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Analysis of mutations in the *rpoB* and *katG* gene through the study of multiplex PCR and nucleotide sequence analysis in patients with TB in Jayapura-Indonesia

Hendrikus M. B. Bolly[1] and Yohanis Ngili[2]

[1]Biochemistry Research Group, Biochemistry Division, Faculty of Medicine, University of Cenderawasih, Jalan Raya Sentani Abepura, Jayapura-Indonesia
[2]Biochemistry Research Group, Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Cenderawasih, Jalan Kamp Wolker Kampus Baru Waena, Jayapura-Indonesia

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ABSTRACT

Research related to MDR-TB have been done either to determine the nature of resistance, nucleotide sequence analysis, determining the position of a mutation in the region Mtb, phylogenetic analysis, and determination of the mechanism of drug resistance through in silico analysis of biomolecular. Due to a mutation of rpoB, particularly in the hotspot or RRDR (rifampin resistance-determining region), then RIF could 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. Currently identified katG mutations cause only resistance mutations in codon 315. A small part of INH resistance can occur because of a gene mutation InhA, ahpC and kasA, as well as other genes that correlate. Here, we reported that results of analysis performed by determining the nucleotide sequence of DNA segments using the forward and reverse primer amplification product on multiplex PCR. The primer pair amplifying the 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. This research has the potential to determine the appropriate drugs and vaccines in the future through cooperation among various fields of science: medical clinics, pharmacology, molecular biochemistry, biostatistics, and molecular microbiology ,

Keywords: *rpoB*, *katG*, multiplex PCR, patients with TB, and Jayapura in Indonesia.

INTRODUCTION

Tuberculosis (TB) is an infectious disease in humans caused by *Mycobacterium tuberculosis* bacteria. 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 reactions of T lymphocytes cells [1-3].

The main problem that continues to increase in the treatment and control of TB is the *Multidrug-Resistant M. tuberculosis* (MDR-TB) *isolates*, which is defined by the World Health Organization, WHO as *M. tuberculosis* isolates were resistant to RIF and INH. Treatment of TB patients are usually carried out by administering three types

of anti-tuberculosis medicines with primary choice 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 subunit-β and INH resistance is largely due to mutations in the *katG* gene. With the increasing number of people with HIV/AIDS cause TB disease WHO categorizes as *re-emerging disease* [4].

Research related to MDR-TB have been done by previous researchers. 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 [5-7].

Currently, mutation *katG* identified only cause resistance mutations at codon 315. A small part of INH resistance can occur because of a gene mutation *inhA*, *ahpC* and *kasA*, as well as other genes that correlated [3,7]. 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 that phenotypically *M. tuberculosis* isolates that are resistant to RIF or INH but there are mutations in the *rpoB* or *katG* gene [8-11].

MATERIALS AND METHODS

Equipment and materials used on stage phenotype include Löwenstein-Jensen medium in test tubes and *laminar flow* as a sterile for culturing *M. tuberculosis*, and ose sterile pipette, as well as an incubator 37 ºC as a culture incubation. Additionally, other materials used on stage phenotype is RIF, INH, streptomycin, kanamycin, ethambutol, and pyrazinamide. The tools and materials used in the test phase of the genotype of which is *thermal cycler Perkin-Elmer GeneAmp PCR System 2400* for DNA amplification, electrophoresis apparatus, and a UV lamp (*transluminator*) were used to analyze the results of amplification. Centrifuges and a water bath is widely used at the stage of DNA isolation, while the micro-tubes, along with the micro pipette tip is a tool often used in the analysis of genotypes. The materials used were Taq DNA polymerase enzyme, the nucleotide sequence of primer, dNTP, and agarose [12-14].

The collection of clinical specimens

M. tuberculosis isolates obtained from sputum, lung fluid, or other body fluids of patients with TB were obtained from the General Hospital Dok II Jayapura, Papua Province-Indonesia. The collection of clinical specimens, followed by isolation and identification conducted during January-June 2016.

Isolation and identification

Isolation of *M. tuberculosis* performed with the culture method using Löwenstein-Jensen medium. The media were inoculated and then incubated at 37 ºC for 4-6 weeks or until you see the growth of the colony. Colonies that grew *M. tuberculosis* can not be confirmed due to other mycobacteria can grow well on this medium [15].

Genotype characterization

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 [16-17].

