Malaysian Journal of Microbiology, Vol 19(4) 2023, pp. 380-384
DOI: http://dx.doi.org/10.21161/mjm.220133

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Detection of rpoB gene mutations in clinical isolates of Rifampicin-resistant *Mycobacterium tuberculosis* in Makassar City, South Sulawesi, Indonesia

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Received 16 November 2022; Received in revised form 2 February 2023; Accepted 6 June 2023

ABSTRACT

Aims: This study was aimed to detect mutations in rpoB gene codons 511, 526, 531 and 516 that cause rifampicin resistance and to analyze the sensitivity and specificity of Multiple Allele Specific Polymerase Chain Reaction (MAS-PCR) in detecting rpoB gene mutations.
Methodology and results: This study used samples of clinical isolates of *Mycobacterium tuberculosis* which had been tested for their antibiotic sensitivity to first-line anti-tuberculosis drugs. Extraction of *M. tuberculosis* bacterial DNA using the boiling method, amplification of the genes encoding rpoB 511, 526, 531 and 516 using the MAS-PCR methods. The results revealed 100% mutations in codons 526, 531 and 516, with sensitivity and specificity values of 90.5% and 100%, respectively.
Conclusion, significance and impact of study: With the detection of mutations in the rpoB gene using the MAS-PCR method, it is hoped that this can be a clinical consideration for the selection of new TB drugs in Multi-Drug Resistant Tuberculosis (MDRTB) patients. In addition, MAS-PCR can be used to detect drug resistance quickly and accurately.

Keywords: Mutation, rpoB gene, tuberculosis, *Mycobacterium tuberculosis*, resistance

INTRODUCTION

Tuberculosis (TB) is an infectious disease that is still a global concern. Tuberculosis (TB) most often attacks lung tissue, caused by the bacterium *M. tuberculosis* (Kenedyanti and Sulistyorini, 2017). The prevalence of TB cases in Southeast Asia is 44%. There are 8 countries with the highest number of TB cases, namely India (26%), Indonesia (8.5%), China (8.4%), Philippines (6%), Pakistan (5.7%), Nigeria (4.4%), Bangladesh (3.6%) and South Africa (3.6%) (Indonesian Association of Pulmonary Doctors, 2021). Transmission of TB disease happens through the intermediary of the patient's saliva or sputum containing the *M. tuberculosis* bacteria. *Mycobacterium tuberculosis* is a rod-shaped and acid-fast bacterium (Widayanti *et al*., 2013).

Currently, tuberculosis (TB) is treated with combination therapy consisting of 3 or more drugs. During treatment, patients with active TB are generally given isoniazid (INH), rifampicin (RIF) pyrazinamide (PZA) and ethambutol (EMB) for the first 2 months as an intensive phase, then followed by isoniazid and rifampicin for another 4 months as a continuation phase. However, repeated use of the same drug and the long therapy duration often lead to low patient compliance. As a result, drug-resistant strains emerged (Irianti *et al*., 2016). Resistance to first-line anti-tuberculosis drugs is one of the biggest challenges in treating tuberculosis patients. It is also a threat to the success of tuberculosis control programs.

The rpoB is a gene that encodes the β subunit of RNA polymerase in the transcription process of *M.*

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(4) 2023, pp. 380-384
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Table 1: Primers used in this research.

Gen target	Primer	Sequence (5'-3')
rpoB	rpoB DR-F	GGG AGC GGA TGA CCA CCC A
	rpoB DR-R	GCG GTA CGG CGT TTC GAT GAA C
	rpoB 511-F	GAG TTC TTC GGC ACC AGC CAG CT
	rpoB 526-F	CCG CTG TCG GGG TTG ACC CA
	rpoB 531-R	CCG CCG GGG CCC CAG CGC CG
	rpoB 516-R	GAC AGC GGG TTG TTC TGG TA

tuberculosis. The rpoB gene is a gene that is the target of rifampicin to kill *M. tuberculosis* bacteria. In previous research conducted by Umar *et al.* (2020) to look at mutations in the rpoB gene in drug-resistant *M. tuberculosis* isolates the results showed that mutations occurred in the rpoB gene, to be precise in the hot spot region (Rifampicin Resistance Determining Region), namely at the codon 507-533.

