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Molecular Analysis of *rrs* Gene Mutation on Detecting The Resistance of Kanamycin from the Clinical Isolates of Tuberculosis Patient

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Abstract

The resistance of kanamycin (KAN) as a second-line drug treatment for tuberculosis is associated with mutations in the 16S rRNA gene (*rrs*gene).*rrs*gene mutations occur at different locus causing changes in codons and amino acids.This study aimed to analyze mutations in the *rrs* gene and its relationship with the resistance of KANin isolate clinical sample of*Mycobacterium tuberculosis*(MTB). The isolate clinical sample of MTB was obtained from the TB HUM-RC laboratory. The analysis of *rrs* gene mutation was conducted using PCR sequencing.The results described that from 93 samples isolates, 51 (54.8%) samples shown multidrug resistant (MDR) while 42 samples shown (45.2%) non-MDR. Drug sensitivity test (DST) from the second line drugs samples isolates7 of 93 samples (7.5%) that shown resistance with KAN.

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The result of PCR sequencing shown6 (6.4%) of 93 isolates samples had mutations *rrs*genein the locus of T1521C (1.1%), G1484C (1.1%), A1401G (2.1%), G940T (1.1%), and A514C (1.1%). Based on that gene locus, an unreported new mutation in the locus of *rrs* gene, called G940T,was discovered.The results of statistical analysis based on the number of mutations in each of anti-tuberculosis drugs indicate the $p<0.05$,described a relatioship between the mutation of *rrs* gene with the resistance of KAN. In conclusion, significance and impact of study, there is an association between the mutation in the *rrs* gene and the resistance to the second-line drugs of KAN in clinical isolates of MTB.Thus, samples of clinical isolates of MTB could be used to detect any resistance of KAN in advance.

Keywords: Molecular; *rrs* Gene Mutation; Resistance of Kanamycin; Tuberculosis Patient.

1. Introduction

Tuberculosis is a major global health problem caused by *Mycobacterium tuberculosis* (MTB). In 2014, World Health Organization (WHO) estimated 9.6 million cases of tuberculosis worldwide and 1.5 million mortality from the disease.Referring to those numbers, 480.000 cases are predicted as the case ofmultidrug-resistant TB (MDR TB). One of the efforts initiated by WHO was the directly observed treatment short program (DOTS) (World Health Organization, 2015). Despite the progress in the area, there are still numerous cases which have not been discovered nor treated.The resistance against anti-tuberculosis medicine, especially multi-drug resistant TB (MDR-TB) and extensively-drug resistant TB (XDR-TB), considered as a serious threat in countering TB globally [1].

The detection of drug-resistant TB is conducted by drug susceptibility testing (DST) either using media ofmycobacterium growth indicator tube (MGIT) orlowenstein-jensen (LJ). However, this procedure is constrained by the slow growth of MTBup to 2-4 weeks;thus requires longer construction.The rapid development of molecular medicine encourages the idea to identify mutationrelated to drug resistance that can reduce setback in diagnosisand proven to be more specific than the phenotype DST in several cases [1, 2].

Basic understanding of the molecular resistance could help the discovery of a new methodtodetect the drug resistance towardsMTBstrain [3, 4,]. Spontaneous chromosomal mutation is a genetic basis for drug resistance in MTB.Various reports indicate the presence of anti-tuberculosis antibiotic resistancecaused by mutations in the mycobacteria gene (Engström and his colleagues 2012) [1]. Rifampicin resistance is associated with the changes in gene rpoB, while isoniazid resistance is associated with mutations in the gene of katG, inhA or aphC. Approximately, 80% of the strains become resistant to streptomycin due to mutations in the gene rpsL or rrs.Mutations in the gene of pncA is responsible for the resistance of pyrazinamide. Another mutation such as the gene of gyrA and B are used to detect the fluoroquinolones resistance such as ofloxacin, while*rrs* gene is used to detect resistance against aminoglycoside class (KAN, AMK, CAP). However, methods used to detect resistance against KAN especially in Indonesia have not yet extensively studied.