Analysis *efpA* gene, Rv1877, as well as segments of *rpoB* and *katG* performed 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, namely the forward primer, reverse primer, and the inner primer. Primer is used to detect mutations in codon *katG315*, *rpoB531*, and *rpoB526* thus generated by the inner primer segments called variable of DNA. 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 [15,17-18]. The results of amplification primer pairs are called non-variable band because there must always be to mark the passage of multiplex PCR process.

PCR reactions *rpoB531* and *rpoB526* at initial denaturation on conditions 96°C for 3 min; 5 cycles at 95°C for 45 sec, 60°C for 1 min, and 72°C for 30 sec; 5 cycles for 40 sec 95°C, 59°C for 50 sec, and 72°C for 30 sec; 22 cycles for 50 sec 94°C, 55°C for 40 sec, and 70°C for 30 sec; final elongation at 72°C for 3 min. *KatG315* multiplex PCR

reaction is conducted initial denaturation under 96°C for 3 min; 5 cycles for 1 min 95°C, 62°C for 1 min, and 72°C for 30 sec; 5 cycles for 1 min 95°C, 60°C for 40 sec, and 72°C for 30 sec; 22 cycles for 1 min 94°C, 58°C for 40 sec, and 72°C for 30 sec; 72°C final elongation for 3 min. A total of 10 μL amplification of DNA fragments electrophoresed on a 1.5% agarose gel with 1% TAE buffer, stained with ethidium bromide, and visualized under UV light [18].

RESULTS AND DISCUSSION

The diagnosis of pulmonary tuberculosis, especially tuberculosis, can be established by clinical examination, analysis of patient complaints and physical examination, laboratory tests and radiological examination. Three examination results were united for the diagnosis of tuberculosis. One of the laboratory examination is to detect *M. tuberculosis* as the cause. In general, the method used is the conventional method such as acid-fast bacilli by microscopic examination and culture examination. Microscopic examination is quite fast and economical but still lack sensitivity and specificity [7-10].

Microscopic examination of sputum is still considered important in diagnosis using the Ziehl-Neelsen method. Sputum microscopy is highly dependent on the concentration of *M. tuberculosis* in sputum. To get a positive acid-fast bacilli under a microscope the number of *M. tuberculosis* at least 5,000 stems/1 mL of sputum. As for checks on culture/culture of *M. tuberculosis* is the number of stem 50-100/mL of sputum. Sputum good to be inspected are those of the discharge brochus by 5-10 ml with minimum content of nasal or oral secretions. Taken a series three times in succession, wherever possible third is phlegm morning of the first day because it is estimated bronchial secretions accumulate in the lungs while sleeping at night (**Fig 1**).

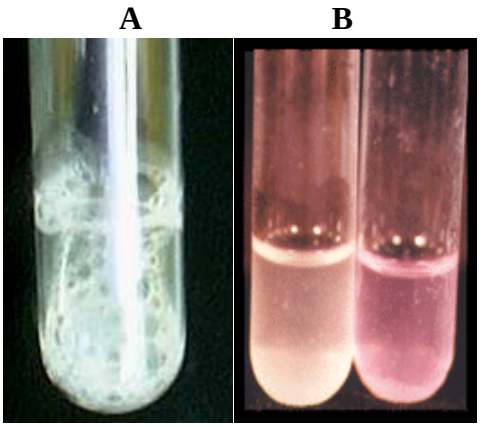

Fig 1. (A) The culture of M. tuberculosis Löwenstein-Jensen medium, at the *M. tuberculosis* catalase test. The enzyme *catalase-peroxidase* is one of the virulence factors of *M. tuberculosis* because the enzymes help the defense system against peroxide *M. tuberculosis* inside the macrophage cell; and (B) Test the reduction of nitrate to nitrite in M. tuberculosis. This reaction is used to distinguish *M. tuberculosis* complex with non-tuberculous mycobacteria. A positive reaction when it changes color to red

After phenotypic characterization of 24 isolates were MDR-TB, then performed a variety of further tests to determine the characteristics of the genotype, ie the multiplex PCR method and determination of nucleotide sequences. Characterization of *rpoB* genotypes aimed at RRDR, mainly codons *rpoB526* and *rpoB531*, characterization *katG* only aimed at codon *katG315*.

A total of eight samples to amplify two bands fagmen DNA for the entire multiplex PCR were performed, namely DNA samples isolates P1, P2, P3, P4, P5, P6, P7 and P8 (P = isolates from Papua) so that a subsequent analysis, the determination of the order nucleotides (**Fig 2**). In addition, several samples to amplify a DNA segment of band on multiplex PCR *rpoB526*, *rpoB531*, and *katG315* also included in the determination of the nucleotide sequence as confirmation.

