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Original Article

Molecular characterisation of mutations associated with resistance to first- and second-line drugs among Indonesian patients with tuberculosis

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الملخص

أهداف البحث: تهدف هذه الدراسة لتحديد الخصائص الجزيئية لتسلسل الجينات *rrs*، *katG*، *rpoB* و*gyrA* في السل الفطري التي تم عزلها من مجموعة من مرضى إندونيسيين يعانون من السل.

طرق البحث: تم تحليل خمسين من عزلات السل الفطري باستخدام اختبار حساسية الدواء لأدوية الخط الأول والثاني باستخدام الطريقة النسبية في وسط سائل. تم استخراج المادة الجينومية لأداء تفاعل سلسلة البلمرة المتعددة من أجل تحديد وتسلسل الجينات ل *rrs*، *katG*، rpoB و*gyrA*.

النتائج: تقريبا ٨٠٪ (٤٠/٥٠) من طفرات *rpoB* حدثت خارج منطقة النقطة الساخنة تمنح مقاومة الريفامبيسين، ١١.٤٢٪ (٤/٣٥). كانت الطفرات *katG* S315T مقاومة للريفامبيسين، بدلا من مقاومة ايزونيازيد. توجد الطفرات الجينية katG أيضا في أماكن مختلفة وكان هناك ٤٢٪ (٢١/٥٠) سلالة مقاومة لستربتومايسين، ولكن ٢ فقط من السلالات لديها*rrs* طفرات جينية (G878A و / أو S514R). وكانت تقريبا ١٤٪ (٧ / ٥٠) من عزلات السل مقاومة لكاناميسين وكابريوميسين، ولم تأو طفرات في تسلسل منطقة الجين ٤٠ /٥٠ (٨٠٪، *rrs*) من العزلات لديها طفرات في كوينولون منطقة تحديد المقاومة للجين gyrA بما فيها عموم السلالة الحساسة.

الاستنتاجات: من بين ٥٠ سلالة تم تحليلها، معظم الطفرات المرتبطة بمقاومة الريفامبيسين في الجين *rpoB* وجدت في الجينات *katG* و*gyrA*. النسبة الجزيئية باستخدام تقنيات تسلسل الحمض النووي لديها حساسية عالية في اكتشاف الطفرة.

الكلمات المفتاحية: وحدة وراثية؛ الطفرات؛ السل الفطري؛ مقاومة الأدوية المتعددة؛ مقاومة الريفامبيسين

Abstract

Objectives: This study aimed to determine molecular characteristics of *rpoB, katG, rrs*, and *gyrA* genes in *Mycobacterium tuberculosis* isolated from a cohort of Indonesian patients with tuberculosis.

Methods: Fifty isolates of *M. tuberculosis* were analysed by testing (DST) for susceptibility to first- and second-line drugs using the proportional method in a liquid medium. The genomic material was extracted to perform multiplex polymerase chain reaction (PCR) for identification and gene sequencing of *rpoB, katG, rrs*, and *gyrA*.

Results: Approximately 80% (40/50) of the *rpoB* mutations that were detected outside the hot-spot region (S450L, H445D, D435V, S441L, I491F, and Q432P)

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conferred rifampicin-resistance on *M. tuberculosis*. Approximately 11.42% (4/35) of isolates with S315T mutation in *katG* led to rifampicin-resistance instead of isoniazid-resistance. The mutation in *katG* gene was found at various locations (P280P, G279R, E340Q, T271I, E340*stop codon, R373G, and S315N). Streptomycin-resistance was detected in 42% (21/50) of the strains, but only two strains had *rrs* gene mutations (G878A and/or S514R). Approximately 14% (7/50) of *M. tuberculosis* isolates were kanamycin- and capreomycin-resistant but did not harbour mutations in the *rrs* gene, while 80% (40/50) of the strains had mutations in the quinolone-resistance determining region (QRDR) of the *gyrA* gene (S95T, D94V, A90V, and S91P) including the pan-susceptible strain.

Conclusions: Of the 50 strains analysed, most of the mutations in the *rpoB* gene associated with rifampicin-resistance were also detected in the *katG* and *gyrA* genes. Molecular characterisation using DNA sequencing techniques is a highly sensitive approach for detecting mutations.

