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Molecular Analysis of Rifampin-Resistant Mycobacterium tuberculosis Strains Isolated from Papua, Indonesia

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Abstract: Resistance to RIF (Rifampin) is caused by mutations in the gene *rpoB* that coding RNA polymerase (RNAP) on β-subunit, determinant of RIF resistance (codons 507-533), with the highest frequency at codon 526 and 531. In one strain of clinical isolates of MDR *M.tuberculosis* from Jayapura, Papua province, which is on the research group of *M.tuberculosis* Laboratory of Biochemistry, University of Cenderawasih, there are isolates that have mutations in codon 315 of *katG* gene causes resistance to isoniazid, but do not have a mutation at codon *rpoB*526 and *rpoB*531. In this research, determining the causes of genotype-level RIF resistance in clinical isolates of MDR *M.tuberculosis* of Papuan isolates and also sought an explanation of the relationship with the nature of the resistance mutations. Sequencing results were analyzed *in silico* that is aligned with the nucleotide sequence of the standard strains of *M. tuberculosis* H37Rv, using the program SeqMan™ and MegAlign™. Here we showed that the result of multiplex PCR and agarose gel electrophoresis of clinical isolates of MDR *M. tuberculosis* gave good results of two DNA bands on allele specific multiplex PCR results *rpoB*526 and *rpoB*531. Results *In silico* translation analysis showed that the CAA codon coding for the amino acid glutamine (Gln) is mutated to CTA which encodes leucine (Leu). Protein modeling results using the program PyMOL showed that Gln513Leu changes alter the distance between the side chain residues with hydroxyl groups of RIF from 2.63 Å to 3.71 Å. Sequencing and alignment shows the mutation in other positions isolates Papua *rpoB* gene, which is at codon 513. These mutations are thought to cause resistance to RIF, as has been reported previously. It is expected that knowledge of this resistance mechanism could be used as the basis for design of new drugs to treat MDR-TB problem.

Keywords: Kata kunci: MDR-TB, DNA, Mutation, Rifampin, Papuan Isolates.

Introduction

Tuberculosis (TB) is a contagious infectious disease in humans caused by the bacterium *Mycobacterium tuberculosis*. The number of tuberculosis patients in Papua has increased every year due to poor sanitation and inadequate health services (Table 1). Until now, there are many drugs to treat tuberculosis which is an antibiotic such as rifampin, isoniazid, pyrazinamide, ethambutol, streptomycin, fluoroquinolone, and others. However, although there are a lot of drugs againts tuberculosis, TB remains a disease that is difficult to overcome. This is mainly due to the properties owned by TB resistance to antibiotics. TB resistance is divided into two kinds: one type of antibiotic resistance, and resistance to more than one type of antibiotic. WHO has defined the TB that is resistant to at least two types of antibiotics as well as the rifampin (RIF) and isoniazid (INH) as a multidrug-resistant TB (MDR-TB). MDR-TB of course caused by strains of *M. tuberculosis* who have the trait. The emergence of cases of MDR-TB is a global problem that must be addressed to eradicate TB.

Resistance *M.tuberculosis* to antibiotics caused by mutations in the bacterial chromosome. This causes the sensitivity of *M. tuberculosis* to anti-tuberculosis drugs is reduced. These mutations occur in genes that code for antibiotic or gene targets that play a role in the interaction of antibiotics with target in *M. tuberculosis.* Resistance to INH occurs largely due to a mutation in the gene encoding the catalase-peroxidase *katG* that play a role in changing the INH into its active form inside cells (1, 2). Resistance to RIF occur due to mutations in the gene *rpoB* encoding the RNA polymerase (RNAP) subunit β, which causes the RIF was not able to function in inhibiting the transcription initiation process (1-3).
The main causes of INH resistance mutations in the *katG* gene is a mutation at codon 315, while the main cause of RIF resistance mutations are located in areas along the 81 base pairs (bp) in the *rpoB* gene, called RIF resistance determining region, namely codons 507-533, with frequency highest mutation at codon 526 and 531. The codon numbering system uses a number of Escherichia coli *rpoB* codon, not codon actual number *M.tuberculosis* (3). Mutations cause both types of resistance can be detected above has been simply and quickly using the method of *Polymerase Chain Reaction* (PCR) allele-specific multiplex (4).

