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Molecular characteristics of the gyrA gene among rifampicin-resistant *Mycobacterium tuberculosis* isolates

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ABSTRACT

Background: Drug-resistant tuberculosis (TB) remains a public health threat, especially during this pandemic. Meanwhile, fluoroquinolone is used as a second-line multidrug-resistant TB (MDR-TB) treatment since this drug was previously prescribed for respiratory, urinary, and genital tract infections. However, unregulated and excessive use of fluoroquinolones leads to resistance.

Methods: The design of this study is a descriptive observational study with a cross sectional approach. This study aims to determine the pattern of *gyr*A gene mutation in fluoroquinolone resistance among rifampicin-resistant *Mycobacterium tuberculosis* isolates during the COVID-19 pandemic in Sumatra, Indonesia. The *Mycobacterium tuberculosis* isolates were stored in the Palembang Health Center Laboratory as the referral laboratory in Sumatra from January to December 2020. Out of the 233 isolates that were tested phenotypically by BACTEC MGIT, 8 isolates of fluoroquinolone resistance (ofloxacin or moxifloxacin or both) were obtained and sequenced using an ABI PRISM 3730XL analyzer for Single Nucleotide Polymorphism analysis (SNP).

Results: Among the six fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates, the *gyr*A mutations were identified in 5/6 isolates (84%), A90V (34%), D94A (16%), and D94G (34%), while 1/6 isolates (16%) had no mutation in *gyr*A gene among *Mycobacterium tuberculosis* that were fluoroquinolone resistance.

Conclusion: The *gyr*A gene mutation in fluoroquinolone resistance among rifampicin-resistant *Mycobacterium tuberculosis* was commonly present in codon 90 (2/6 isolates =32%) and 94 (3/6 isolates=68%).

Keywords: gyrA Gene Mutation, MDR-TB, fluoroquinolone resistance, rifampicin resistance.
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INTRODUCTION

Drug-resistant TB continues to be a public health threat. MDR-TB is drug-resistant TB against rifampin and isoniazid. At the same time, XDR-TB is MDR-TB plus resistance to second-line TB drugs, such as the fluoroquinolone group (levofloxacin, ofloxacin, and moxifloxacin) and one of the second-line injectable drugs, the aminoglycoside group (amikacin, kanamycin, and capreomycin).[1–3] Worldwide in 2019, nearly half a million people suffered from rifampin-resistant TB (TB-RR), of whom 78% had multidrug-resistant TB (MDR-TB).[4] Indonesia was the 2nd in the TB ranks in the world after India.[4] The number of new TB cases in Indonesia was 420,994 cases.[5] Data on XDR-TB cases in the world in 2016 were around 490,000 people, with an estimated 6.2% of MDR-TB cases to XDR-TB.[1] Fluoroquinolone resistance as a second-line MDR-TB therapy occurs because this drug was previously prescribed frequently and has become an option in other infections, such as the respiratory tract, urinary tract, and genitalia. Unregulated and excessive use of fluoroquinolones can lead to resistance.[6] Some studies have reported that the majority (about 50-90%) of fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates carry mutations in the QRDR (Quinolon Resistance Determining Region) *gyr*A gene.[7,8] However, the genetic involvement of several *gyr*A gene mutations against fluoroquinolone resistance in *Mycobacterium tuberculosis* is unknown, so it is necessary to conduct research to identify the proportion of mutations in the *gyr*A gene in *Mycobacterium tuberculosis* and assess its significance in determining the level of fluoroquinolone resistance.[6]

