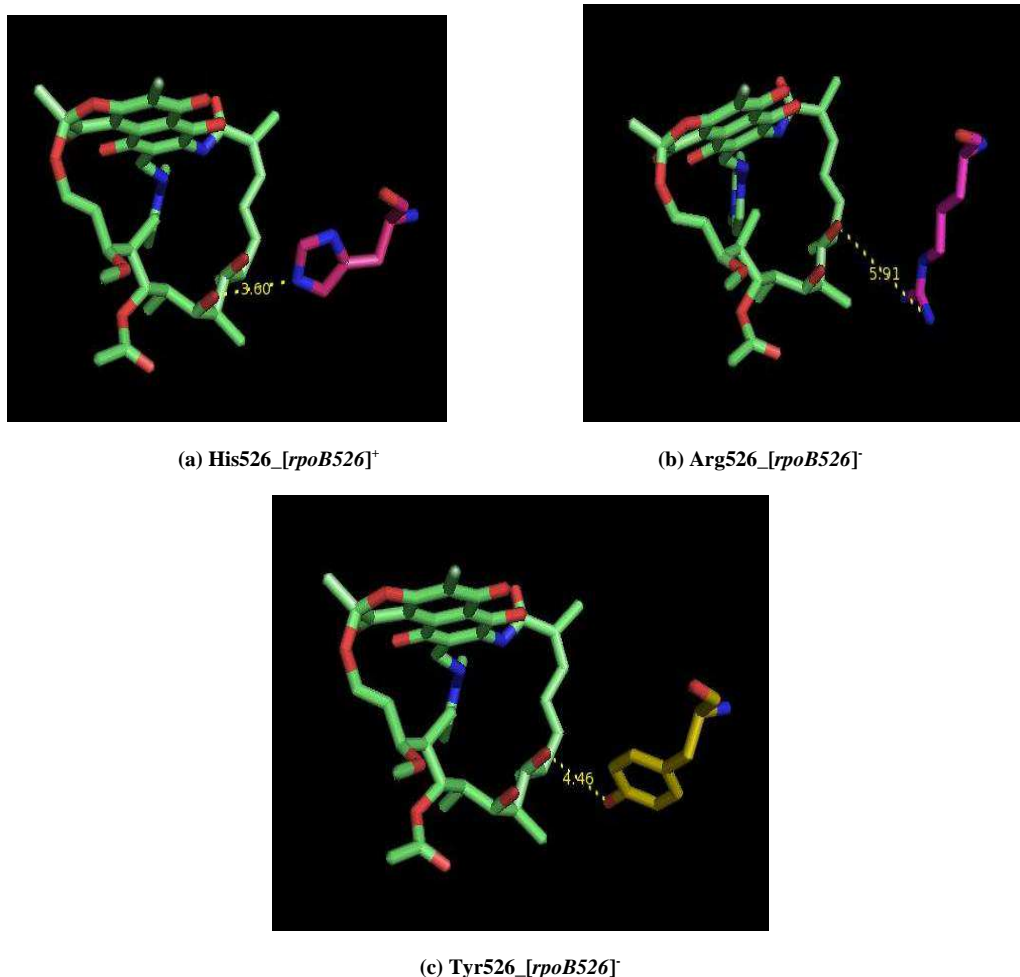


Fig 3. The distance and the possibility of the type of bond between RIF with the residue *rpoB526*⁺ (a) or *rpoB526*⁻ (b and c). Modelling shows the type of bond on *rpoB526*⁺ and *rpoB526*⁻ no different. Only the bond distance which indicates the difference so that the increased resistance due to mutations in codon *rpoB526* not by mutations in codon *rpoB531*. The structure of the cluster in blue is nitrogen, red is oxygen, while the color green, purple, and yellow is hydrogen

Isolates P1 (Papua 1) and Papua 5 consecutive have mutations that alter amino acid residues and Ser512Trp Gln513Leu, while isolates P12 double mutated residue Asp516Gly and Pro552Ser. Mutations in the P1 isolates found by other students in the same research group [12]. The level of resistance which is owned by the three isolates were very high, ≥ 300 $\mu\text{g/mL}$. This shows that the amino acid changes that were located very close to the RIF, the change in residue 512, 516, and 531 cause isolates of *M. tuberculosis* has a high level of resistance to RIF.