KAN(2-deoxy aminoglycosides streptomycin) are classified as aminoglycoside class of antibiotics which have a broad spectrumand used as an alternative medicine for MDR-TB (Bauskenieks and his colleagues 2015). KAN

of the main second-line drugs for tuberculosis treatment given to TB patientswhen MTBstrains are resistant to first-line anti-tuberculosis drugs, such asrifampicin, isoniazid, ethambutol, streptomycin or pyrazinamide.KAN works by binding 16S rRNA with the 30S ribosomal unitand inhibit protein synthesis of bacteria [1,3]. Nevertheless, the resistance of this drug againstMTBbecomes greater, ranging from 67.4–85.9% in KAN [4-6].

Mutations that generate resistance to aminoglycosides group have not been fully understood. KAN resistance has three mechanisms,the first mechanism is conducted by modifying aminoglycosides enzyme carried by transposons.The second mechanism is performed with the specific methylation of rRNA whereas the third mechanism involves changes of nucleotides in the 3'fromthe 16S rRNA gene [5]. Of these three mechanism, the most reported KAN resistance is the mutation of 16S rRNA gene (rrs) especially at nucleotide A1401G, C1402T, and G1484T from the clinical isolates [4-6].

Mutations number 1401 and 1484 are associated with resistance to all of the genes. Meanwhile,mutation at nucleotide 1484 is related with the resistance of capreomycin (CAP) and kanamicin (KAN). The most common mechanism of the resistance against KAN is mutation with the point from A to G at nucleotide 1400. In very rare cases, mutation founds from G to T in nucleotide 1483 at the 16S ribosomal *rrs* gene which leads to high levels of resistance to both drugs [4].

Based on preceding description, studies related to the rapid detection of molecular diagnostic techniquesis needed in order to determine the mutation in the *rrs* gene as a sign of KAN resistance. This research is important to understand how the detection of *rrs* gene in clinical isolatescould determine the diagnosis of the KAN resistance. The results of this study are expected to be applicable on identifying clinical isolates of MTBwith KAN resistant phenotype.

2. Materials and Methods

2.1 Research populations

The study populations were pulmonary tuberculosis patients, suspected with MDR, examined in TB Hasanuddin University Medical Research Center (HUM-RC) laboratory on 2015.

2.2 Research samples

Samples of this research are MTBisolate clinical samples of MDR-suspected pulmonary tuberculosis patientswith the results of positive AFB examination and culture.Clinical isolates samples are collections stored at TB HUM-RC Laboratory.All samples had been passing through the stages of decontamination using a decontamination solution (NaOH 4% + Natrium citrate 2.9% + N-Acetyl L-Cysteine), smeared with Ziehl-Neelsen, cultured in a liquid medium MGIT 960 (*Mycobacteria Growth Indicator Tube*) and finally identified using SD bioline.Thus, all clinical isolates samples used to detect resistance against KAN is positive culture of MTB.

2.3 Resistance test at medium MGIT 960

The first stage in examining drug resistance is the preparation of medium MGIT 960.Stock concentrations of drugs being used are: streptomycin 83 µg/mL; isoniazid 8.3 µg/mL; rifampicin 83µg/mL, ethambutol 415 µg/mL; kanamycin 210 µg/mL. Six tubes of MGIT 960 MGIT were labeled, including control.Each tube was added by 100 uL of antibioticsbased on the label of the tube.Then, 800 uL of oleic acid dexrose catalaseOADC were added into the MGIT tube.Finally, 500 µLof positive culture isolates were added into each tubeand incubated at37 °C for approximately 42 d. The reading was conducted using the BACTEC MGIT 960 Reader.

2.4 Mutation analysis (polymorphisms)of rrs gene

Mutations in the *rrs* gene as a sign of the antibiotic resistance against KANis detected using sequencing techniques.This technique consists of several stages such asDNA extraction, DNA amplification by polymerase chain reaction (PCR), electrophoresis agarose gel and sequencing method.