METHODS

The design of this study is a descriptive observational study with a cross sectional approach to determine the types of mutations that occur in the *gyr*A gene in fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates. This research was conducted at the Public Health Centre Laboratory Palembang (Regional Reference Laboratory of the Sumatra Region). Samples were resistant *Mycobacterium tuberculosis* fluoroquinolone isolates in the Public Health Centre Laboratory Palembang from January - December 2020, which had previously been identified by phenotypic testing with the MGIT second-line

sensitivity test, and the exclusion criteria were defective isolates. From 233 resistant rifampicin *Mycobacterium tuberculosis* isolates, there were obtained 8 isolates of resistant fluoroquinolone *Mycobacterium tuberculosis were obtained* for sequencing test to determine the gryA gene mutation. The isolates studied came from various regions of Sumatra and were sent to the Health Centre Laboratory Palembang as a regional reference laboratory for culture and sensitivity testing for *Mycobacterium tuberculosis* and stored as a standard. In this study, eight isolates *of Mycobacterium tuberculosis resistant to fluoroquinolones consist of three isolates from South Sumatra, two isolates from Riau, two isolates from Lampung,* and one isolate from Bangka Belitung. For this study, the H37Rv isolate was used as a negative control.

Sample processing

Resuscitation of fluoroquinolone-resistant and sensitive Mycobacterium tuberculosis isolates was done. Cyrotube containing fluoroquinolone-resistant and sensitive *Mycobacterium tuberculosis* isolates from the deep freezer -70^{0}C was taken and left at room temperature until thawed.

PCR amplification

Isolated DNA was amplified by the PCR method. Identification of the *gyr*A gene using *gyr*AF (5'-CAGCTACATCGACTATGCGA) and *gyr*AR (5'-GGGCTICGGTGTACCTCAT) primers in a 320 bp.[9–11] The PCR mix composition (50 plI) consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 10% glycerol, 200 jiM (each) dATP, dTTP, dCTP, and dGTP; 0.5 jiM (each) primer; and 1.25 U of Taq DNA polymerase (Boehringer Mannheim). The master mix is left at room temperature and vortexed, then spun in a microcentrifuge so that the material collects at the bottom of the tube. The master mix volume was 25 µl, the forward and reverse primers were 10 µM each with a volume of 2.5 µl each, and 2 µl of template DNA was mixed. The reaction mix was added with ddH2O until the final volume reached 50 µl. Amplification was carried out in a thermocycler with conditions, pre-denaturation at 94^{0}C for 1 minute, denaturation at 94^{0}C for 1 minute, annealing at 55^{0}C for 1 minute, and extension at 72^{0}C for 10 minutes for 40 cycles. PCR products were analyzed by electrophoresis on a 1.5% agarose gel with a voltage of 300 volts for 20 minutes, then documented PCR products using Gel Doc (UV translumination).

Sequencing

Purified DNA was directly sequenced by SNP-mutation analysis of the ABI PRISM 3730XL sequencer using the same forward or reverse primers as PCR amplification, gyrAF (5'-CAGCTACATCGACTATGCGA) and gyrAR (5'-GGGCTICGGTGTACCTCAT) to detect the mutation of the *gyr*A gene.

Statistical analysis

Statistical analysis was conducted using SPSS 26.

RESULTS

A total of eight isolates of *Mycobacterium tuberculosis* that were resistant to fluoroquinolones and eight sensitive isolates of fluoroquinolones that had been tested for phenotypic BACTEC MGIT were sequenced using ABI PRISM 3730XL sequencers, including H37RV isolates as negative controls. The result was two isolates being damaged; they could not be sequenced, so they were excluded. Five fluoroquinolone-resistant Mycobacterium tuberculosis isolates showed a missense mutation in the gyrA gene, two isolates had A90V mutations, one isolate had a D94A mutation, and two isolates had D94G mutations. One fluoroquinolone-resistant Mycobacterium tuberculosis isolate did not have mutations in the *gyr*A gene amplification area (QRDR). There were found a missense T95S mutation was found in *Mycobacterium tuberculosis* H37RV as a negative control. Meanwhile, eight isolates of *Mycobacterium tuberculosis* sensitive to fluoroquinolone showed no mutations in the *gyr*A gene. SNP-sequencing results can be seen in Table 1. The percentage isolates FQ resistance by phenotypic BACTEC MGIT, compared to the sequencing result, can be seen in Table 2. The PCR gel electrophoresis from the target primers is shown in Figure 1.