In the collection of 20 strains of clinical isolates of MDR *M.tuberculosis* in Papua, Indonesia, which is on the *M. tuberculosis* research group of Biochemistry, Faculty Mathematics and Natural Sciences, University of Cenderawasih, there is a genotype test isolates by using multiplex PCR is known mutated at codon katG315 but do not have mutations at codon rpoB526 and rpoB531. Therefore, it has RIF resistance phenotype must be caused by other factors. Presumably the nature of it caused by the mutation in codon positions other than the codon above.

Based on existing problems, the study was conducted to determine the cause of RIF resistance levels of genotypes in clinical isolates of MDR *M.tuberculosis* above, in addition to mutation at codon *rpoB*526 and *rpoB*531. It also sought clarification on the relationship of mutations with RIF resistance.

Materials and Method

Polymerase Chain Reaction (PCR) multiplex Specific *rpoB* alleles

Clinical isolates of MDR *M.tuberculosis* used in this study is isolate P1, P2, P3, and P4, which originated from the Laboratory of Biochemistry, University of Cenderawasih, Jayapura, Papua Province, Indonesia. The method used is a modified method of allele-specific multiplex PCR test for detection of *rpoB* RIF-resistant *M. tuberculosis* (RIFr) conducted by previous researcher (4).

In this study, first done reconfirmation multiplex PCR *rpoB*531 and *rpoB*526 above that have been conducted on the four isolates. For each type of multiplex PCR used the outer primer pair, namely RF forward primer (5'-GTCGCCGCGATCAAGGA) RR and a reverse primer (5'-TGACCCGCGCGTACAC), and one inner primer R526 (5'-GTCGGGGTTGACCCA) or R531 (5'-ACAAGCGCCGACTGTC). Template DNA form of DNA lysis *M. tuberculosis* clinical isolates (5 µL) was added to the PCR reaction mixture (final volume of 20 µL for PCR-*rpoB*526 and 15 µL for PCR-*rpoB*531) containing 10x PCR buffer without $MgCl_2$ (2.5 µL for PCR-*rpoB*526 and 2 µL for PCR-*rpoB*531), $MgCl_2$ (3 µM for PCR-*rpoB*526 and 4 µM for PCR *rpoB*531), 0.5U Taq DNA polymerase, 200 µM each dNTP, 1 pmol outer primer RF, 20 pmol outer primer RR, and one of the specific primers in allele-R526 (30 pmol) or R531 (30 pmol). There are four primers that used, synthesized by Proligo, Singapore. As a positive control used the same PCR reaction mixture, with template DNA containing results of the standard strains of lysis *M.tuberculosis*

H37Rv, whereas the negative control contains no template DNA (ddH_2O replaced with the same volume).

Both PCR reactions performed on GeneAmp® PCR System instrument 2700, Perkin Elmer, with conditions as follows: initial denaturation at 96 °C for 3 min, 5 cycles consisting of 95 °C for 45 seconds, 60 °C for 1 min, and 72 °C for 30 seconds, 5 cycles consisting of 95 °C for 40 seconds, 59 °C for 50 seconds, and 72 °C for 30 seconds, 25 cycles consisting of 94 °C for 50 seconds, 55 °C for 40 seconds, and 70 °C for 30 seconds, and final elongation at 72 °C for 3 minutes. PCR results were then analyzed by agarose gel electrophoresis 1.5% w/v (0.6 g agarose in 40 mL 1X TAE buffer containing 2 mL EtBr 10 mg/mL). PCR samples (5 µL) added with 2 µL loading buffer and electrophoresed in 1X TAE running buffer, at 75 volts for 50 minutes. The results of electrophoresis and then visualized with the aid of UV light. As a standard DNA (markers) used pUC19/HinfI containing DNA fragments sized 1419 bp, 517 bp, 396 bp, 214 bp, 75 bp and 65 bp.

Multiplex PCR to confirm the results above, the determination of the nucleotide sequence 249 bp fragment containing RIF resistance determining region. The four clinical isolates on their respective first amplified using outer primer pair only (RF and RR), with the same PCR reaction conditions with multiplex PCR, so that the resulting fragment size of 249 bp, with a quantity of about 800 ng. The same was done on strains of *M. tuberculosis* H37Rv, which will then be used as a standard. 249 bp fragment of PCR and standard four clinical isolates and then sent to Macrogen Inc, Seoul, South Korea, for the nucleotide sequence determined using the RR primer as sequencing primer. The method used is an extension Macrogen single primary (single primer method), with the help of Automatic Sequencer 3730xl.

