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# Mixed Multiplex Allele-Specific PCR (MiMAS-PCR) test for rapid detection of MDR and XDR -TB from the sputum of pulmonary TB patients

M N Massi<sup>1,\*</sup>, Subair<sup>2</sup>, N Hidayah<sup>2</sup>, A A Wulandari<sup>1</sup>, R S Abidin<sup>1</sup>, I Handayani<sup>2</sup>, S Rafiah<sup>3</sup> and H Halik<sup>1</sup>

<sup>1</sup>Microbiology Department, Medical Faculty, Hasanuddin University, Makassar, Indonesia.

<sup>2</sup>Postgraduate Program, Hasanuddin University, Makassar, Indonesia.

<sup>3</sup>Anatomy Department, Hasanuddin University, Makassar, Indonesia.

\*E-mail: vicerektor4@unhas.ac.id; nasrumm2000@yahoo.com

**Abstract.** Until now, Multi-Drug Resistant TB (MDR-TB) and Extended Drug-Resistant TB (XDR-TB) are still challenging to detect rapidly, since the gold standard culture method takes at least eight weeks to achieve diagnostic results. In this research, we designed a rapid and accurate detection method to detect MDR and XDR-TB using a technique which identifies specific genes encoding susceptibility to first and second-line anti-TB drugs combined in a PCR technique called Mixed Multiplex Allele-Specific Polymerase Chain Reaction (MiMAS-PCR) directly from sputum specimens. From total 117 sputum samples, The MiMAS-PCR test demonstrated 98.4% and 100% specificity in MDR-TB and XDR-TB detection, respectively, but the sensitivity is only suitable for rapid detection of XDR-TB (100%), not for MDR-TB (43.9%). The MiMAS-PCR technique is suitable for the rapid detection of XDR-TB (in this study, only one sample), but not for MDR-TB from the sputum specimens. Other methods are required to identify MDR-TB rapidly for early treatment and to prevent spread to their surrounding contacts.

## 1. Introduction

Resistance to TB drugs (DR-TB) is the main threat to TB control in the world. A global estimation accounts for 3.5% of new cases and 18% of previously treated TB patients to have MDR-TB[1]. This data is almost the same result with our data that have been published in 2011[2]. In 2017, it was estimated that there were 558,000 new MDR-TB/RR-TB cases in the world, and about 230,000 deaths caused by MDR-TB[1].

To determine the presence of drug resistance rapidly from clinical isolates of *Mycobacterium*



*tuberculosis* (MTB) is an essential prerequisite to effective and successful treatment as well as prevention of the spread of the resistant pathogens in the society. Isoniazid (INH) and rifampin (RIF) are the most important first-line anti-tuberculosis (anti-TB) drugs to date, and resistance to these drugs will result in failure in treatment and the disease getting worse[3].

Resistance to anti-TB drugs in MTB isolates is related to a random genetic mutation in specific genes. Several studies revealed that resistance to INH might be related to a mutation in the 315 codons of the *katG* gene, which encodes the peroxidase catalase and in the *inhA* promoter region[4]. On the other hand, missense mutation, which is a small deletion or insertion into the *rpoB* gene encoding the  $\beta$ -subunit polymerase from the RNA, is known to be responsible for RIF resistance[5]. Several kinds of literature explained that the presence of point mutation in the “hot spot” region, which comprises 81 bp in this *rpoB* region, is related to RIF resistance[6–9].

Resistance to kanamycin usually occurs due to cross-reaction to amikacin and involves the *rrs* gene. Changes in 1400, 1401, and 1483 nucleotides in the *rrs* gene are specifically related to the resistance to KAN. The change from A to G at the 1401 codon in the *rrs* gene shows resistance against KAN from more than 200 mg/ml MICs[10].

Ciprofloxacin (CIP) and ofloxacin (OFL) are two types of quinolones used as second-line drugs in the treatment of MDR-TB. Quinolones targets and inactivated the DNA gyrase, a topoisomerase DNA II type. DNA gyrase is encoded by the *gyrA* and *gyrB* genes and it introduces negative supercoil into the closed circular DNA molecule. Quinolone resistance is located in the (QRDR) region of the *gyrA* (220 bp) and *gyrB* (375 bp) genes which are the point of interaction between quinolone and gyrase. Mutation at the 90, 91, and 94 codon of *gyrA* is related to resistance to quinolone[10].

Molecular examination different from basic PCR and sequencing techniques has been used to identify drug resistance of MTB isolates rapidly. Furthermore, phenotypic drug susceptibility test (DST) method using MGIT 960 has also been extensively used to detect resistance. Therefore, this research is focused on the standardization and application of the rapid method to detect MTB from clinically collected TB suspected sputum samples using the MiMAS-PCR rapid method and to identify mutations responsible for drug resistance (MDR /XDR) in sputum specimens derived from TB patients in Makassar.

