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

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Abstract

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

Methods: Fifty isolates of \textit{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 \textit{rpoB}, \textit{katG}, \textit{rrs}, and \textit{gyrA}.

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

Keywords: Mycobacterium tuberculosis; drug resistance; drug susceptibility; \textit{rpoB}, \textit{katG}, \textit{rrs}, \textit{gyrA}
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 resistance genes through transduction or transformation is a highly sensitive approach for detecting mutations.

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

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Multiple PCR amplification for species identification

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

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.

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 Gln340Gln 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 (°C) | Length (bp) |
|--------------|------|--------|-----------|--------|------------|
| RIF          | \( rpoB \) | BDR-F5 | 5’-GGGAGCGGTACCGGTGGATAC-3’ | 62.4 | 350 |
| INH          | \( katG \) | katG-F | 5’-GGTCCGACATTGGCCGAGGTT-3 | 45.2 | 518 |
| FQs          | \( gyrA \) | gyrA-F5 | 5’-ATGACAGACGCGGTGCGTCCCGC-3’ | 57.9 | - |
| Aminoglycosides | \( Rrs \) | rrs-F5 | 5’-GACATTGCCTTCTTACCATCGACG-3’ | 54.7 | 556 |

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              | 2 (4%)        |
| RIF+MOXI         | 1 (2%)        |
| RIF+KANA+CAP     | 1 (2%)        |
| STR+INH+RIF+ETB  | 4 (8%)        |
| STR+INH+RIF      | 3 (6%)        |
| INH+RIF          | 1 (2%)        |
| 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%) |
| STR+INH+RIF+ETB+KANA+OFX+CAP+MOXI | 1 (2%) |
| STR+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 |
| Total (n)        | 50 (100%)     |

STRI: streptomycin, INHI: isoniazid, RIFI: rifampicin, ETB: ethambutol, KANA: kanamycin, OFX: ofloxacin, CAP: capreomycin, MOXI: moxifloxacin.
genes. Several studies have shown that in the RIF-resistant phenotype and harboured mutations.13 This was in line with the findings of our study, which showed 70% (35/50) of the isolates having Ser315Thr mutation exhibited INH-resistance rather than RIF-resistance. The Ser315Thr mutation generates high levels of INH resistance in the enzyme encoded by the katG gene, which leads to INH-NAD product formation, which then inhibits the antimicrobial action of INH. The Ser315Thr katG mutation contained 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).

### Discussion

**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.

### 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         | Gin/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            | TGG → TAG        | Trp/Stop codon     | 1                        |                             |
|      | 514            | 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.

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 isolates.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).

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### 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, RSS, 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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