Analysis *in silico*

Nucleotide sequence of clinical isolates and standard sequencing result was analyzed *in silico*, or using a computer program. I*n silico* analysis of the first is the alignment sequences of clinical isolates and standard strains of sequences *M.tuberculosis* H37Rv using the program DNASTAR SeqMan™ and MegAlign™. Alignment is intended to detect the presence of mutations in the sequences of clinical isolates. Analysis in silico which is next done used the protein modeling program PyMOL version of *Open-Source*, Delano Scientific, USA. This model is aimed to observe the effect of mutations on the interaction of RIF with RNAP, to explain the relationship between the mutations with RIF resistance. As a model of interaction used RNAP-RIF complex crystal structure of RNAP core *T. aquaticus* (Taq) and RIF with the code ID Protein Data Bank (PDB) 1I6V (Campbell et al., 2001). This structure is used because of Taq, *M. tuberculosis*, and *E. coli*, nucleotide sequence regions that contain mutations RIF^r the three organisms are identical for 91% (5). In this model, there will be changes in the amino acid residues are mutated.

Results and Discussion

Specific Multiplex PCR *rpoB* alleles

In this study, first, done reconfirmation *rpoB*526 allele specific multiplex PCR and *rpoB*531, which has been done before, the isolates P1, P2, P3 and P4. This multiplex PCR aimed to detect the mutation at codon *rpoB*526 and *rpoB*531. Multiplex PCR results four isolates that were electrophoresed with agarose gel 1.5% (w/v) and visualized using UV light gave the following results. P1 isolates produced two bands of DNA size 0.25 kb and 0.18 kb for PCR-*rpoB*526 and 0.25 kb and 0.17 kb for PCR-*rpoB*531. While the other isolates, P2, P3 and P4, also gives the results of two DNA band size 0.25 kb and 0.18 kb for PCR-*rpoB*526, but only returns a single DNA band size of 0.25 kb for PCR-*rpoB*531.

PCR results are validated with positive controls and negative controls used. Both controls work well, indicated by the absence of DNA bands in the PCR negative control and the presence of two DNA bands sized 0.25 and 0.18 and 0.17 kb or 0.25 kb (according to the type of multiplex PCR is performed) on the results PCR positive control. The meaning of the formation of one or two bands in multiplex PCR and electrophoresis results above can be explained by looking at the target schema in Figure following multiplex PCR. Outer primer RF and RR will amplify 249 bp fragment remains, both in PCR and PCR-*rpoB*526-*rpoB*531. Inner primer (R526 and R531), each has been designed for 3'-end of his stick on the second base codon *rpoB*531 and *rpoB*526 and wild-type allele (4) (Fig 1). If the two codons are not mutated, the primer will stick well and together with the RR primer will amplify specific fragments of wild-type allele, the size of 181 bp for PCR-*rpoB*526

and 167 bp for PCR-*rpoB*531. Conversely, if there are mutations in the first two bases of each codon, the 3-'end primer cannot stick and will not be formed allele-specific fragment.

DNA bands of PCR multiplex and the size of 0.25 kb electrophoresis showed the formation of 249 bp fragment remains, while the band with 0.18 and 0.17 kb respectively indicate the formation of specific fragments of wild-type 181-bp alleles (PCR-*rpoB*526) and 167 bp (PCR-*rpoB*531). Therefore, multiplex PCR results of two DNA bands 0.25 and 0.18 or 0.25 kb and 0.17 kb, respectively indicate that the codon *rpoB*526 or *rpoB*531 a wild-type allele. While the results of multiplex PCR of DNA only one band (0.25 kb) showed a mutation at codon concerned.
Summary on the following table shows that the four isolates had wild-type allele codons *rpoB*526 (not mutated) and only isolates that have codon *rpoB*531 P1 wild-type allele, whereas the three other isolates (P2, P3, and P4) the mutated codon (**Table 2).** On the basis of data multiplex PCR results four isolates, only isolates P1 confirmed no major mutations causing RIF resistance, whereas isolates P2, P3, and P4 appeared to have a mutation in codon *rpoB*531 which is the main cause of RIF resistance (1, 2).