Keywords: Gene; Mutations; Multi-drug resistant (MDR); *Mycobacterium tuberculosis*; Rifampicin-resistance

Introduction

Genetic resistance among groups of bacteria can be caused by transfer of genetic elements through transduction or transformation. In *Mycobacterium tuberculosis* (MTBC), genetic resistance arises as a result of chromosomal mutations. However, the transfer of genetic elements such as insertion of IS6110 sequence to MTBC is considered to be related to resistance through the inactivation of important genes.[1]

With the increase in the number of multi-drug-resistant tuberculosis (MDR-TB) cases worldwide, the use of anti-TB drugs such as fluoroquinolones (FQs) and aminoglycosides for treatment has also increased. A study showed that anti-TB drug resistance in mycobacterium tuberculosis (MTBC) is related to competitive drug compatibility, which depends on specific resistance mutations.[2]

The main mechanism underlying FQ resistance is a point mutation in the *gyrA* and *gyrB* genes that encode two subunits of DNA gyrase. The *gyrA* mutation commonly occurs at codons 90 and 94 and is rarely found at codons 88 and 91, whereas the *gyrB* mutation, commonly occurs at codons 472 and 510.[3]

In Indonesia (especially in Makassar), like in other developing countries, the high burden of TB has contributed to the development of resistance towards first- and second-line antimicrobials drugs, as many patients diagnosed with TB are not tested for drug susceptibility. Moreover, extensively drug resistant (XDR) cases and mutations related to FQs and second-line drugs have emerged. This study aimed to determine the molecular characteristics of *rpoB*, *katG*, *rrs*, and *gyrA* genes in *M. tuberculosis* isolated from patients with TB in Makassar, Indonesia.

Materials and Methods

Clinical isolates

This study was approved by the Institutional Research Board of Medical Faculty of Hasanuddin University, Makassar, Indonesia (registration number: 42/H4.8.4.5.31/PP36-KOMETIK/2018, dated January 18, 2018). This research was conducted from January to November 2018.

Forty clinical strains of *M. tuberculosis* were recovered from different sputum samples of patients with TB and ten clinical strains of *M. tuberculosis* were isolated from patients diagnosed with pulmonary TB. The patients were registered at a centre for TB treatment in Makassar. The resistant and drug-susceptible strains of *M. tuberculosis* isolated from patients with MDR-TB or from patients newly diagnosed with TB were analysed by gene sequencing based on the results of drug susceptibility testing (DST) performed using first- and second-line anti-TB drugs.

The drug susceptibility profiles of the isolates were evaluated by the proportional method using Mycobacterium Growth Indicator Tube (MGIT) 960 System (BD Biosciences, Becton, Dickinson and Company, MD, USA) with the following critical drug concentrations: streptomycin 1.00 μg/mL (STR), isoniazid 0.10 μg/mL (INH), rifampicin 1.00 μg/mL (RIF), ethambutol 5.00 μg/mL (ETB), kanamycin 2.5 μg/mL (KANA), ofloxacin 2.0 μg/mL (OFX), capreomycin 2.5 μg/mL (CAP), and moxifloxacin 0.25 μg/mL (MOXI). The critical proportion of resistant bacillus necessary to define a resistant strain is 1% for all tested drugs.[4,5]

Multiplex PCR amplification for species identification

Genomic DNA was extracted from 50 isolates of *M. tuberculosis* cultured on Lowenstein-Jensen (LJ) slants using guanidium thiocyanate.[5] The PCR mixture contained 12.5 μL of 2X Kapa2G fast ready-mix and 2 μL of primers (HT1: 5′-CCTGCGAGCGTAGGCGTCGG-3′ and HT2: 5′-CTCGTCCAGCGCCGCTTCGG-3′). The length of PCR products for HT1/HT2 was 123 base pairs (bp).[5–7]

M. tuberculosis DNA isolation

Scrapped bacterial colonies were recovered in 200 μL of nuclease free water, which was then boiled at 90 °C for 30 min to terminate bacteria and discharge the mycobacterial DNA.[8]

PCR and sequencing

The drug-resistant genes, *rpoB*, *katG*, *rrs*, and *gyrA*, were amplified by PCR using specific primers listed in Table 1. The thermal cycling conditions were as follows: *pre-denaturation* at 95 °C for 15 min; 45 cycles of *annealing* for *rpoB* and *katG* genes at 95 °C for 15 sec, 65 °C for 15 sec, and 72 °C for

1 min; and *final extension* at 72 °C for 5 min. The annealing phase for *rrs* and *gyrA* genes consisted of 40 cycles at 95 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 1 min.

