Evaluation of *Mycobacterium tuberculosis* ripA gene to detect antibiotic resistance

M P Koentjoro¹, D S Rahayu¹, A Donastin³ and E N Prasetyo³*

¹Medical Technology Laboratory Study Program, Universitas Nahdlatul Ulama Surabaya, Jl. Jemursari No. 51-57, Surabaya 60293, Indonesia
²Departement of Biology, Institut Teknologi Sepuluh Nopember Surabaya, Jl. Teknik Mesin No. 173, Surabaya 60115, Indonesia
³Faculty of Medicine, Universitas Nahdlatul Ulama Surabaya, Jl. Jemursari No. 51-57, Surabaya 60293, Indonesia

*Corresponding author: endry@bio.its.ac.id

Abstract. *Mycobacterium tuberculosis* infection has remained a public health threat in Indonesia. This infection is complicated by the antibiotic-resistant of *M. tuberculosis* strains. The common essential in resistance comes from a mutation in genomic DNA. ripA gene is one of the regions critical in the replication and persistence of *M. tuberculosis* in their resistance. This gene has responsible for cell wall polymer peptidoglycan. The objective of this research was to evaluate the ripA gene in antibiotic resistance. This is to investigate and compare the ripA gene sequences of *M. tuberculosis* at an unprecedented rate. A total of five specimens of *M. tuberculosis* were isolated from tuberculosis patients with rifampicin resistance. The ripA gene from *M. tuberculosis* was isolated and amplified using a design primer for ripA N-terminal domain of peptidoglycan hydrolase. Further, ripA gene was analyzed using the Sanger method sequencing. The data were analyzed and compared using *M. tuberculosis* H37Rv from the National Center for Biotechnology Information (NCBI). In addition, the sequence was analyzed with multiple sequence alignment (Clustal IW) to identify the mutation. Our result suggests that the evaluation of genes in *M. tuberculosis* isolates revealed sequence variation in ripA regions (Ala701Gly). Understanding these mutations implies an evaluation of antibiotic-resistant. Furthermore, this information implies local diagnostic and treatment guidelines to cell-wall targeting antibiotics.

1. Introduction

*Mycobacterium tuberculosis* infection is a human pathogen and causing tuberculosis (TB). This bacteria persistence's in pulmonary and transmitted by aerosol. The routes of transmission can be done from a droplet containing spread infections from the patient. It can be infected with healthy people when inhaled [1]. In 2019, Indonesia had the third-highest TB cases in the global total. This case increases from 2015 to 2018, recognized by the World Health Organization [2]. Indonesian Ministry of Health (KEMENKES) reported that the disease's mortality rate is 13 people per hour.

Globally, as much as 3.4% of new TB cases had Multi Drug-Resistant TB (MDR-TB). In Indonesia, MDR-TB is rising with a high number of deaths [3]. MDR-TB is drug-resistant TB against at least 2 (two) of the most potent anti-TB drugs, namely INH and Rifampicin (RIF), together with resistance to other first-line TB drugs such as ethambutol, streptomycin, and pyrazinamide. The other type of drug-
resistant is Extensively Drug-Resistant TB, or XDR-TB is MDR-TB accompanied by immunity to second-line anti-TB drugs, namely the fluoroquinolone group and at least one injection of a second-line anti-TB drug such as kanamycin, amikacin, or capreomycin [4].

The incidence of RIF resistance is largely detected in Indonesia [5]. The inability of RIF causes this resistance to inhibit the cell division of M. tuberculosis [6]. M. tuberculosis is a Gram-positive bacterium and exhibits acid-resistant staining characteristics. The cell membrane sheath of M. tuberculosis comprises three components: the plasma membrane, walls, and capsule. The cell wall is resembling a layer of mycolic acid-lipid esters, arabinogalactants (AG), glycolipids, and peptidoglycan (PG), which play a role in physiology and pathogenicity [7]. PG consists of glycan strands of N-acetylglucosamine and N-acetyl / N-glycolylmuramic acids with tight interpeptide cross-linking. Therefore, the cell wall structure is impermeable and useful for M. tuberculosis to survive from phagocytic cells host cell immune system or antibiotics [8]. Therefore, the cell wall is becoming a target of antibacterial drugs [9].

The PG of the M. tuberculosis layer is encoded by a series of ripA and ripB genes [10]. The biosistronic operon encodes the ripA and ripB genes with the proximal ripA located in front of the promoter, both of which are controlled by the two components of the MtrAB system. These genes' role in cell wall physiology is unclear because no single mutant of the two genes has been studied. This study aims to evaluate the ripA gene sequence related to antibiotic resistance. We have used the N-terminal domain of ripA peptidoglycan hydrolase (Figure 1.). Evaluation of genes in samples can identify a mutation within ripA regions. Understanding these mutations implies an evaluation of antibiotic-resistant. Furthermore, this information implies local diagnostic and treatment guidelines to cell-wall targeting antibiotics.

