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Research Article

IDENTIFICATION GENE MUTATIONS *RPOB* CAUSE OF MULTIDRUG RESISTANCE TUBERCULOSIS IN HAJI ADAM MALIK HOSPITAL

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ABSTRACT

Objective: The study aimed to investigate gene mutations in *Mycobacterium tuberculosis* causing resistance to rifampicin (RIF) in multidrug resistance TB patients in Haji Adam Malik Hospital and determined the suitability of the general pattern of mutations that occur in patients with multidrug-resistant TB (MDR-TB) in the hospital with global mutation pattern.

Methods: This observational study was performed prospectively in 31 patients with MDR-TB who had data medical record results of GeneXpert MTB/RIF positive for resistant to RIF period August–November 2016 in the hospital by taking sputum from patients. DNA extracted from sputum and then purified to be detected using polymerase chain reaction (PCR). PCR products were examined by electrophoresis.

Results: The highest percentage of 81 bp *rpoB* gene mutation MTB is called as RIF -resistance determining region which was located at codon 516 (100%) and then was followed by mutation at codon 531 (96.77%) and codon 526 (90.32%), respectively, while the smallest percentage of mutation (12.90%) MTB at codon was 533.

Conclusion: The *rpoB* gene shows a positive mutated causes resistance to rifampicin to RIF.

Keywords: Multidrug resistance tuberculosis, Mutations, Gene *rpoB*, Polymerase chain reaction.

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INTRODUCTION

Tuberculosis (TB) treatment is inseparable from multidrug-resistant TB (MDR-TB), a condition where TB patients cannot be treated with a first-line anti-TB drug regimen, because resistance to rifampicin (RIF) and isoniazid has occurred with or without resistance to other anti-TB drugs. Globally, of 6.3 million TB patients, as many as 5% of them (+300,000 cases) have been detected with MDR-TB. Facts indicated that from 480,000 patients with MDR-TB caused 190,000 of deaths [1,2].

Based on MDR-TB Global Report 2010, Indonesia is a country with the eight highest burden of MDR-TB in the world with estimated cases of new MDR-TB as many as 8900 people per year [3]. Meanwhile, according to the WHO data in 2013, Indonesia has been ranked as the 9th highest prevalent of 30 countries. [3]

According to Rattan in MDR-TB molecular perspectives, genetic analysis and drug resistance molecules in MDR-TB show that resistance is usually obtained from bacilli with changes in drug targets through mutation or through an excess of drug titration to the target. MDR-TB is mainly due to the accumulation of individual gene mutations of RIF drug target reported in the *rpoB* gene that produces B-subunit RNA polymerase (>95% of resistant strains) [4].

Research on conservative areas of mutation in MTB has not been widely practiced in Indonesia. Therefore, this study aimed to identify mutations in the *rpoB* MTB gene that includes the RIF resistance determining region (RRDR) area using the polymerase chain reaction (PCR) multiplex method. The results of this study are expected to help the development of molecular techniques in MDR-TB diagnosis in Indonesia in particular.

MATERIALS AND METHODS

This research procedure has received permission from the Ethics Committee of the Faculty of Medicine, University of Sumatera Utara/RSUP H. Adam Malik, and the patients included in the research have signed the written informed consent.

MATERIALS

The materials used as samples in this study were sputum obtained from 31 patients with MDR-TB admitted to RSUP H. Adam Malik Medan. The chemicals used were 4% NaOH, sterile phosphate buffer pH 6.8, DNA extract kit PureLink ™ Genomic DNA, PBS (Phosphate Buffer Saline), GoTaq® Green Master mix, DMSO, nuclease-free water, M. TB H37RV, agarose gel (Invitrogen), TAE ×10 (Invitrogen) buffer, ethidium bromide, and BenchTop 1000 bp DNA ladder (Promega). Instrument used in this experiment was PCR Thermal Cycler (Applied Biosystem Veriti 96 well, Temecula, California).