2.5 Extraction of DNA from isolates ofMTB

MTB DNA extraction was performed using sonication and boiling method. A total of 1 mL of clinical isolates from MGIT tube is put into a sterile 1.5 mL eppendorf tube.Then, it is centrifuged at a speed of 13000 rpm for 15 min.Supernatant was removed and added with 100 µL of sterile water (ddH_2O). Eppendorf tube containing isolates were then inserted into the water bath (at 95 °C) for 25 min. After that, itwas being sonicated for 20 min and centrifuged at 13000 rpm for 5 min.The DNA-containing supernatant was transferred to a new tube and stored at temperatures of -20°C (If it is not directly put into PCR).

2.6 DNA amplification by PCR and electrophoresis

The results of DNA extraction were inserted into the PCR Mix, such as 5 µL 5× buffer, 2 µL $MgCl_2$, 1 µL dNTP mix, 1 µL Primer*rrs* forward F (5'–TAAACCTCTTTCACCATCGACG-3'), 1 µL primer*rrs* reverse 10 µM R(5'-CCTGGTAAGGTTCTTCGCGTTG-3'). It is also inserted into the 0.25 µL Hotstart taq enzyme (Qiagen), 5 µL DNA template and nuclease free water, resulted into the total volume of 50µL for PCR.Amplification conditions consisted of 95 °C pre-denaturation for 5 min 1 cycle, denaturation 94 °C for 1 min, followed by annealing at 60 °C for 30 s, extension at 72 °C for 1 min, and being repeated for 40 cycles. The last cycle was the final extension at 72 °C for 10 min.The results of DNA amplification was continued using electrophoresis with 2% agarose gel contains ethidium bromide.Finally, electrophoresis results were observed under UV light.

2.7 Sequencing Examination

Mutational Analysis using direct sequencing method was performed at the 1st Base Laboratory of Malaysia.The PCR products was being sequenced to detect mutations in the *rrs* genewhich is then analyzed using software "Bioedit" and compared with data on "Gene Bank"in the NCBI database using methods of basic local alignment search tool (BLAST).

2.8 Data Analysis

Data results of DNA sequencing in the form electrophenogram was aligned with normal sequences derived from GenBank, and then being analyzed using sequence alignment editor bioedit software version 7.0.5.1.Statistical analysis of the data was processed using SPSS version 21.

3. Results

Of the 93 samples examined by the first line anti-tuberculosis drug (FLD), 26 (28%) were found susceptible, 8 (8.6%)were monoresistant to INH, 7 (7.5%) weremonoresistant to rifampicin, 1 (1.1%) shown to bepolyresistant, and 51(54.8%) were MDR, as described in Table 1.

Table 1: Result of DST first line drug (FLD) andsecond line drug (SLD) Isolate

Result ofFLD		Result ofSLD	
DST	n	DST	n
Suscept	26	Suscept	86
Mono INH	8	Mono KAN	7
Mono Rifampisin	7		
Polyresisten	1		
MDR	51		
Total	93	Total	93

Furthermore, MDR Category was examined through its DST and *rrs* gene for the second-line-anti-tuberculosis (SLD) which isKAN.

Following DSTexamination of KAN, we found 86 samples (92.4%) as susceptible, 7(7.5%) wasresistantagainst KAN, as shown in Table 2.

Table 2: Relationship between mutation gene*rrs* with SLD resistance from clinicalMTB isolates

Mutation of*rrs* gene	SLDResistance				Total		p
	Susceptible		Resistance				
	n	%	n	%	n	%	
Non mutation	85	91.4	2	2.2	87	93.6	0.000
Mutation	1	1.1	5	5.3	6	6.4	
Total	86	92.5	7	7.5	93	100.00	

($p<0.05$)

Electrophoresis results of PCR products presented *rrs* gene amplification at 1200bp.Of the 93 isolate samples carried out *rrs* gene examination, 6 (6.4 %) observed mutation against KAN (Table 2). From the obtained

results, 1 new mutation locus was discovered as G940T. The variation of mutational pattern on isolate samples towards KAN was depicted on Table 3.