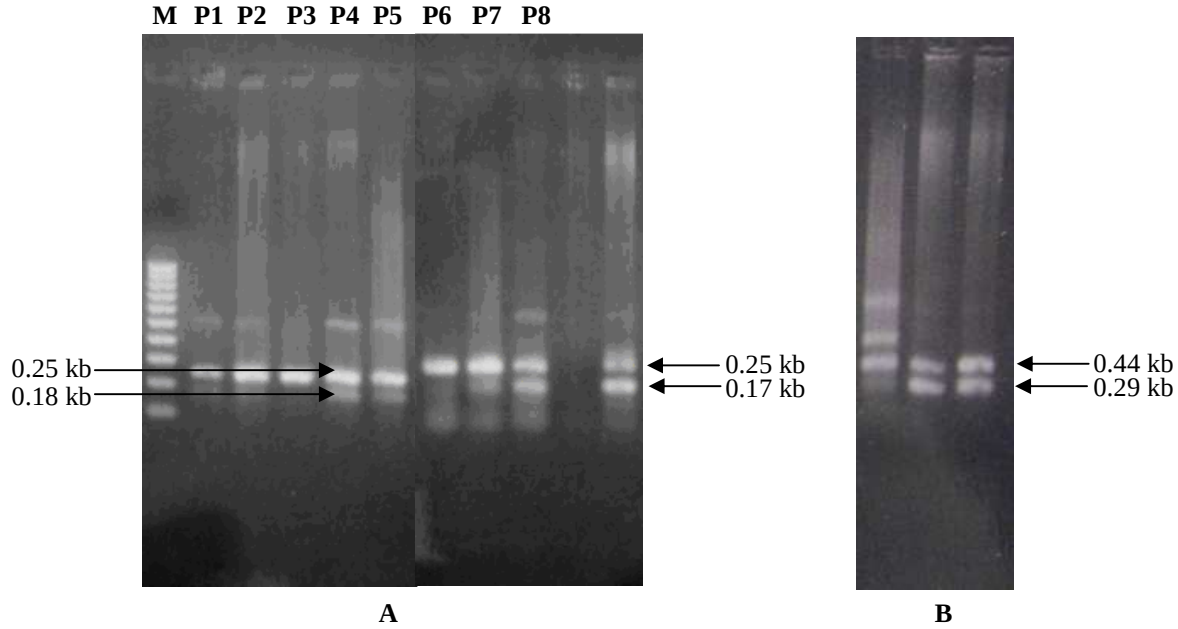

Fig 2. (A) The results of multiplex PCR *rpoB526* and *rpoB531* gene. Amplified two bands of 0.17 or 0.18 kb, and 0.25 kb indicates the isolate is *wild-type rpoB531* or *rpoB526* as seen isolates P7, P8, and other isolates while the result of amplification of the 0.25 kb band indicates the mutant isolates *rpoB526* or *rpoB531* as P1 isolates and isolates the other; (M) band marker 100 pb. (B) The results of multiplex PCR *katG315*. Amplified two bands of 0.29 and 0.44 kb indicates the isolate is *wild-type* isolates *katG315* as shown in P6, P7, and other isolates, while the result of amplification of the 0.44 kb band indicates the mutant isolates *katG315* like other isolates

Multiplex PCR and determining the nucleotide sequence of the *rpoB* and *katG*

Characterization of genotypes by PCR multiplex and electropherogram *rpoB* and *katG* gene showed almost the same results. Both of these methods show as much as 4 isolates did not have mutations *katG315*, 6 isolates have mutations *rpoB526*, 12 isolates mutations *rpoB531*, 1 double mutation *rpoB531-rpoB526* isolates and one of them is a mutant *rpoB526-rpoB531-katG315*.

A total of nine isolates amplifying the two band of DNA segments on multiplex PCR *rpoB526*, *rpoB531*, and *katG315*. But one of the isolates, namely P3, shows the electropherogram different. The presence of mutations in isolates P3 *rpoB526* not detected by multiplex PCR although repetition twice (*duplo*). After determining the nucleotide sequence analysis, mutation *rpoB526* on these isolates can be detected. Electropherogram showed *rpoB* nucleotide mutation in the other three of the nine isolates amplify DNA segments *rpoB526* two bands, *rpoB531*, and *katG315*. The results of the determination of the nucleotide sequence revealed a number of new mutations, ie A1534T and C1536G on P2 isolates, and mutation C1548T and C1654T mutations in isolates P5, as shown in Fig. 3 below. Five other isolates of MDR-TB is not mutated, but there is a silent mutation in isolates P2, P3, and P4 as shown in the following figure (**Fig. 3**).