## 2. Material and Methods

### 2.1 Ethics Statement

This study was approved by the Research Ethical Committee of Medical Faculty, Hasanuddin University, Makassar, Indonesia. Written informed consent was obtained from all the participants in this study.

### 2.2 Samples

In this research, sputum samples of TB suspect patients from the HUM-RC (Hasanuddin University Medical Research Center) Makassar laboratory were used. All sputum samples were decontaminated using the standard N-acetyl-L-cysteine-sodium hydroxide method. The decontaminated samples were

smear on a glass slide and Ziehl Neelsen staining was performed for the identification of acid-fast bacilli (AFB). The decontaminated sputum samples were cultured using the MGIT 960 medium. For crosschecking, we used Nutrient Agar medium for contamination.

### 2.3 Resistance test on MGIT 960 medium

The initial step in the drug-resistant analysis is MGIT 960 medium preparation. The concentration of drug stock used are as follows: isoniazid 8.3 µg/mL; rifampicin 83 µg/mL; ofloxacin 164 µg/mL; and kanamycin 210 µg/mL. Five MGIT 960 tubes, including controls, were labeled, and each was added with 100 µL antibiotic according to the label. The MGIT tube was added with 800 µL OADC (Oleic Acid Dextrose Catalase). Subsequently, 500 µL of culture-positive isolates were inoculated into each tube and then incubated at 37°C for  $\pm$  12 days. Readings were done using the MGIT BACTEC 960 READER (Becton, Dickinson and Company, New Jersey, USA).

### 2.4 DNA Extraction

As much as 200 µL decontaminated sputum sample was pipetted into 1.5 mL sterile microcentrifuge tube and added with 20 µL Proteinase K and homogenized through pipetting and then incubated at 60°C for 5 minutes. Afterward, 200 µL GSB buffer (Geneaid Biotech Ltd, Taiwan) was added and the mixtures were then vortexed before reincubation at the same temperature for 2 minutes. After incubation, absolute ethanol (96%) was added and then vortexed for 10 seconds. The mixture was then transferred to a spin column and centrifuged at 14000 x g for 1 minute. The collection tube below the spin column was discarded and replaced with a new collection tube. Buffer W1 was added followed by 30 seconds centrifugation at the same speed. The liquid in the collection tube was then discarded. Then, 600 µL wash buffer was added followed by further centrifugation for 30 seconds, and another 3 minutes centrifugation after the liquid in the collection tube was discarded. After centrifugation, the collection tube was replaced with a sterile microcentrifuge below the spin column to collect the extracted DNA. Elution buffer (100 µL) was then added and was left still for 3 minutes followed by centrifugation at the same speed for 30 seconds. The extracted DNA contained in the liquid collected inside the microcentrifuge tube was kept at -20°C to be used as a PCR template.

### 2.5 MiMAS-PCR

MiMAS-PCR is a kind of PCR that mix several primers directly from genes representative of four TB drugs treatment (FLD and SLD) in one PCR process. Extracted DNA was pipetted into the PCR Mix composed of 5 µL 5X buffer, 2 µL MgCl<sub>2</sub>, 1 µL dNTP mix, 1 µL Primer *rpoB* F, 1 µL Primer *gyrA* R, 1 µL Primer *rrs* F, 1 µL Primer *rrs* R, 0.25 µL Hotstart Taq enzyme (Qiagen, Germany), 5 µL DNA template, and nuclease-free water to a total volume of 50 µL for PCR. Amplification condition consists of pre-denaturation phase at 95°C for 15 minutes 1 cycle, denaturation phase at 95°C for 1 minute, followed by annealing phase at 65°C for 30 seconds, and extension phase at 72°C for 1 minute, repeated for 40 cycles. The last cycle, which is a final extension at 72°C for 10 minutes. Afterward, the amplified DNA underwent electrophoresis using 20% agarose gel containing ethidium bromide. Electrophoresis gel was then observed under UV light.

### 2.6 Sequencing Examination

Analysis of mutation with direct sequencing was performed at 1<sup>st</sup> Base Laboratorium in Malaysia. The PCR product was sequenced to detect mutation on the *rpoB*, *katG*, *gyrA*, and *rrs* gene which further was analyzed with the “Bioedit” software to compare with data from the Gene Bank from the NCBI database using the Basic Local Alignment Search Tool (BLAST) method.