Extraction and purification of MTB DNA from standard sputum and strain H37RV

DNA extraction and purification of MTB samples and standard strains of H37RV as a positive control were performed using PureLink™ Genomic DNA (Invitrogen) with procedures according to the manufacturer.

Amplification of *rpoB* gene codons 516, 526, and 531 multiplex-PCR [5]

5 µL DNA extract was added with 12.5 µL of GoTaq® Green Master mix, 0.5 µL of DMSO, 3 µL of nuclease-free water, and primer *rpoB* codons 516, 526, and 531 (Table 1) each 1 µL in PCR tube, then amplification the samples on PCR Thermal cycler machines under conditions as shown in Table 2. PCR products were examined by electrophoresis gel. Wild-type MTB H37RV will form DNA bands measuring 218 base pair (bp), 185 bp, and 170 bp, and a sample is said to be mutant if no DNA bands are formed in 218 bp, 185 bp, and 170 bp or one of those DNA bands.

Amplification of *rpoB* gene codons 507, 518, and 533 multiplex-PCR [5]

5 µL DNA extract was added with 12.5 µL of GoTaq® Green Master mix, 0.5 µL of DMSO, 3 µL of nuclease-free water, and primer *rpoB* codons 507, 518, and 533 (Table 3) each 1 µL in PCR tube, then amplification of

rpoB gene fragments on PCR Thermal cycler machines under conditions as shown in Table 2. PCR products were examined by electrophoresis gel. Wild-type M. TB H37RV will form DNA bands measuring 244 bp, 213 bp, and 163 bp, and a sample is said to be mutant if no DNA bands are formed in 244 bp, 213 bp, and 163 bp or one of those DNA bands.

Amplification of *rpoB* gene codons 511, 513, and 522 multiplex-PCR [5]

5 μL DNA extract was added with 12.5 μL of GoTaq® Green Master mix, 0.5 μL of DMSO, 3 μL of nuclease-free water, and primer *rpoB* codons 511, 513, and 522 (Table 4) each 1 μL in PCR tube, then amplification of *rpoB* gene fragments on PCR thermal cycler machines under conditions as shown in Table 2. PCR products were examined by electrophoresis gel. Wild-type MTB H37RV will form DNA bands measuring 230 bp, 251 bp, and 199 bp, and a sample is said to be mutant if no DNA bands are formed in 230 bp, 251 bp, and 199 bp or one of those DNA bands.

Detection of PCR results by agarose gel electrophoresis [6]

A total of 3.9 g of agarose in 130 mL trisasetate EDTA (TAE) ×10 was heated until dissolved, and then, the solution was kept warm and added 1 μL ethidium bromide (EtBr). The mixture was shaken until homogen and then poured into the mold and allowed to freeze completely. A total of 5 μL PCR and marker samples were incorporated into a 3% agarose gel well. The electrophoresis process was performed with a potential difference of 80 V, 400 A for 70 min. The DNA of the electrophoretic amplification was visualized using gel documentation. The DNA bands will be visible and can be measured according to the molecular size marker expressed by the base pair.

RESULTS

Based on the distribution of mutation of *rpoB* MTB gene in the study subjects, all samples had mutation of *rpoB* MTB gene at codon 516 (100%), and no wild-type MTB was found. Of 31 samples, 30 people (96.77%) had mutations of *rpoB* gene in multiple codons (multiple mutations), and only 1 sample had mutated rpoB MTB gene at codon 516 (Table 5).

The highest percentage of gene mutation of 81 bp *rpoB* MTB or at RRDR area was at codon 516 (100%), followed by codon 531 (96.77%) and codon 526 (90.32%). The smallest percentage of 81 bp *rpoB* MTB gene mutation was present at codon 533 (12.90%). This mutation occurrence is similar to the research of Da Silva and Palomino [7], and the most frequent mutations in codons 531, 526, and 516 often reported in most studies [7,8]. The mutations found in this study were consistent with the study found by Lingala *et al*. [9] in India that the 81 bp *rpoB* MTB gene mutation was found in codon 531 (47%), 526 (17%), and 516 (13%). While Yao in 2010 used isolates from Chongqing, China, the percentage of 81 bp *rpoB* MTB gene mutation occurred at codon 531 (73%) and codon 513 (27%), but no mutations were found in codons 511, 516, 526, and 533 [10]. Nghiem's [11] study using the Vietnamese isolate MTB also showed similar results with Lingala *et al*. findings in India with the highest percentage of *rpoB* mutations at codon 531 (37.8%), 526 (23%), and 516 (9, 46%).