By not mutated *rpoB*526 and *rpoB*531 codon in isolate P1, then owned isolates RIF resistance must be caused by other factors, such as *rpoB* gene mutations at codon positions other than the two above. To find out, has been sequenced 249 bp fragment of *rpoB* gene isolates P1, which contains the determinant of RIF resistance. Sequencing was also performed on three other isolates to confirm the mutation rpoB531 above.

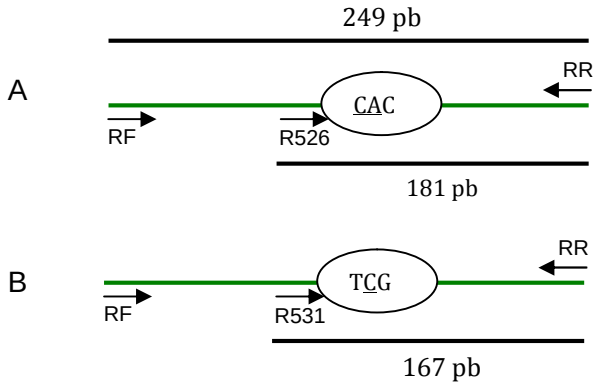

Fig 1. The scheme targets specific multiplex PCR and allele *rpoB*526 and *rpoB*531. (A) PCR-*rpoB*526, *up*. (B) PCR-*rpoB*531, *down*. Short arrows represent the primer, long arrows represent PCR fragments that remain (249 bp), and fragment-specific wild-type alleles (181 and 167 bp). If the mutated codon 526 and 531 produced only 249 bp fragments.

Table 1.The number of tuberculosis patients in Papua Province on years 2007-2010

Type of diseases	2007	2008	2009	2010
Tuberculosis	1,448	1,523	6,747	2,569
Clinical tuberculosis	2,203	3,427	3,027	3,583
Total	3,651	4,950	9,774	6,152

Table 2. Results of allele specific multiplex PCR *rpoB*

Papua Isolates	PCR-*rpoB*526			PCR-*rpoB*531		
	249 pb	181 pb	*rpoB526*	249 pb	167 pb	*rpoB531*
P1	√	√	+	√	√	+
P2	√	√	+	√	χ	-
P3	√	√	+	√	χ	-
P4	√	√	+	√	χ	-

Note: mark (√) and (χ) in a row indicate that the fragment is formed and not formed, while the sign (+) and (-) respectively indicate codon *wild-type* and mutant alleles.

Determination of Nucleotide Sequences

249 bp fragment of PCR isolates P1, P2, P3 and P4, as well as standard *M. tuberculosis* H37Rv, which contains the determinant of RIF resistance characteristics, nucleotide sequence determined with the helped by Macrogen Inc., South Korea. The method used was a single primer extension, with RR as the sequencing primers. Results obtained for each isolate in the form of electropherogram data (in the form of ab1 files). In the following picture looks isolates P1 nucleotide sequence (Fig 2). Electropherogram three other isolates and H37Rv standard.

Analysis of Alignment

To identify the mutations in positions other than *rpoB*526 and *rpoB*531 in clinical isolates P1 and confirm *rpoB*531 mutations in isolates P2, P3, and P4, do the alignment between the nucleotide sequence of sequencing results of clinical isolates and standard *M. tuberculosis* H37Rv. Alignment performed with the program DNASTAR SeqMan™ and MegAlign™.

SeqMan™ DNASTAR is programs that align the nucleotide sequence with the display data electrophoregram. Complement sequence alignment isolate P1 (P1-RR) and H37Rv sequence (H37R-RR) are shown in Figure below in sequence (Fig. 3). The alignment results showed the presence of one substitution mutation of adenine (A) to thymine (T), which is the nucleotide number 1295 *rpoB* gene or genome nucleotide number 761101 *M.tuberculosis* H37Rv. Nucleotide at this position is second base codon *rpoB*513, so that CAA mutated into CTA.

Sequencing and alignment has been done to confirm the data of allele-specific multiplex PCR and *rpoB*531 and *rpoB*526. The data obtained show consistency with the results of multiplex PCR, namely that the P1 isolates, codon *rpoB*526 and *rpoB*531 is wild-type allele, while the isolates P2, P3, and P4, codon *rpoB*526 a wild type allele while *rpoB*531 mutated codon (Fig 4). Mutations at codon RIF^r *rpoB*531 explain the nature of the three isolates.