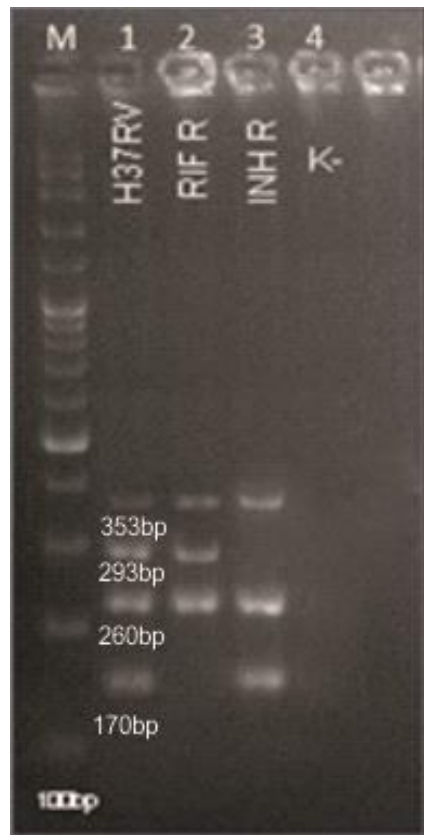
### 3. Results

In this research, sputum specimens from TB suspect patients in the HUM-RC Laboratory Makassar were used. DST examination of first-line drugs (FLD) with MGIT 960 culture method revealed that amongst 117 sputum samples, 52 samples (44.4%) were susceptible, six samples (5.1%) were mono-resistant R, three samples (2.6%) were mono-resistant I and 56 samples (47.9%) were MDR. All MDR and mono-resistant R samples were followed up by the second-line drug (SLD) DST.

Based on SLD-DST, among 65 samples resistant to FLD, 53 samples (81.5%) were susceptible to SLD, six samples (9.2%) were mono-resistant to OFX, five samples (7.7%) mono-resistant to KAN, and one sample (1.5%) were resistant to both OFX and KAN.

All positive cultures with MGIT media and DST were reconfirmed with smear microscopy using the Ziehl-Nielsen to detect whether the colony growing on MGIT was, in fact, *M. tuberculosis* or was a contaminant instead. The presence of *M. tuberculosis* growth colony was marked by cord formation on smear BTA.

Extracted DNA from previously decontaminated sputum samples then underwent molecular examination using the Mixed Multiple Allelic Specific PCR (MiMAS-PCR) method to detect anti-TB drug resistance rapidly. Amplification of *M. tuberculosis* DNA using the MiMAS-PCR to detect mutations on the *rpoB* 531 gene as a marker for RIF resistance, *katG* 315 for INH resistance, *gyrA* 94 for OFX resistance, and *rrs* 1401 for KAN resistance. Mutations in those genes are marked by the presence or absence of bands on electrophoresis gels (Figures 1). Bacterial isolates collection of *M. tuberculosis* strain H37RV were used as a control in this study.



**Figure 1.** MiMAS-PCR amplification on susceptible H37RV, INH resistant, and RIF resistant control isolates. Lines: M, marker 100bp; 1: H37RV control isolate; 2: RIF resistant control with mutation at 170bp; 3: INH resistant control with mutation at 293bp; 4: negative control.

The MiMAS-PCR amplicons were visualized with agarose gel electrophoresis and this resulted in distinct visualization according to their respective mutation profiles. Band formation on the gel for amplicons sizes 353 bp, 293 bp, 260 bp and 170 bp for control samples (non-resistant), in this case, H37RV, represents specific band patterns for susceptible samples (Figure 1). Mutations in the *rpoB* 531, *katG* 315, *gyrA* 94 and *rrs* 1401 genes are indicated by the absence of the bands of 170 bp, 293 bp, 260 bp, and 353 bp amplicons on the gel, respectively.

The MiMAS-PCR amplification on 43 susceptible sputum samples based on MGIT 960 culture examination (Figure 2) did not show any mutation in all of the genes, which indicates that all of those samples were also susceptible (100%). However, several unspecific bands other than the target bands (4 target bands) were found in sputum samples, which may influence the visualization needed for the interpretation of results. Besides, sputum sample-derived amplicons for the target bands 260 bp and 170 bp were very thin in comparison to target bands 353 bp and 293 bp.



**Figure 2.** MiMAS-PCR amplification of susceptible sputum samples as visualized on an electrophoresis gel. Line 1, H37RV control; line 2, negative control; line 3, Marker 100bp; line 4 to 20, sputum samples where 1- 17 showed amplification at 353bp, 293 bp, 260bp dan 170bp.