DISCUSSION

This study aims to determine the mutation pattern of the *gyr*A gene in resistant fluoroquinolone *Mycobacterium tuberculosis* isolates in Sumatra, Indonesia. Fluoroquinolones are widely used for the treatment of bacterial infections. In cases of pneumonia, the IDSA / ATS guidelines recommend fluoroquinolones as a therapeutic option for CAP or HAP.[12] The low-level risk of fluoroquinolone resistance in *Mycobacterium tuberculosis* occurred starting at more than 13 days of

Table 1. Point mutations of the *gyr*A gene in QRDR of five resistant fluoroquinolone *Mycobacterium tuberculosis* isolates

Codon	Nucleotide mutation	Type mutation	Sequencing	Amino acid changes	Mutation frequency isolates(percentage)
90	GCGàGTG	substitution	A90V	Alaàvalin	2 (40%)
94	GACàGCC	substitution	D94A	AspàAla	1(20%)
94	GACàGGC	substitution	D94G	AspàGly	2 (40%)

Table 2. The percentage of fluoroquinolone resistant according to phenotypic MGIT and the mutation pattern of the *gyr*A gene from five isolates

Phenotypic DST BACTEC MGIT		Sequencing	Mutation frequency (isolates)(percentage)
Ofloxacin	Moxifloxacin		
Resistant	sensitive	A90V	2 (40%)
Resistant	sensitive	D94A	1(20%)
Resistant	Resistant	D94G	2 (40%)

Figure 1. PCR products with a 320 bp amplicon, the result of 17 isolates (8 resistant, 8 sensitive to fluoroquinolone, and 1 H37RV isolate as a control). Samples 2 and 17 did not form a band because the isolates were damaged.

exposure to the use of fluoroquinolones for therapy, while the high-level risk of exposure to fluoroquinolone use was more than 60 days before tuberculosis was diagnosed.[13]

In this study, two fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates were excluded because they were damaged. Two defective isolates were possible at the pre-analysis; sputum sample transport does not comply with standards, for example, did not use an ice box at the time of delivery, or there was a long delay in the original laboratory with storage using a refrigerator that was not -700 °C. One fluoroquinolone-resistant *Mycobacterium tuberculosis* isolate with sequencing results did not show mutations; this may be due to: 1) there is a change in target DNA *gyrase*; the presence of MfpA (Mycobacterial FQ resistance protein A) protein that resembles DNA *gyr*ase mimicry.[14,15] 2) mutations are not in the QRDR amplification area (Quinolon Resistance Determining Region), 3) other resistance mechanisms, such as the presence of MmpL (Mycobacterial membrane protein large) protein with an efflux mechanism that releases drugs from the cell walls.[16]

In the SNP-mutation analysis using the ABI PRISM 3730XL sequencer, from five isolates *of Mycobacterium tuberculosis, FQ resistance was found to have mutation patterns of gyrA; A90V, D94A, and D94G, all of which were missense mutations*. In the A90V mutation, there is a change in the amino acid alanine to valine in codon 90 of two fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates. The type of mutation is substitution; the change in the nucleotide base of cytosine is substituted for thymine in base pair 269. The results of this study are in accordance with the Quinolon Resistance Determining Region.[9,11,17–21] These mutations lead to resistance to low doses ofloxacin and moxifloxacin, but higher doses of moxifloxacin are sensitive. The D94A missense mutation changes the amino acid aspartic acid to alanine at codon 94 found in one fluoroquinolone-resistant *Mycobacterium tuberculosis* isolate. The type of substitution mutation with a change in the nucleotide base of adenine is substituted for cytosine in the base pair 281. The results of this study are in accordance with the Quinolon Resistance Determining Region.[11,17,19,20] This mutation causes resistance to ofloxacin (MIC 2 ug/ml) and low dose of moxifloxacin (0.25 ug/ml), but high dose of moxifloxacin is still sensitive (MIC 1 ug/ml) in this study.