Detection of gene mutations by sequencing

Mutational analysis was performed using a direct sequencing method at the 1st BASE Laboratory, Malaysia. The PCR products were sequenced to detect mutations in the target genes (*rpoB*, *katG*, *rrs*, and *gyrA*) and were then analysed using Bio-Edit software version 7.0.5.1.[9]

Results

Among 50 *M. tuberculosis* isolates tested by DST for susceptibility to first- and second-line anti-TB drugs, we found that 8% (4/50) were RIF-resistant (RR). Next, 62% of the isolates (31/50) showed MDR phenotype with resistance to INH and RIF, and 10% (5/50) showed XDR phenotype with resistance to first-line drugs (RIF and INH), FQs (OFX and/or MOXI), and aminoglycosides (CAP and/or KANA), which are injectable second-line drugs (Table 2). Approximately 20% of the isolates (10/50) were susceptible to all drugs.

RIF-resistance in isolates of M. tuberculosis

The following mutations were detected in four RIF-resistant strains (Tables 2 and 3): Asp435Val, His445Asp, and Ser450Leu in *rpoB*; Ser315Thr and E3408*stop codon in *katG*; and Ser95Thr in *gyrA*.

A. MDR phenotype in isolates of *M. tuberculosis*

There were 31 MDR strains (Table 3) identified in this study. Of the 31 MDR strains, 14% (7/50) were resistant to INH and RIF and had the following mutations: His445Asp and Ser450Leu in *rpoB*; Ser315Thr, Gly279Arg, and Glu340Gln in *katG*; and Ser95Thr in *gyrA*. Another 14% (7/50) of the strains were resistant to STR, INH, RIF, ETB, OFX, and MOXI and had the following mutations: His445Asp and Ser450Leu in *rpoB*; Ser315Thr and Gly279Arg in *katG*; and Ser95Thr, Asp94Val, Ala90Val, and Ser91Pro in *gyrA*.

Table 1: Primer sequences and positions used to amplify related genes in Anti-TB drug.

Anti-TB drug	Gene	Primer	Sequences	Tm	Length
RIF	*rpoB*	BDR-F5	5′-GGGAGCGGATGACCACCCA-3′	62.4 °C	350 bp
		BDR-R5	5′-GCGGTACGGCGTTTCGATGAAC-3′	61.0 °C	
INH	*katG*	katG-F	5′-GGTCGACATTCGCGAGTT-3	45.2 °C	518 bp
		katG-R	5′-CGGTGGATCAGCTTGTACCCAG-3′	51.2 °C	
FQs	*gyrA*	gyrA-F5	5′-ATGACAGACACGACGTTGCC-3′	57.9 °C	
		gyrA-R5	5′-GGTAGCACCGTCGGCTCTTG-3′	60.5 °C	504 bp
Aminoglycosides	*Rrs*	rrs-F5	5′-TAAACCTCTTTCACCATCGACG-3′	54.7 °C	556 bp
		rrs-R5	5′-CCACGTAAGGTTCTTCGCGTTG-3′	58.2 °C	

TB: Tuberculosis, F: forward, R: reverse, *bp*: base pair, *Tm*: Melting time, RIF: rifampicin, INH: isoniazid, FQs: fluoroquinolones.

Table 2: Drug resistance profiles of clinical isolates of *Mycobacterium tuberculosis*.