The prevalence of MDRTB in Indonesia in October 2022 is close to 70%. Based on laboratory data from the Hasanuddin University Medical Research Center tuberculosis unit, the number of cases of rifampicin resistance in Makassar city from 2017 to 2019 was 274 cases. Due to the increasing number of cases of MDRTB, it is necessary to research to detect mutations in the rpoB gene, which encodes rifampicin resistance in MDRTB. We detected mutations in the rpoB gene in this study using the MAS-PCR technique. This technique is a fast and simple detection technique to detect the presence of MDRTB. The amplification method using MAS-PCR has been reported to have high accuracy for detecting genes from organisms, where this method can be applied for the diagnosis of drug-resistant TB. This study uses samples from several places in the city of Makassar, namely Labuang Baji Hospital, Hasanuddin University Hospital, Dr. Wahidin Sudirohusodo Hospital, Siloam Hospital Makassar, Makassar Public Lung Health Center, Center for Makassar Health Laboratory and Makassar Health Research and Development Center.

MATERIALS AND METHODS

Samples collection

This study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. We used samples of clinical isolates of *M. tuberculosis* stored at the Hasanuddin University Medical Research Center (HUM RC) Laboratory. The samples used in this study were 281 samples that had been subjected to the Drug Susceptibility Test (DST) and showed positive results for resistance to anti-tuberculosis drugs.

Research materials

Clinical isolates of *M. tuberculosis* on Mycobacteria Growth Indicator Tube (MGIT) media, wild type *M. tuberculosis* H37Rv, primer rpoBDR-F, rpoBDR-R, rpoB 511, 526, 531 and 516, Phosphate Buffer Saline (PBS), 70% alcohol, distilled water, Nuclease free water, MyTaq HS Redmix DNA Polymerase, Ethidium Bromide (EtBr).

Subculture of *Mycobacterium tuberculosis* isolates

As much as 200 µL of *M. tuberculosis* isolates on Mycobacteria Growth Indicator Tube (MGIT) media were inoculated into PBS (Phosphate Buffer Saline) in Eppendorf stored in the freezer at -20 °C.

DNA extraction by boiling method

The DNA extraction method refers to the modified Ahmed and Dablool (2017) method. Pipette 200 µL of *M. tuberculosis* isolated from PBS media to new Eppendorf. The next step is heating the isolate into a thermoblock at a temperature of 95 °C for 30 min. DNA purification was carried out by centrifugation at 13,200 rpm for 10 min. Then, the supernatant was pipetted into a new Eppendorf as pure DNA stock (Ahmed and Dablool, 2017).

Amplification of rpoB 511, 526, 531 and 516 genes by MAS-PCR

The amplification of the rpoB gene in this study was carried out using the Multiple Allele Specific Polymerase Chain Reaction (MAS-PCR) methods for 23 cycles consisting of initial denaturation at 95 °C for 15 min, denaturation at 95 °C for 50 sec, annealing at 68 °C for 40 sec, extension at 72 °C for 1 min and final extension at 72 °C for 7 min. The primers used in this study are listed in Table 1. The amplified fragments were then electrophoresed in 2% agarose gel and visualized under UV light.

RESULTS

Distribution of *Mycobacterium tuberculosis* resistance

The distribution of resistance in clinical isolates of rifampicin-resistant *M. tuberculosis* (RIF) is listed in Table 2.

Based on Table 2, the dominant resistance category in this study is MDR TB (Multiple Drug Resistance Tuberculosis). MDR TB is a condition when *M. tuberculosis* has become resistant to isoniazid and rifampicin or other first-line drugs. Table 3 shows that 16% of clinical isolates were rifampicin-resistant, 41%

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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MDR IR, 9% MDR IRE, 21% MDR SIR and 13% MDR SIRE.

Table 2: Distribution of resistance in clinical isolates of rifampicin-resistant *Mycobacterium tuberculosis*.

No.	Antituberculosis drugs	Percentage (%)
1	R (mono)	16
2	IR (MDR)	41
3	IRE (MDR)	9
4	SIR (MDR)	21
5	SIRE (MDR)	13

I (Isoniazid); R (Rifampicin); E (Ethambutol); S (Streptomycin); MDR (Multiple Drug Resistance).

Table 3: Distribution of rpoB gene mutations 511, 526, 531 and 516.

No.	Codons	Percentage (%)	
		Monoresistance	MDRTB
1	511	-	-
2	526	36	64
3	531	11	89
4	516	20	80

MDR-TB (Multiple Drug Resistance Tuberculosis).

Table 4: MAS-PCR sensitivity and specificity in detecting rpoB gene mutations.

No.	PCR	Culture		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		Resistance	Sensitive				
1	Resistance	229	24	90,5	100	100	53,85
2	Sensitive	0	28				

PCR (Polymerase Chain Reaction); PPV (Positive P Value); NPV (Negative P Value).

Percentage of rpoB gene mutations 511, 526, 531 and 516

The percentage of rpoB gene mutations 511, 526, 531 and 516 are listed in Table 3.