In this research, the D94G missense mutation changes the amino acid aspartic acid to glycine in codon 94 in two isolates. The type of substitution mutation with a change in the nucleotide base of adenine is substituted for guanine in the 281st base pair. The results of this study are in accordance with the Quinolon Resistance Determining Region.[11,17,19–21] This mutation leads to ofloxacin and moxifloxacin resistance. In Farhat et al's study of 240 MDR-TB isolates after sequencing the *gyr*A and *gyr*B genes, it was found that the *gyr*A mutations in QRDR, A90V and D94A had the smallest effect on MIC (Minimum Inhibitory Concentration) moxifloxacin, 2.2 times more resistant than without mutation This effect is different from the D94G *gyr*A mutation, with changes in the MIC of moxifloxacin 3.1 times more resistant than without the mutation. Whereas in the MIC ofloxacin, the A90V and D94A mutations have a resistance effect comparable to the D94G mutation.[22] In Farhat et al study, it was also mentioned that there was a *gyr*B mutation against high-dose resistant moxifloxacin, which was not studied in this research.[22] Based on this study, the *gyr*A mutation pattern at codons 90 and 94, which were A90V, D94G, and D94G, with the same OFX and MFX resistance results as previous research, but the role of *gyr*B in resistance levels in this study was unknown because it was not studied.[22,23]

Mutations in codons 88 to 94 of the *gyr*A gene are associated with OFX and MFX resistance in East Asian (Beijing), Euro-American, and Indo-Oceanic strains.[23] Based on the results of this study, for H37Rv sequencing as a negative control, missense T95S mutations were found, changes in threonine to serine at codon 95. The type of substitution mutation from nucleotide bases from cytosine to guanine in base pair 284 was also found in the previous research, QRDR region of Mycobacterium tuberculosis H37Rv.3. But for the phenotypic sensitivity test of BACTEC MGIT, the results were sensitive to fluoroquinolones. This mutation is a natural polymorphism of *Mycobacterium tuberculosis* H37Rv.[3] This is a limitation of this study. Possible mutations that occur in *Mycobacterium tuberculosis* H37Rv due to repeated subculture processes in the laboratory. The H37Rv strain was originally chosen for sequencing. It is a widely used laboratory strain because it has maintained its virulence. H37Rv was originally derived from a clinical isolate, H37, obtained from a patient with pulmonary tuberculosis in 1905. There were 72 polymorphisms that were similar among the six variants of H37Rv plus H37Ra, compared to the reference sequence H37Rv.[24] The natural polymorphism of T95S in *Mycobacterium tuberculosis* H37Rv was found in 7/46 strains (15.2%) and 10/104 MDR-TB isolates (9.6%) in the previous research,

while in this study, 1/6 isolates (16%) were found.[3,25]

The limitations of this study are the small number of samples, and this can be overcome by collaboration between various institutions that have cultured and tested TB samples.

CONCLUSION

The molecular characteristics of the gyrA gene are very important to determine resistance to fluoroquinolone in *Mycobacterium tuberculosis*. It can be used as a marker for the quick diagnostic of MDR-TB second-line drugs in molecular methods and also to get faster therapy for the patient. From this study, we can find out the mutation profile of FQ-resistant Mycobacterium tuberculosis isolates in Sumatra, where the hot spot codons of QRDR were in positions 90 and 94. It leads to the effect of those mutations on fluoroquinolone therapy for MDR-TB, and it can give information to clinicians about the pattern of resistance to FQ Mycobacterium tuberculosis in the Sumatra region of Indonesia. There are five missense mutations that can be detected in this study. The mutations of the *gyr*A gene, A90V and D94G, were resistance to ofloxacin but still sensitive to moxifloxacin, whereas the D94G was resistance to ofloxacin and moxifloxacin, which we might have been concerned about.

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AUTHOR CONTRIBUTION

Febriana Aquaresta and Kuntaman were involved in concepting, designing, and supervising the manuscript. Febriana Aquaresta and Lisa Dewi conducted the study. Febriana Aquaresta, Kuntaman Kuntaman dan Irbasmantini analyses the data. All authors prepare the manuscript and agree to this final version of the manuscript being submitted to this journal.

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CONFLICT OF INTEREST

The authors declare that no competing financial, professional, or personal interests might have affected the performance or presentation of the work described in this manuscript.

ETHICAL APPROVAL

This research was approved by the Committee of Ethical Medicine's faculty, Universitas Airlangga, with the number 16/EC/KEPK/FKUA/2021

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