	Resistance Pattern	No. of strains
RIF Resistant (RR)	RIF	2 (4%)
	RIF+MOXI	1 (2%)
	RIF+KANA+CAP	1 (2%)
Multi Drug Resistant (MDR)	STR+INH+RIF+ETB	4 (8%)
	STR+INH+RIF	3 (6%)
	INH+RIF	7 (14%)
	STR+INH+RIF+ETB+OFX+MOXI	7 (14%)
	INH+RIF+ETB+OFX+MOXI	6 (12%)
	STR+INH+RIF+OFX+MOXI	1 (2%)
	INH+RIF+ETB+OFX	1 (2%)
	STR+INH+RIF+KANA+CAP	1 (2%)
	STR+INH+RIF+ETB+KANA+CAP	1 (2%)
Extensively Drug Resistant (XDR)	STR+INH+RIF+ETB+KANA+OFX+CAP+MOXI	1 (2%)
	STR+INH+RIF+KANA+OFX	1 (2%)
	INH+RIF+KANA+OFX+CAP	1 (2%)
	STR+INH+RIF+ETB+OFX+CAP+MOXI	1 (2%)
	STR+INH+RIF+KANA+OFX+CAP+MOXI	1 (2%)
Pan-sensitive	Susceptible to all drugs	10 (20%)
Total (n)		50 (100%)

STR: streptomycin, *INH*: isoniazid, *RIF*: rifampicin, *ETB*: ethambutol, *KANA*: kanamycin, *OFX*: ofloxacin, *CAP*: capreomycin, *MOXI*: moxifloxacin.

Table 3: Frequency of mutations identified by sequencing the *rpoB*, *katG*, *rrs*, and *gyrA* genes of *Mycobacterium tuberculosis* isolates.

Gene	Codon position	Type of mutation	Amino acid changes	No. of resistant isolates	No. of pan-sensitive isolates
rpoB	435	GAC → GTC	Asp/Val	3	
	445	CAC → GAC	His/Asp	6	
	450	TCG → TTG	Ser/Leu	26	
	491	ATC → TTC	Ile/Phe	1	
	441	TCG → TTG	Ser/Leu	2	
	432	CAA → CCA	Gln/Pro	1	
	445	CAC → TAC	His/Tyr	1	
katG	315	AGC → ACC	Ser/Thr	35	
	280	CCG → CCT	Pro/Pro		1
	279	GGA → CGA	Gly/Arg	4	
	340	GAA → CAA	Glu/Gln	1	
	271	ACC → ATC	Thr/Ile	1	
	340	GAG → TAG	Glu/stop codon	2	
	373	CGT → GGT	Arg/Gly	2	
	315	AGC → AAC	Ser/Asn	1	
rrs	878[a]	TGG → TAG	Trp/Stop codon	1	
	514[a]	AGC → CGC	Ser/Arg	2	
gyrA	95	AGC → ACC	Ser/Thr	31	6
	94	GAC → GTC	Asp/Val	9	
	90	GCG → GTG	Ala/Val	5	
	91	TCG → CCG	Ser/Pro	1	

[a] Nucleotide position.

B. XDR phenotype in isolates of *M. tuberculosis*

Of the 5 XDR strains (Table 3) identified in the study, 2% (1/50) were resistant to STR, INH, RIF, ETB, KANA, OFX, CAP, and MOXI and harboured the following mutations, Ser441Leu in *rpoB*, Ser315Thr in *katG*, and Ser95Thr and Asp94Val in *gyrA*.

C. Pan-susceptible phenotype in isolates of *M. tuberculosis*

The 10 pan-susceptible isolates of *M. tuberculosis* harboured mutations in the *katG* (Pro280Pro (1/10) and Ser315Thr (1/10)) and *gyrA* (Ser95Thr (6/10)) genes.

Discussion

RIF is a very potent first-line drug and an important drug for determining the effectiveness of TB treatment regimens.[10,11] In this study, 40 of the 50 *M. tuberculosis* isolates showed RIF-resistant phenotype and harboured mutations in the *rpoB* gene accompanied by alterations in *katG* and *gyrA* genes. Several studies have shown that *rpoB* gene mutations in RIF-resistant isolates occur within the hot-spot region of 81 bp (codon 507 to 533), which is referred to as the RIF resistance-determining region (RRDR). In RRDR, mutations at codons 516, 526, and 531 are the origin of 90% of RIF-resistant strains.[12] In this study, all the *M. tuberculosis* isolates with RIF-resistant phenotype harboured mutations in the *rpoB* gene outside the RRDR region (Table 3).