The PCR product was examined by gel electrophoresis. Wild-type MTB H37RV as a positive control will form the specific fragment of the allele according to the primary target used, whereas the PCR multiplex sample results are said to mutate if no DNA bands are formed according to the specific fragment of the allele, which is validated with positive control and the negative controls used. Both controls performed well, indicated by the absence of DNA bands on negative PCR control results and the presence of DNA bands according to PCR multiplex performed on positive PCR control results. The electrophoretic results of PCR products resistant to RIF are shown in Figs. 1-4.

DISCUSSION

RIF resistance is associated with mutations in the *rpoB* gene, which encodes the β-subunit of MTB RNA polymerase [12]. With GeneXpert MTB/RIF, the resistance to RIF is detected as failure of one or more specific

Table 1: Primer used for *rpoB* gene amplification codons 516, 526, and 531 multiplex-PCR and detection of MDR strains MTB

Target	Primer	Sequence (5'-3')	Product size (base pair)
rpoB 516	*rpoB* 516	CAGCTGAGCCAATTCATGGA	218
	RIRm	TTGACCCGCGCGTACAC	
rpoB 526	*rpoB* 526	CTGTCGGGGTTGACCCA	185
	RIRm	TTGACCCGCGCGTACAC	
rpoB 531	*rpoB* 531	CACAAGCGCCGACTGTC	170
	RIRm	TTGACCCGCGCGTACAC	

PCR: Polymerase chain reaction, MDR: Multidrug-resistant, MTB: *Mycobacterium tuberculosis*

Table 2: Multiplex PCR thermal cycler genes *rpoB*

Initial denaturation 5 min 95°C		
40 cycles		
Step 1	30 sec	95°C
Step 2	30 sec	68°C
Step 3	30 sec	72°C

Final extension 7 min 72°C, PCR: Polymerase chain reaction

Table 3: Primer used for *rpoB* gene amplification codons 507, 518, and 533 multiplex-PCR and detection of MDR strains MTB

Target	Primer	Sequence (5'-3')	Product size (base pair)
rpoB 507	*rpoB* 507	GCGATCAAGGAGTTCGG	244
	RIRm	TTGACCCGCGCGTACAC	
rpoB 518	*rpoB* 518	TGAGCCAATTCATGGACCAGA	213
	RIRm	TTGACCCGCGCGTACAC	
rpoB 533	*rpoB* 533	CGCCGACTGTCGGCGCT	163
	RIRm	TTGACCCGCGCGTACAC	

PCR: Polymerase chain reaction, MDR: Multi-drug-resistant, MTB: *Mycobacterium tuberculosis*

Table 4: Primer used for *rpoB* gene amplification codons 511, 513, and 522 multiplex-PCR and detection of MDR strains MTB

Target	Primer	Sequence (5'-3')	Product size (base pair)
rpoB 511	*rpoB* 511	TTCGGCACCAGCCAGCT	230
	RIRm	TTGACCCGCGCGTACAC	
rpoB 513	*rpoB* 513	CACCAGCCAGCTGAGCC	251
	RIRm	TTGACCCGCGCGTACAC	
rpoB 522	*rpoB* 522	GACCAGAACAACCCGCTGT	199
	RIRm	TTGACCCGCGCGTACAC	

PCR: Polymerase chain reaction, MDR: Multi-drug-resistant, MTB: *Mycobacterium tuberculosis*