Tabel 3: Change of mutational patterns, codon and amino acid at KAN mutation

Anti-tuberculosis drugs	Gene	Mutational pattern	Codon	Amino acid
Kanamycin	rrs	514 A →C	AGC → CGC	Serine→ arginine
		940 G→T	GCA → TCA	Alanine → serine
		1401 A →G	TCA → TCG	Serine→serine
		1484 G→C	CGA → CCA	Arginine→proline
		1521 T→C	GCT → GCC	Alanine →alanine

The results of statistical analysis based on the number of mutations in each of *rrs*gene shownthe p-value ($p<0.05$), proving the relationship between *rrs* gene mutations with KAN resistance in isolates (Table 2).

4. Discussion

Kanamycin is types of aminoglycoside. Aminoglycosides bind with ribosomes and disrupt the peptide chain elongation in bacteria.Mutation in the *rrs* gene with the code of 16S rRNA is associated with resistance against KAN.In the scope of microbiology, resistance is caused by genetic mutations, and it creates inefficacy in combating mycobacteria. Mutations occur spontaneously and produces resistance towards anti-tuberculosis drugs.Resistance to antimicrobials is the innate characteristic (inborn) of MTB. It is associated with a genetic mutation that occurs naturally in most populations ofMTBwild type. The emergence antimicrobial resistance is precipitated by the use of wrong antimicrobial andit is a man-made phenomenon. Resistance towards more than one antimicrobial drugs usually arises when the drugs are continuously consumed, increasing the numbers of drug-resistant MTB [7].

In this study, we obtained the smaller percentage of*rrs* gene mutations againstKANthananother studies [1,3,8]. Study conducted by Campbell [2] showed thatof 314 isolate samples, 181 (57.6%) samplesare found to be resistantwith the second-linedrugs, and127 (40.4%) samples were mutated.

In this study, the KAN-resistant mutated gene was found at the locus of T1521C, G1484C, A1401G, G940T and A514C. Whereas the research carried out by Jugheli(2009) showed that mutatedisolate samples on gene locus were found in locus A1401G and C1402T.Previous studies about locus mutations have been reported before [2,6,8], however, this study found a new locus mutation called G940T.Nucleotide changes in 1400, 1401 and 1483 of the *rrs* gene have been particularly associated with resistance against KAN. The change of A → G in codon 1400 at*rrs* geneindicates resistanceagainst KAN from over 200 mg/mL MICs (Johnson and his colleagues 2009). Indeed, resistance against KAN also occurs in nucleotide 1402 and 1484 [1].

According to thegene locus, mutationdue to resistanceagainst KANare located inT1521C, G1484C, A1401G, G940T and A514C. In addition, study conducted by Jugheli [6] showed that mutational pattern in isolate sampelsare located in A1041G and C1402T. The similar mutational pattern also observed in another studies. In this study, relationship between the *rrs* gene mutation and KAN resistance was found.With these results, the isolate clinical samples can be used to detect (genetic examination using PCR andsequencing) the occurence of KAN resistance in advance.

This study described 1 sample mutation (T1521C) in the *rrs* gene examination but not resistant against KAN at the DST examination. This phenomenon occurred because mutation locus is considered as a nonsense mutation, in which there are no alteration in the amino acid (GTC/GCC= Alanine/Alanine). There is also two sample that shown resistance in the DST examination without any evidence of mutation. It is assumed that mutation occurred outside the locus target. Research carried out by Campbell [2] found that 54 of 181 sampels that resistant to anti-tuberculosis drugs shown no mutation. Molecular tests have several limitations, this test is incapable to include all types of mutations that occur in each resistance. Although some types of mutations that occur in every resistance has been widely known, some new mutations could occur and not necessarily associated with the pre-existing resistance. This phenomenon is the cause of resistance without accompanied with gene mutation [2, 10,11].

5. Conclusion

In conclusion, significance and impact of study, there is an association between the mutation in the *rrs* gene and the resistance to the second-line drugs of KAN in clinical isolates of MTB.Thus, samples of clinical isolates of MTB could be used to detect any resistance of KAN in advance.

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