Based on the sensitivity calculation of MiMAS-PCR for DST MGIT of sputum samples sensitivity values for MiMAS-PCR were 48.3% for INH, 69.8% for RIF, and 43.9% for MDR. Whereas, MiMAS-PCR specificity values to DST MGIT on sputum samples were 98.2% for INH, 98.1% for RIF, and 98.3% for MDR (Table 1). The sensitivity of MiMAS-PCR for DST MGIT on sputum samples were 28.6% for KAN, 75% for OFX, and 100% for XDR. The specificity values were 98.3% for KAN, 96.5% for OFX, and 100% for XDR (Table 1).

**Table 1.** Sensitivity and specificity values of MiMAS-PCR in detecting INH, RIF, MDR, KAN, OFX, and XDR MTB from sputum samples.

ANTIBIOTIC	MiMAS-PCR RESULT	DST-MGIT		Sensitivity (%)	Specificity (%)
		Resistant	Susceptible		
INH	Mutation	29	1	48.3	98.2
	No mutation	31	56		
RIF	Mutation	44	1	69.8	98.1
	No mutation	19	53		
MDR	Mutation	25	1	43.9	98.3
	No mutation	32	59		
KAN	Mutation	2	1	33.3	98.3
	No mutation	4	57		
OFX	Mutation	6	2	85.7	96.5
	No mutation	1	55		
XDR	Mutation	1	0	100	100
	No mutation	0	65		

#### 4. Discussion

Samples used in this research are sputum specimens from suspect TB patients with a positive BTA in direct examination. All sputum samples used for molecular analysis have undergone a decontamination process. The process of sputum decontamination aims to kill or eliminate microbes other than mycobacteria, such as gram-negative bacteria, fungi, and other normal flora. Besides, decontamination was also performed to concentrate dispersed microbes in the preparation to ease the collection of a large number of mycobacteria.

The presence of a single gene mutation in TB can cause drug resistance. We designed the MiMAS-PCR method to detect mutation on *rpoB* 531, *katG* 315, *gyrA* 94, and *rrs* 1401, which are related to drug resistance (MDR and XDR). This method is rapid and relatively cheap in detecting MDR-TB and even XDR-TB incidences, which makes it suitable to be applied in Indonesia, especially in Makassar, where the number of TB cases is high. The application of this technique may reduce the delay in the diagnosis and treatment of MDR and XDR-TB. Primer design was based on prior research which showed that the most common mutation in the world for *rpoB* gene is located at the 531 codon position (85.67%)[11], and codon position 315 for the *katG* gene (around 82%)[12]. For second-line, mutation *gyrA* gene is located at the 94 codon position (79%)[13], and codon position 1401 for *rrs* gene (60%)[14].

Currently, the DST method to detect the presence of anti-TB drug resistance is still rarely done in TB examination laboratories. It causes ineffective drug prescription to TB patients and allows further spread of TB pathogen. The DST test conducted by several standardized laboratories still uses the LJ culture method, which requires quite a long time to achieve results. The PCR method is one that can be used to detect resistance rapidly and with specific outcomes.

The MiMAS-PCR method used in this research achieved a high specificity value to detect incidences of RIF resistance (98.1%), and the same goes for INH resistance and MDR (98.2% and 98.3%). That condition was the same as OFX, KAN, and XDR (96.5%, 98.3%, and 100% respectively). However, the sensitivity is very low for INH (48.3%), rifampicin (68.9%), and MDR (43.9%) compared with other results[15,16], but the sensitivity of OFX, KAN and XDR were 75 %, 28.6% and 100%, respectively. The low sensitivity of the MiMAS-PCR technique in detecting INH and MDR-TB can be caused by the fact that besides the *katG* gene, there are other hot spot mutation regions such as at *-15inhA*, *ahpC*, and *kasA*. OFX was represented by *gyrA*, *gyrB*, *parC*, and *pare*, but in this method, we only use *gyrA*, the same with KAN was represented by *rrs* and *eis* gene in several hot spot mutation.

The presence of unspecific bands in sputum samples resulted in the hindrance of result interpretation, and may have been caused by very few numbers of MTB cells in direct sputum samples and also by the presence of normal flora and inhibitory substance in the sputum which affects the concentration and purity of the DNA extracted from those samples. Thus, MiMAS-PCR results directly from sputum cannot find high sensitivity for MDR-TB detection.

Generally, the time needed to identify MDR-TB with the MGIT culture method requires  $\pm 1.5$  months, while the molecular MiMAS-PCR method allows the reduction of MDR-TB and XDR-TB

identification time to 2 days if using direct sputum samples. Therefore, this molecular approach is very effective as early detection of the incidence of anti-TB drug resistance if the sensitivity results is high.