Table 5: Distribution of mutations of *rpoB* MTB gene in the study subjects

Mutated codon	n (%)
507	24 (77.42)
511	22 (70.97)
513	22 (70.97)
516	31 (100.00)
518	25 (80.65)
522	27 (87.10)
526	28 (90.32)
531	30 (96.77)
533	4 (12.90)
Wild type	0 (0.00)

n was number of samples, MTB: *Mycobacterium tuberculosis*

rpoB-molecular beacons to hybridize the *rpoB* polymorphism amplicon and most commonly in codons 516, 526, and 531. Some studies show that 95% of MTB who are resistant to RIF have mutations in the 81bp rpoB gene at codons 507–533 [12,13]. An estimated 5% of MTB that is resistant to RIF has mutations outside the hotspots of the *rpoB* region [12].

Mutation of the *rpoB* MTB gene at codon 516 has been reported to occur Asp516 mutation to Val and Gly, the residue termination into Val and Gly causes the hydrogen bond not formed, and the amino acid residue change with polar side chain becomes non polar. The loss of the initially formed hydrogen bonds reduces the binding affinity of the RIF so that the RIF is weaker in the RNAP β subunit, consequently the RIF cannot work effectively, whereas the RIF activity is more dependent on its ability to bind to RNAP. In addition, rigid RIF conformations are also suspected to cause RIFs to adapt to mutations that alter the shape and chemical environment of the bonding site. If the RIF is weakly bound to RNAP, it results in a change of RIF position, especially in dynamic enzyme conditions and also influenced by the presence of water molecules. When the RIF position changes in such a way that it no longer blocks the RNA extension path, it is likely that the transcription process will continue to run and the bacteria will be resistant to RIF [14].

One study of the *rpoB* gene in RIF -resistant isolates has identified various short mutations and deletions in genes. Of the reported studies, there were 69 single nucleotide changes, 3 insertions, 16 deletions, and 38 multiple nucleotide changes. Over 95% of all mutations are located at 81bp of the *rpoB* gene core region between codon 507 and 533 with the most frequent changes in the Ser531Leu codon, His526Tyr, and Asp516Val. Serine amino acid changes at codon 531 and histidine at codon 526 were found in 70% of RIF -resistant isolates [10,11]. However, further research is needed to confirm the amino acids that are mutated in the results of this study through sequencing.

Based on the theory, if the resistance of RIF and MDR of TB has a strong correlation, then the detection of MDR TB is sufficient only with single rapid test that detects RIF resistance such as GeneXpert MTB/RIF. In countries with low RIF monoresyant events but high TB MDR prevalence, this correlation can be used. In countries with high RIF

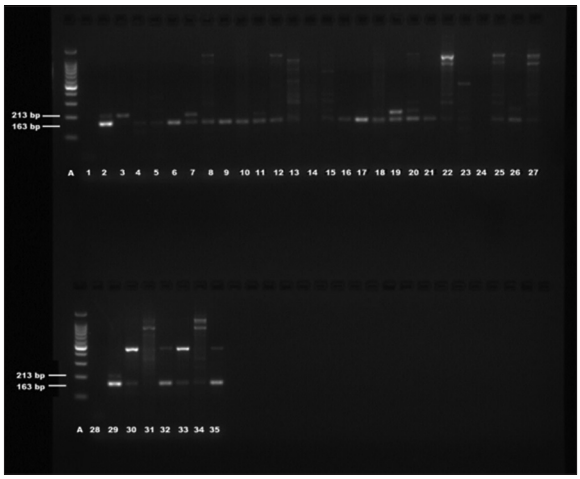

Fig. 1: Electrophoresis of polymerase chain reaction (PCR) products 213 bp and 163 bp of *rpoB* MTB gene fragments with primers rpoB518, rpoB533, and RIRm in agarose 3%. Lane A: 1000 bp DNA Ladder/M arker; Lanes 1 and 28: negative controls; Lanes 2 and 29: H37RV wild-type PCR results (positive control); Lanes 3–27 and 30–35: PCR result sample 1–31