INH is a prodrug activated by catalase-peroxidase enzyme encoded by the *katG* gene. The most common resistance mechanism is Ser315Thr mutation in *katG* gene, which leads to INH-NAD product formation, which then inhibits the antimicrobial action of INH. The Ser315Thr katG mutation generates high levels of INH resistance in MDR isolates.[13] This was in line with the findings of our current study, which showed that 70% (35/50) of the *katG* gene mutations occurred at codon 315 G → C, causing a change in amino acid from serine to threonine. However, 11.42% of *M. tuberculosis* isolates having Ser315Thr mutation (4/35) exhibited INH-resistance rather than RIF-resistance (Tables 2 and 3).

We sequenced small parts of *rrs* gene (556 bp). Alteration in *rrs* gene at locus 915 is related to STR-resistance, specifically the point mutations at the following positions of codon, 491, 512, 514, 516, 904, and 905.[14] Of the 50 *M. tuberculosis* isolates, there were 21 *M. tuberculosis* isolates that were STR-resistant, but only one isolate harboured a mutation at 878 G → A, which resulted in the change of tryptophan into stop codon, and one isolate carried A → C point mutation (Ser514Arg) at codon 514. Globally, 70%–80% of 1401 A → G polymorphisms in the *rrs* gene are the primary molecular mechanism underlying CAP and AMK resistances and 60% of KANA resistance.[15] In this study, 14% (7/50) of the MDR isolates were KANA- and CAP-resistant without any mutations in the *rrs* gene.

The FQ-resistant strains of *M. tuberculosis* harbour the most frequent mutations at codons 90, 91, and 94 in the *gyrA* gene. Mutations at codons 74, 88, and 91 are also associated with FQ resistance.[16] In this study, 21 OFX and/or MOXI-resistant isolates (Table 2) showed an increasing frequency of pre-XDR or XDR strains (the antibiotics were tested at a TB laboratory).

Conclusion

Of the 50 isolates examined in this study, 80% (40/50) of the isolates harboured mutations in the *rpoB* gene that were present outside the RRDR hot-spot. We found that these mutations (Ser450Leu, His445Asp, Asp435Val, Ser441Leu, Ile491Phe, and Gln432Pro) were more likely to confer RIF resistance. However, 11.42% (4/35) of *M. tuberculosis* isolates with the Ser315Thr mutation exhibited INH-resistance rather than RIF-resistance. The *katG* gene contained

mutations at various locations (Pro280Pro, Gly279Arg, Glu340Gln, Thr271Ile, E340*stop codon, Arg373Gly, and Ser315Asn). Although 42% (21/50) of the *M. tuberculosis* isolates were STR-resistant, only two isolates harboured a mutation in the *rrs* gene (G878A and/or Ser514Arg), and 14% (7/50) of the *M. tuberculosis* isolates were KANA- and CAP-resistant but did not carry mutations in the sequenced *rrs* gene. Molecular analysis showed that 80% (40/50) of the strains had mutations in the QRDR region of the *gyrA* gene (Ser95Thr, Asp94Val, Ala90Val, and Ser91Pro), including the pan-susceptible strains.

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Conflict of interest

There is no conflict of interest.

Ethical approval

This study was approved by the Institutional Research Board of Medical Faculty of Hasanuddin University, Makassar, Indonesia. Registered approval number 42/H4.8.4.5.31/PP36-KOMETIK/2018, dated January 18th 2018. The informed consent for this study was obtained written from all participants or their parents/guardians accompanied by the authorized nurses who were in charged of managing TB patients.

Authors contributions

FFU, DRH, MMH, RRN, and RSS conceived and designed the study, conducted research, provided materials, and collected and organised the data. FFU, DRH, MMH, RRN, RRS, RRD, ARJ, and MRP drafted the manuscript. FFU analysed and interpreted the data. FFU, DRH, RRD, ARJ, MRP, and MMH wrote the initial and final drafts of the manuscript and provided logistic support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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