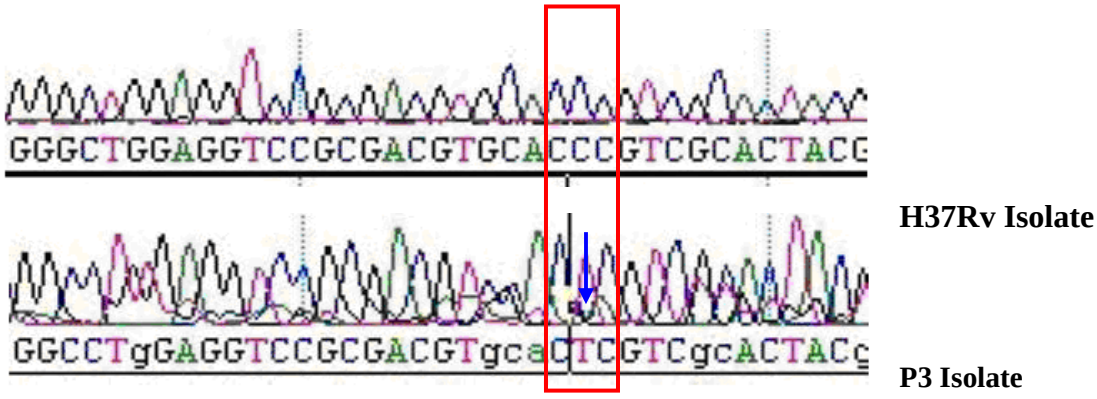

Fig 3. P3 isolate *rpoB* gene electropherogram showing the codon *rpoB552*. On the spectral shows the change C1654T nucleotides (codons *rpoB552*) and alter Pro (CCG) → Ser (TCG) compared with standard isolates of *M. tuberculosis* H37Rv. Analysis using DNASTAR Program on Seqmen application

Isolates P5 which is a multi-sensitive isolates turned out to have mutations outside RRDR, namely the nucleotide sequence C1604A (codon *rpoB535*) that converts histidine amino acid into a proline [3,11,15-17].

Genotype characterization of *rpoB* and *katG*

rpoB and *katG* genes each having a length of 4810 bp and 3516 bp while the segment analysis of the nucleotide sequence of the *rpoB* and *katG* performed successively in order nucleotide 1521-1730 and 675-1104 for *katG* segment. On the results of multiplex PCR *rpoB526*, *rpoB531*, and *katG315*, in addition to band 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 primary 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 band 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 are different.

The nature resistant to RIF occurs because of the *rpoB* gene mutation, the gene that produces RNA polymerase β-subunit. 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 [7,17-18]. Mutations that often happens is a change Ser531Leu (TCG→TTG) and His526Asp (CAC→GAC). *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 those reported others researcher, ie more than 70% of RIF-resistant properties of the *M. tuberculosis* in the region are caused by mutations *rpoB526* and *rpoB531* [17-18].

Determining the nucleotide sequence of the *rpoB* segment showed a very varied in the samples to amplify two DNA segments band 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 electrophoregram results showed A1576G mutation (codon *rpoB526*) as shown in Fig. 3. *rpoB526* mutations that are not detected by multiplex PCR amplification may be caused by other segments as a result of imprecision annealing. This imprecision generate DNA bands other but with the same size.

RIF is anti-TB 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. Researcher found 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 [19-22].

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, A1534T and C1536G, and C1548T and C1654T (P9), are present in the sample P2, P5, and P7. G1389C silent mutations occur at P4 and P9 isolates that allegedly silent mutations are forms of *M. tuberculosis rpoB* gene polymorphism. This research could be developed by associating the mutation in certain areas to the emergence of diseases caused by mutations in the coding region of a gene in a sequence, locus, organisms, and others in the province [23-27].

CONCLUSION

Analysis of determining the nucleotide sequence in the Mtb isolates from Jayapura, Papua province, Indonesia, conducted by using band segments of DNA amplification product primer forward and reverse 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.

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