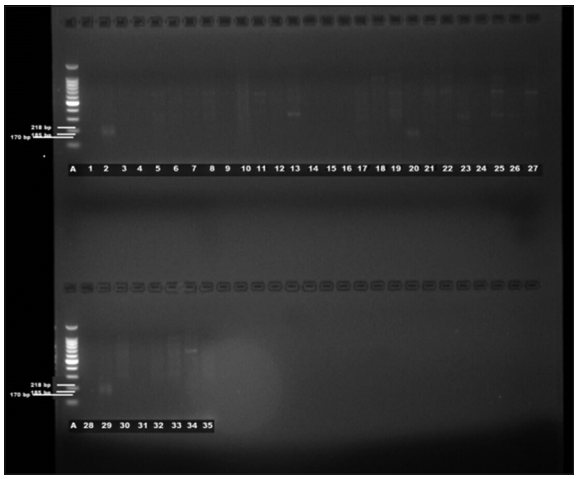

Fig. 3: Electrophoresis of polymerase chain reaction (PCR) product 218 bp, 185 bp, and 170 bp of *rpoB* MTB gene fragment with primer *rpoB516, rpoB526, rpoB531,* and RIRm in agarose 3%. Lane A: 1000 bp DNA Ladder/Marker; Lanes 1 and 28: negative controls; Lanes 2 and 29: H37RV wild-type PCR results (positive control); Lanes 3–27 and 30–35: PCR result sample 1–3

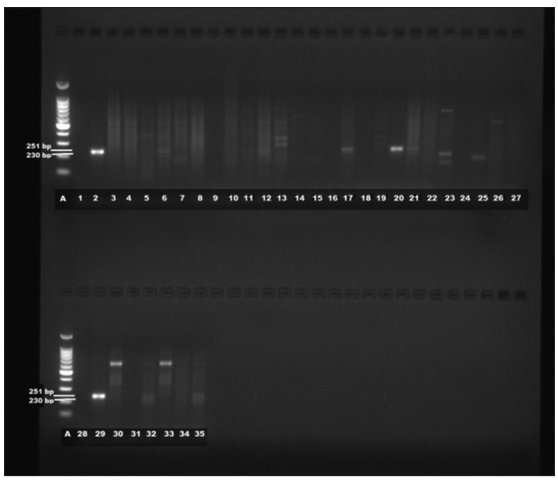

Fig. 2: Electrophoresis of polymerase chain reaction (PCR) product 251 bp and 230 bp of *rpoB* MTB gene fragment with primer rpoB511, rpoB513, and RIRm in agarose 3%. Lane A: 1000 bp DNA Ladder/Marker; Lanes 1 and 28: negative controls; Lanes 2 and 29: H37RV wild-type PCR results (positive control); Lanes 3–27 and 30–35: PCR result sample 1–31

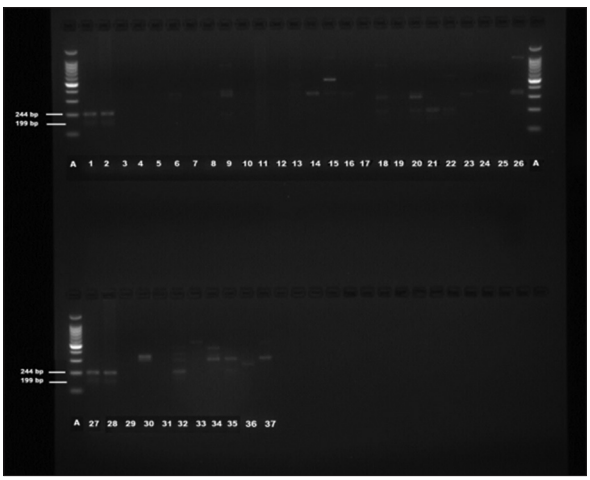

Fig. 4: Electrophoresis of polymerase chain reaction (PCR) product 244 bp and 199 bp of *rpoB* MTB gene fragment with primer *rpoB507*, *rpoB522*, and RIRm in agarose 3%. Lane A: 1000 bp DNA Ladder/Marker; Lanes 1 and 28: negative controls; Lanes 2 and 29: H37RV wild-type PCR results (positive control); Lanes 3–27 and 30–35: PCR result sample 1–31

monoresistant events, the correlation is questionable and not always usable [15]. Based on data from the WHO [3], Indonesia is one of the countries with high TB burden of MDR with estimated MDR-TB patients of 6,900 cases, i.e., 1.9% of new cases and 12% of re-treatment cases. Therefore, GeneXpert MTB/RIF can be used to detect or diagnose MDR-TB for cases in Indonesia.