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### References

- [1] World Health Organization 2018 *Global Tuberculosis Report 2018* (Geneva: World Health Organization)
- [2] Massi M N, Wahyuni S, Halik H, Anita, Yusuf I, Leong F J, Dick T and Phyu S 2011 Drug resistance among tuberculosis patients attending diagnostic and treatment centres in Makassar, Indonesia *Int. J. Tuberc. Lung Dis.* **15** 489–95
- [3] Hillemann D, Ru S, Richter E and Icrobiol J C L I N M 2007 Evaluation of the GenoType MTBDRplus Assay for Rifampin and Isoniazid Susceptibility Testing of Mycobacterium tuberculosis Strains and Clinical Specimens *J. Clin. Microbiol.* **45** 2635–40
- [4] Aurin T H, Munshi S K, Kamal S M M, Rahman M, Hossain S, Marma T, Rahman F and Noor R 2014 Molecular Approaches for Detection of the Multi-Drug Resistant Tuberculosis (MDR-TB) in Bangladesh *PLoS One* **9**
- [5] Bang H, Park S, Hwang J, Jin H, Cho E, Kim D Y, Song T, Shamputa I C, Via L E, Barry C E, Cho S and Lee H 2011 Improved rapid molecular diagnosis of multidrug-resistant tuberculosis using a new reverse hybridization assay, REBA MTB-MDR *J. Med. Microbiol.* **60** 1447–54
- [6] Evans J, Stead M C, Nicol M P, Segal H, Town C, Africa S, Microbiology M and Health N 2009 Rapid genotypic assays to identify drug-resistant Mycobacterium tuberculosis in South Africa *J. Antimicrob. Chemother.* **63** 11–6
- [7] Mokrousov I, Otten T, Vyshnevskiy B and Narvskaya O 2003 Allele-Specific rpoB PCR Assays for Detection of Rifampin-Resistant Mycobacterium tuberculosis in Sputum Smears *Antimicrob. Agents Chemother.* **47** 2231–5
- [8] Morcillo N, Zumarraga M, Alito A, Dolmann A, Schouls L, Cataldi A, Kremer K and Soolingen V D 2002 A low cost, home-made, reverse-line blot hybridisation assay for rapid detection of rifampicin resistance in Mycobacterium tuberculosis *Int. J. Tuberc. Lung Dis.* **6** 959–65
- [9] Telenti A, Imboden P, Marchesi F, Matter L, Schopfer K, Bodmer T, Lowrie D, Colston M and Cole S 1993 Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis *Lancet* **341** 647–51
- [10] Johnson R, Streicher E M, Louw E, Warren R M, Helden P D Van and Victor T C 2003 Drug Resistance in Mycobacterium tuberculosis *Curr. Issues Mol. Biol.* **8** 97–112
- [11] Allegui Z, Ghariani A and Slim-saidi L 2012 Detection of isoniazid and rifampin resistance of Mycobacterium tuberculosis by a multiplex allele-specific polymerase chain reaction (PCR)

- assay *Int. J. Mycobacteriology* **1** 34–9
- [12] Torres J N, Paul L V, Rodwell T C, Victor T C, Amallraja A M, Elghraoui A, Goodmanson A P, Ramirez-busby S M, Chawla A, Zadorozhny V, Streicher E M, Sirgel F A, Catanzaro D, Rodrigues C, Gler M T, Crudu V, Catanzaro A and Valafar F 2015 Novel katG mutations causing isoniazid resistance in clinical *M. tuberculosis* isolates
- [13] Singh P, Jain A, Dixit P, Prakash S, Jaiswal I, Venkatesh V and Singh M 2015 Prevalence of gyrA and B gene mutations in fluoroquinolone-resistant and -sensitive clinical isolates of *Mycobacterium tuberculosis* and their relationship with MIC of ofloxacin *J. Antibiot. (Tokyo)*. **68** 63–6
- [14] Georghiou S B, Magana M, Garfein R S, Catanzaro D G, Catanzaro A and Rodwell T C 2012 Evaluation of Genetic Mutations Associated with *Mycobacterium tuberculosis* Resistance to Amikacin , Kanamycin and Capreomycin : A Systematic Review **7**
- [15] Evans J and Segal H 2010 Novel multiplex allele-specific PCR assays for the detection of resistance to second-line drugs in *Mycobacterium tuberculosis* 897–900
- [16] Imperiale B R, Cataldi A A and Morcillo N S 2011 Rapid detection of multidrug-resistant *Mycobacterium tuberculosis* by multiplex allele-specific polymerase chain reaction **15** 496–501