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GATCGGGC ACATCCGGCC GTAGTGCGAC GGGTGCACGT 70
CGCGGACCTC CAGCCCGGCA CGCTCACGTG ACAGACCGCC 110
GGGCCCCAGC GCCGACAGTC GGCGCTTGTG GGTCAACCCC 150
GACAGCGGGT TGTTCTGGTC CATGAATAGG CTCAGCTGGC 190
TGGTGCCGAA GAACTCCTTG ATCGCG              216
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Fig 2. Nucleotides sequence of isolate P1. Published partial nucleotide sequence of isolate P1 sequencing results, in accordance with electrophoregram.

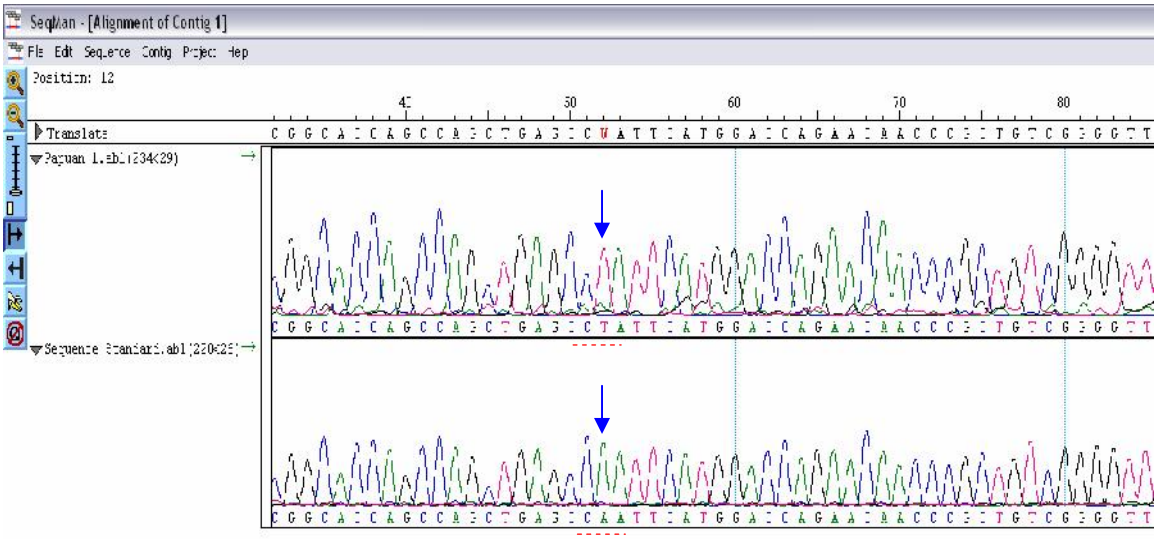

Fig 3. Alignment SeqMan™ application on DNASTAR program. Shown in the picture complements the nucleotide sequence of isolate P1 aligned with nucleotide sequences complementary to the standard strains of *M. tuberculosis* H37Rv. Arrows indicate mutation bases adenine (A) on the H37Rv into thymine (T) in isolate P1. Fields marked with red color indicates the mutated codon *rpoB*513 from CAA to CTA.

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H37R-RR: … ACC AGC CAG CTG AGC CAA TTC ATG GAC CAG AAC …
P1-RR:   … ACC AGC CAG CTG AGC CTA TTC ATG GAC CAG AAC …
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Fig 4. Nucleotide sequence alignment partially isolate P1 (P1-RR) with standard strains of *M. tuberculosis* H37Rv (H37R-RR). Shown in the picture is *rpoB*513 the mutated codon from CAA to CTA (blue).

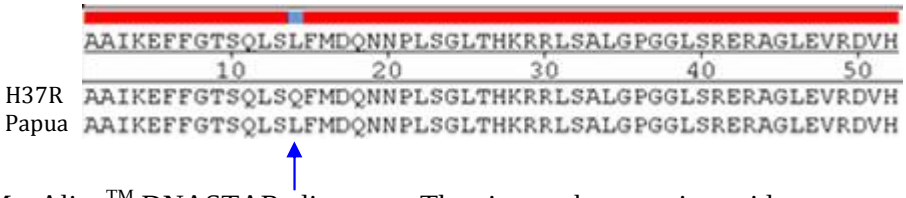

Fig 5. MegAlign™ DNASTAR alignment. The picture shows amino acid sequence alignment of isolate P1 with the amino acid sequence of the standard strains of *M. tuberculosis* H37Rv. The position of the marked blue arrow indicates the mutation Q (glutamine) in H37Rv to L (leucine) at P1 isolates.