CONCLUSION

The highest percentage of gene mutation 81 bp *rpoB* MTB or RRDR is located at codon 516 (100%) then at codon 531 (96.77%) and codon 526 (90.32%), while the smallest percentage of the gene mutation 81 bp *rpoB* MTB at codon 533 (12.90%).

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REFERENCES

1. World Health Organization. Multidrug-Resistant Tuberculosis. Geneva, Switzerland: World Health Organization; 2015.
2. World Health Organization. Companion Handbook to the WHO Guidelines for the Programmatic Management of Drug-resistant Tuberculosis. Geneva: (WHO/HTM/TB/2014.11); 2014. p. 77-94.
3. World Health Organization. Global Tuberculosis Report. Geneva: (WHO/HTM/TB/2013.11); 2013. p. 6-67.
4. Rattan A, Kalia A, Ahmad N. Multidrug-resistant *Mycobacterial tuberculosis*: Molecular perspective. Emerg Infect Disease. 1998;4:195- 206.
5. Farnia P. Susceptibility Testing: Multiplex -PCR, PCR-SSCP and PCR–ELISA Mycobacteriology Research Center (MRC). Tehran: Iranian National Reference TB Laboratory, National Research Institute of Tuberculosis & Lung Disease (NRITLD). Protocol No:2. 2012. p. 21- 49.
6. Made DW, Wirajana IN, Yowani SC. Amplifikasi dan identifikasi mutasi pada fragmen 0,5 KB gen rpoB Isolat 134 B Isolat 134 *Mycobacterium tuberculosis* multidrug resistant dengan metode nested polymerase chain reaction. J Kimia 2014;8:166-70.
7. Da Silva PE, Palomino JC. Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: Classical and new drugs. Antimicrob Chemother 2011;66:1417-30.
8. Zhang Y, Yew WW. Mechanisms of drug resistance in *Mycobacterium tuberculosis.* Int J Tuber Lung Dis 2009;13:1320-30.
9. Lingala MA, Aparna S, Suman J, Rao MV, Ranganadha RP. Clinical and geographical profiles of rpoB gene mutations in *Mycobacterium tuberculosis* isolates from Hyderabad and Koraput in India. J Microbiol Antimicrob 2010;2:13-8.
10. Yao C, Zhu T, Li Y. Detection of rpoB, katG and inhA mutations in *Mycobacterium tuberculosis* in clinical isolates from chonqing as determined by microarray. Clin Microbiol Infect 2010;16:1639-43.
11. Nghiem NM. Molecular characteristic of rifampisin and isonizid resistant *Mycobacterium tuberculosis* strains isolated in vietnam. J Clin Microbiol 2011;50:598-601.
12. Li J, Xin J, Zhang L, Jiang L, Cao H, Li L. Rapid detection of rpoB in rifampin resistant *M. tuberculosis* from sputum samples by denaturing gradient gel electrophoresis. Int J Med Sci 2012;9:148-56.
13. Castan P, Pablo A, Romero A. Point of care system for detection of *Mycobacterium tuberculosis* and rifampin resistance in sputum samples. J Clin Microb 2014;52:502-7.
14. Ubyaan R. Study of influence of RpoB gene mutation at codon 513; Analysis on isolate papua. Proc Insinas 2012;195:1-5.
15. Coovadia YM, Mahomed S, Pillay M, Werner L, Mlisana K. rifampicin mono-resistance in *Mycobacterium tuberculosis* in kwa zulu-natal, South Africa: A significant phenomenon in a high prevalence TB-HIV region. PLoS One 2013;8:e77712.