Table 3 shows the proportion of mutations in the rpoB gene codons 511, 526, 531 and 516 of monoresistant and MDRTB isolates, respectively. Based on Table 3, there were no mutations at codon 511. At codon 526, mutations occurred in monoresistant isolates with a proportion of 36%, MDRTB 64%. Codon 531 also experienced mutations in isolates in the monoresistant and MDRTB categories, with a proportion of 11% and 89%, respectively. Then, codon 516 also experienced mutations in monoresistant and MDRTB isolates with a successive 20% and 80%. Table 3 shows that the highest proportion of mutations occurred at codon 531, namely 89% in the MDRTB category.

Visualization of rpoB gene amplification results 511, 526, 531 and 516 using MAS-PCR

The visualization of the MAS-PCR results in this study is listed in Figure 1.

This study only looked at substitution mutations by using primers that were designed directly at codon locations that frequently mutate. The band that appears during amplification indicates that the codon has not been mutated. Conversely, a band that does not appear during amplification indicates that the codon has been mutated. To see the existence of other types of mutations, it is necessary to confirm with the sequencing method.

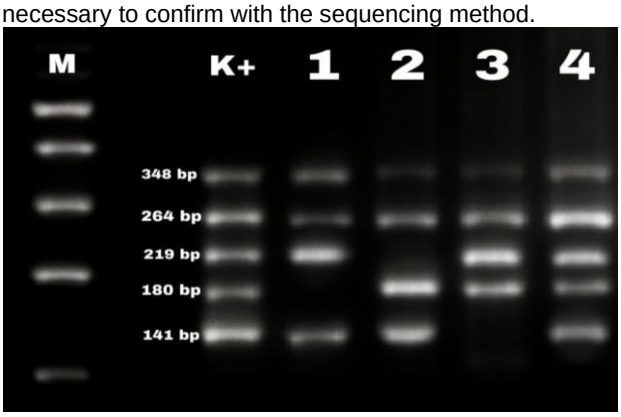

Figure 1: The result of rpoB gene amplification. M: DNA ladder 100 bp; K+: Wild type *M. tuberculosis* H37Rv; 348 bp: RIF internal control; 264 bp: rpoB 511; 219 bp: rpoB 526; 180 bp: rpoB 531; 141 bp: rpoB 516; Lanes 1 mutation of rpoB 531; Lanes 2 mutations rpoB 526; Lanes 3 mutations rpoB 516.

Based on Figure 1, the codon 511 rpoB gene (264 bp) did not experience mutations, which was indicated by the appearance of a band on the amplification results with MAS-PCR. Whereas in codon 526 (219 bp), there is a mutation, to be precise, in lanes 2. The rpoB 531 (180 bp) gene also has mutations in lanes 1 and rpoB 516 (141 bp) shows mutations in lanes 3. Mutations are characterized

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(4) 2023, pp. 380-384
DOI: http://dx.doi.org/10.21161/mjm.220133

by the absence of a band that appears when amplification with MAS-PCR.

Sensitivity and specificity of MAS-PCR in identifying mutations of the rpoB gene

The sensitivity and specificity of MAS-PCR in detecting mutations of the 511, 526, 531 and 516 rpoB genes in clinical isolates of rifampicin-resistant *M. tuberculosis* (RIF) are listed in Table 4.

Table 4 shows that the sensitivity and specificity of MAS-PCR are 90.5% and 100%, the positive p-value (PPV) is 100% and the negative p-value (NPV) is 53.85%. The PPV result of 100% showed that the rpoB gene mutated, while the NPV was 53.85%, indicating that 28 samples did not have mutations in the rpoB gene.

DISCUSSION

This research is a study on the detection of rpoB gene mutations, precisely at codons 511, 526, 531 and 516 using the MAS-PCR method. Thirumurugan *et al*. (2015) reported that MAS-PCR is a standardized technique for detecting mutations in the rpoB gene. The primers used in this study are designed to directly detect the Rifampicin Resistant Determining Region (RRDR) mutation, so sequencing is not necessary to detect mutations (substitutions). Based on the results of the drug susceptibility test (DST), the category of resistance that dominated in this study was MDRTB, followed by rifampicin monoresistance. MDRTB is a condition when *M. tuberculosis* is resistant to isoniazid and rifampicin or other first-line anti-tuberculosis drugs. Each resistant drug has different resistance coding genes. Isoniazid resistance is encoded by the genes katG, inhA, ahpC, ndh and kasA, rifampicin is encoded by rpoB, pyrazinamide is encoded by pncA and rpsA, ethambutol is encoded by embCAB and embR, streptomycin is encoded by rpsL, rrs and gidB (Irianti *et al*., 2016).