In addition to the SeqMan™, sequence alignment of isolate P1 (P1-RR) with standard sequence H37Rv (H37R-RR) were also performed with DNASTAR MegAlign™. Previously, the nucleotide sequence of clinical isolates and sequencing results are stored in the form of first EditSeq™ file (file with extension SEQ fields). Results position MegAlign™ show the same mutation on P1 isolates, the A1295T. Using this program, can be directly aligned amino acid sequence translation of the nucleotide sequence of results which have been aligned previously. The results of amino acid sequence alignment results of translational sequences P1 and standard H37Rv isolates showed mutations Q (glutamine, Gln) to L (leucine, L). Changes in amino acids is located at codon *rpoB*513, resulting from mutations A1295T previously mentioned, which amended the CAA coding for coding for Gln to CTA Leu. Alignment analysis was performed on isolates of P1 MDR *M.tuberculosis*, either by using the program DNASTAR MegAlign™ and SeqMan™ and has shown the mutation in codon *rpoB*513 second base, namely A1295T (**Fig 5).** In the standard strains of *M.tuberculosis* H37Rv, *rpoB*513 codons have nucleotide sequences that encode Gln CAA, while in isolate P1, it mutated into CTA codons coding for amino acid Leu.

If the codon 513 mutation was the only isolate mutations in the *rpoB* gene of P1 MDR *M.tuberculosis*, the nature of RIF resistance isolates were allegedly caused by changes Gln513Leu. Mutations in this position in accordance with the results of research that has been done in various countries (3, 6, 7-11) and is also thought to cause high-level RIF resistance, both in *M. tuberculosis* and *E. coli* (8). Elucidation of the relationship between these mutations with RIF resistance phenotype sought at the protein level.

Interaction of RNA Polymerase-Rifampin Based Protein Modeling

To be able to explain the relationship of mutation Gln513Leu above with RIF resistance phenotype isolates possessed MDR *M.tuberculosis* P1, studied the influence of these mutations on the interaction of RNAP and RIF at the protein level. This is done by modeling the crystal structure of complex proteins using RNAP core Taq and RIF as model of the structure of RNAP interaction-RIF in *M. tuberculosis* (5). The structure of Taq RNAP-RIF was selected as a model for the nucleotide sequence regions that contain mutations cause the organism RIF^r Taq, *M. tuberculosis*, and *E. coli* has a high level of similarity, 91% (5). Information regarding the structure and interactions of RNAP-RIF Taq RNAP is used to describe the interaction-RIF in *M. tuberculosis*. Protein modeling is done with the help of the program PyMOL version of open-source, Delano Scientific, USA.

Residues Gln513 RNAP β-subunits *M. tuberculosis* (432 residues for *M. tuberculosis* numbering), which mutated into Leu at the P1 isolates, a homologous residue Gln393 Taq RNAP β subunit. Gln residues at these positions are known as residues that play a role in the binding of RIF on the RNAP β subunit by forming hydrogen bonds between side chains that are polar Gln with hydroxyl groups of RIF (5). Using the PyMOL program, performed mutation Gln393 residue, Leu Taq become a non-polar side chain, and calculated the distance between the residue side chain hydroxyl group of RIF, before and after mutation. The modeling results show that changing Gln to

Leu resulted in distance between residue side chain with hydroxyl groups of RIF becomes more distant, ie from 2.63 Å to 3.71 Å.

Gln side chain that initially form a hydrogen bond, the nitrogen atom, and hydroxyl group are important for RIF RIF activity (5), was replaced by Leu side chains in non-polar and can not form hydrogen bonds. Modeling using PyMOL with RIF-structure model Taq RNAP also shows that the mutation resulted Gln513Leu distance between the residue side chain with hydroxyl groups of RIF becomes more distant (3.71 Å) (**Fig 6).** These changes are due to mutations Gln513Leu strongly suspected to cause the hydrogen bond is formed on top. It is known that hydrogen bonding occurs between highly electronegative atoms with hydrogen atoms attached to another electronegative atom, with distances of less than 3.5 Å (12-13). The atoms which have a high electronegatifity are fluorine, oxygen, and nitrogen.