The data on the A1534T and C1536G mutation (codon *rpoB*512) on P5 isolates, as well as C1548T mutation (codon *rpoB*516) and C1654T (codon *rpoB*552) in isolates P12 has never been published in previous research journal. A1534T and C1536G mutations in isolates P5 change Ser512Trp residue, and mutation C1548T in isolates P12 which converts residue Asp516Gly still require further analysis. This mutation is only known based on the determination of the nucleotide sequences using a reverse primer and has not been confirmed by the results of determining the nucleotide sequence using forward primer. Meanwhile, C1654T mutation on P12 isolates have been able to know for sure because the results of the analysis determining the nucleotide sequence was performed using forward and reverse primer.

C1654T mutation in Papua isolates 12 leads to changes in the amino acid proline into serine. Proline is a non-polar amino acids serine whereas polar. Both of these amino acids tend to have a neutral pH. In addition, the determination of the nucleotide sequence P12 isolates using reverse primer detect the presence of other mutations, namely *rpoB*516. Modelling interactions with residues *rpoB* RIF showed that replacement of glycine residues *rpoB*516 be due C1548T nucleotide changes will increase the distance between the residue with RIF when compared with the previous residues aspartic acid. Glycine is an amino acid that has the non-polar neutral pH while the polar aspartic acid that tends to sour. Both the nature of these amino acids are so different that their replacement causes a very large effect on the overall interaction RIF and β -subunit of RNA polymerase. Model binding of RNA polymerase RIF in use with the help of a software program PyMOL.

In addition C1548T and C1654T mutations that alter amino acid, P12 isolates also had some silent mutation, ie A1617C and G1632C. G1632C silent mutations are not only found in isolates P12 but P11 is also found in isolates and some silent mutation C1668T, G1671T and G1695A in isolates P5. P9 isolates have double mutations *rpoB*550 and *rpoB*531 that alter valine into methionine residue. Valine and methionine have the same properties, namely the non-polar neutral pH. However, mutations in isolates P9 *rpoB*531 have enough to make it resistant to RIF isolates with resistance levels above 300 $\mu\text{g/mL}$.

C1604A mutation at nucleotide (codon *rpoB*535) on P4 isolates change Pro535His residue. Proline is a type of non-polar amino acid with a pH neutral bring imino group, while the polar histidine with an alkaline pH for carrying a side chain of basic groups [4, 13-21]. Both properties have very different amino acids and amino acid residues location not too far from RRDR should change the phenotype of resistant isolates P4 into RIF. This constraint comes from the possibility of error reading test results resistant properties that only have a limit of 1% difference in the number of colony growth of *M. tuberculosis* control tube and test tube containing RIF. Increase the number of colonies related to the length of incubation time is not yet have a standard (between 4-6 weeks), can lead to differences in results between phenotype with genotype isolates. Another possibility is that not all *rpoB* mutations can cause resistance to RIF. Analysis of determining the nucleotide sequence of P4 isolates carried out in two directions, using forward and reverse primer and both show the same results. Other research has detected *rpoB*535' on MDR-TB isolates were also *rpoB*516.

CONCLUSION

Bioinformatics research is conducted concluded that changes in gene *rpoB* in RRDR residues at positions 511, 512, 515, 521, and 529 did not significantly affect the MIC (*minimum inhibitory concentration*) RIF, but this study shows *rpoB*512 mutations can also cause high levels of resistance. Meanwhile, changes in the 12 amino acid in the area (pocket) protein resulted in the area becoming no direct contact with the RIF. Most mutations in β -subunit RNA polymerase discovered in the region I (the position of amino acid residues 505 to 537) and region II (amino acid position 562 to 572). The results of the analysis of protein structure model β -subunit of RNA polymerase binding RIF on *T. aquaticus*, showed only a few amino acid residues that bind directly with RIF because it has the same polarity and can create a bond between the nitrogen or oxygen, the hydroxyl group of RIF. The amino acid residues are Glu393 (513 homologous to the *M. tuberculosis*), Phe394 (homologous 514), His406 (homologous 526), Arg409 (homologous 529), and Ser411 (homologous 531). The analysis showed a residue that forms a bond with a hydroxyl group of RIF will provide the most impact when there is a change of amino acid residues. Ser411 residue (531 homolog in *M. tuberculosis*), has the shortest distance to the RIF and formed bonds. Changes in the amino acid residues have caused the greatest effect on the phenotype of *M. tuberculosis*. The change of leucine amino acid serine be causing changes in the bond and also the distance between the amino acid residues with the RIF. It is

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Acknowledgements

The authors gratefully acknowledge the support of this work by the Chairperson and colleagues of Papua Biomedical Research and Development Institute, Jayapura-Indonesia, Chairperson of the Laboratory of Biochemistry and Molecular Biology, Faculty of Mathematics and Natural Sciences, University of Cenderawasih that has provided research facilities.

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