Table 4 shows that the highest percentage of mutations was found in codon 531, to be precise, in isolates with the MDRTB (Multi Drug Resistant Tuberculosis) category, at 89%. This indicates that mutations in the rpoB gene at codon 531 cause high resistance levels in *M. tuberculosis* isolates. Several studies have shown that codon 531 is the most frequently mutated codon of the *M. tuberculosis* rpoB gene and dominates worldwide (Tirumurugan *et al*., 2015).

This study is in line with another study by Dymova *et al*. (2014), which analyzed the sequence of hot spot areas from various genetic loci and proved that the most common mutation among *M. tuberculosis* isolates occurred at codon 531 in the rpoB gene. The study by Gupta *et al*. (2013), which detected mutations in the rpoB gene using the MAS-PCR technique, also showed the same results as this study, namely the codons that had the highest percentage of mutations were 531, 526 and 516, respectively. The results of the same study were also revealed by Ajbani *et al*. (2015); namely, the most dominant change observed in the rpoB gene was a mutation at codon 531, which was detected in 70.4% of rifampicin-resistant isolates (Ajbani *et al*., 2015).

This study showed that the percentage of rpoB 531 gene mutations in MDRTB isolates in Makassar city was the highest among the other codons. Based on Table 3, mutations at codons 526, 531 and 516 are strongly associated with the incidence of resistance to antituberculosis drugs, especially rifampicin. Rifampicin resistance has been reported and is caused by mutations in the Rifampicin Resistance Determining Region (RRDR) of the rpoB gene, where the most frequent mutations occur at codon 531, followed by codons 526 and 516. Yoon *et al*. (2012) stated that most mutations in the RRDR (Rifampicin Resistance Determining Region) region occur at codons 526, 531 and 516. Research by Yoon *et al*. (2012) explains that 90.3% of MDRTB isolates have mutations in RRDR and most of them are dominated by 3 specific codons, namely 526, 531 and 516. A study conducted by Thirumurugan *et al*. (2015) also showed the same results as this study; namely the codon that was most frequently mutated in the rifampicin-resistant *M. tuberculosis* rpoB gene was codon 531, followed by codon 526 and codon 516.

The results of the sensitivity and specificity of MAS-PCR in detecting rpoB gene mutations in this study were 90.5% and 100%. These results indicate that MAS-PCR is a molecular diagnostic method that can be used to detect drug resistance early on, because the process does not take a long time compared to culture. The results obtained from this study are in line with research conducted by Salim *et al*. (2021) which detected the sensitivity of the Xpert Mtb/Rif gene and MAS-PCR for diagnosing *M. tuberculosis* resistance. The results show a specificity value of MAS-PCR of 100%. Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR) is a conventional PCR that can be used as a fast and cost-effective method for detecting rifampicin and MDRTB resistance (Salim *et al*., 2021). The PCR-based examination can detect *M. tuberculosis* DNA with high sensitivity and specificity (Gholoobi *et al*., 2014).

Rifampicin (RIF) is a first-line anti-tuberculosis drug that works by inhibiting RNA transcription by binding to the subunit of RNA polymerase. Mutations in the rpoB gene encoding the subunit of RNA polymerase result in reduced rifampicin binding affinity. Studies have demonstrated that *M. tuberculosis* may enhance rpoB activity, cause rpoB to be mistranslated, modify metabolism, and increase efflux pump activity in order to cause tolerance to rifampicin exposure. (Goossens *et al*., 2021).

The efflux pump involved in the MDR case works to remove various molecules with different antibacterial activities. It is known that a number of clinically important mutations in the mycobacterial RNA polymerase subunit result in variable degrees of rifampicin resistance. Resistance arises because of modifications and shifts in the rifampicin binding pocket, which makes the RNA outlet inaccessible to the drug and makes the organism resistant to the drug. The binding between rifampicin and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

RNA polymerase is predominantly hydrophobic (Singh *et al*., 2017).

CONCLUSION

This study examined the presence of mutations in the rpoB gene, which encodes rifampicin resistance in MDRTB. The results of this study indicate that mutations at codons 526, 531 and 516 cause a high degree of resistance to *M. tuberculosis*. This can be a serious concern because it can potentially cause difficulties in treating tuberculosis patients. This research can be used as a guideline for further research on the genes encoding resistance to other classes of anti-tuberculosis drugs. The MAS-PCR method in this study can be used as a fast method for detecting resistance and mutations in several genes encoding drug resistance. Whole genome sequencing is necessary to obtain an overview of mutations in the RRDR region.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538