In some isolates of *M. tuberculosis* is earlier known to have mutations cause RIF^r, also there is a change of amino acid residues with polar side chains into residues with non-polar side chains such as the above. An example is the mutation Asp516 to Val and Gly (3, 6, 9, 10, 11). The residue is homologous with Asp396 Taq hydrogen bonded hydroxyl group of RIF. Mutated this residue into Val and Gly can cause hydrogen bond is not formed. The trend is the formation of hydrogen bonding on an explanation that can be given to the nature of the P1 isolates RIF resistance is caused by mutations Gln513Leu. The loss of hydrogen bonding that was originally formed to reduce the affinity binding of RIF to RIF more weakly bound in RNAP β-subunit. The above will result in RIF cannot work effectively the activity of RIF is more dependent on its ability to bind to RNAP (5). In addition to the above, the rigid conformation expected causes of RIF RIF cannot adapt to mutations that alter the shape and chemical environment binding pocket, so that the binding of RIF on RNAP is very sensitive to the presence of mutations in the above (14-19). RIF is weakly bound to the RNAP expected to result in changes in the position of RIF, particularly on the conditions of this enzyme in the cell, which of course is dynamic and is also influenced by the presence of water molecules. When the position of RIF changed in such a way that is no longer blocking the path extension of the RNA, the transcription process likely will continue to run and bacteria will be resistant to RIF.

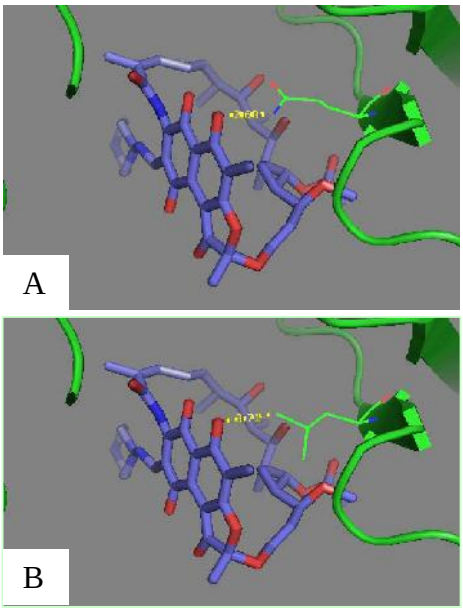

Fig 6. Interaction of RIF-RNAP β-subunit PyMOL modeling results. (A) Interaction of wild-type RNAP Gln393-RIF; (B) Interaction of RNAP mutants Leu393-RIF. There are homologous with Taq Gln393 Gln513 *M.tuberculosis*. Mutations resulting in side chain residues into non-polar and change the distance between the side chain hydroxyl group of RIF from 2.63 Å to 3.71 Å (indicated by yellow numbers). These changes may result in loss of hydrogen bonding that was originally formed. RNAP backbone and carbon atoms are shown in green, carbon atoms RIF purplish blue, nitrogen atoms dark blue, and oxygen is red.

Conclusions

One isolate (P1) of 20 clinical isolates of multidrug-resistant (MDR) *M. tuberculosis* from Papua Province, who have no major mutations causing rifampin resistance (RIF), has successfully found to have mutations Gln513Leu who allegedly causing RIF resistance phenotype. These results are based on the genotype and the mutation rate *in silico* analysis that shows the changing nature of the side chains of polar residues into non-polar side chain and changes the distance the hydroxyl group of RIF. These mutations can cause the binding affinity of RIF in RNA polymerase (RNAP) cannot be reduced so that the RIF working inhibit RNAP in the transcription and *M. tuberculosis* become resistant to these antibiotics. To reinforce the above conclusion, this study recommended continued with experiments *in vitro*, for example by cloning and expression of the mutant *rpoB* gene, or with protein modeling further to see the stability of the mutant protein compared with wild-type protein. It is expected that knowledge about the mechanisms of resistance obtained in this study can be used as the basic for the design of new drugs to overcome resistance to RIF and MDR-TB in general.

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