![Figure 1](image-url).

**Figure 1.** ripA peptidoglycan hydrolase of *Mycobacterium tuberculosis*. The magenta color is the position of 257-471 (predicted by Protein Homology/analogy Recognition Engine V 2.0). The N-terminal domain was used in the study indicated by green color in the position of 3-219 (Protein Data Base ID. 6EWY).

2. Methods

2.1. Specimen collection

The *M. tuberculosis* isolates examined in this study isolated from the sputum sample. The sputum specimens were obtained from patients in Islam Jemursari Hospital in Surabaya (Indonesia) with TB diagnosis and had been through a Gram staining test. The diagnosis of TB was based on the medical record by the Pulmonologist Department of the hospital. M. tuberculosis H37Rv was used as a controlled laboratory in this evaluation.

2.2. Isolation of *Mycobacterium tuberculosis*

*M. tuberculosis* H37Rv isolates as a positive control and sputum were inoculated on Lowenstein – Jensen (LJ) agar slant. They were incubated in CO₂ atmosphere at 37°C for 30 days [11]. A single colony appears taken for isolation of DNA.
2.3. DNA extraction protocol

A bacterial colony that had grown was harvested by picked up and inoculated to 500 µl sterile aquadest. The suspension was centrifugated at 4,000 rpm at 4°C for 5 minutes. Furthermore, the pellets in the form of bacterial cells were washed with 0.9% sterile NaCl physiological solution. The pellets were then dissolved in a TE (Tris–EDTA) 0.01 M buffer, pH 8.0. The cell lysis process was carried out using 1/10 volume of lysozyme (10 mg/ml), incubated for 90 minutes at 37°C. DNA extraction was carried out with a saturated solution of Tris-Phenol/Chloroform-Isoamyl-Alcohol (1:1). The suspension was homogenized by shaking for 10 minutes and then centrifuged again. The supernatant obtained was then transferred to a sterile Eppendorf tube, and the same volume of chloroform solution was added. The tube is homogenized for 5 minutes. The water phase formed was separated by centrifugation at 10,000 rpm for 15 minutes. DNA was precipitated by adding 1/25 the volume of 5.0 M NaCl and 2.5 by volume of absolute cold ethanol. DNA pellets were washed twice with 70% ethanol, centrifuged in the above method for 5 minutes. The supernatant was removed, the DNA precipitate was dissolved into 10 mM TE buffer, pH 8.0. The concentration of isolated DNA was then measured quantitatively using a spectrophotometer using a wavelength of 260 nm. The isolated DNA was then running in gel electrophoresis to test its quality.

2.4. PCR and electrophoresis

The PCR was carried out using the ripA gene-specific primer [12]. A total of 2.5 µl of DNA samples were amplified in a mixture of 12.5 µl GoTaq® Green Master Mix, 1.0 µl of reverse primers (5’–CGGCGGATCACGTATTCAGA–3’) and 1.0 µl of forwarding primers (5’–ATTCCGTCGGCTTTGAGTGT–3’) and 8.0 µl Nuclease free water. Amplification was carried out in 45 cycles with a PCR temperature of 94° C for 1 minute (denaturation), 58° C for 1 minute (annealing), 72° C for 1 minute (elongation). The final phase of the thermal cycle incorporates an extended elongation period of 10 minutes.

DNA concentration measurements were carried out using a spectrophotometer (Shimadzu, UV-2600i). The concentration is determined by the formula \[ [DNA] = A_{260} \times 50 \times \text{dilution factor} \]

Where \( A_{260} \) = absorbance value at a wavelength of 260 nm; 50 = solution with an absorbance value of 1.0 proportional to 50 µg of the DNA double-strand/ml. DNA purity was determined by measuring the absorbance ratio (OD) at a wavelength of 260 and 280 [13].

The amplified DNA of \( M. \) \( \text{tuberculosis} \) was analyzed using agarose gel electrophoresis. Agarose gel was prepared using 1% (w / v) agarose dissolved in TBE buffer (0.09 M Tris-Borate, 0.2 mM EDTA, pH 8). A total of 20 µl of DNA solution and 2 µl of loading buffer (50% glycerol and 0.025% bromophenol blue) were added to agarose. The DNA marker used was the UC 18DNA marker (SIGMA) clanOXI74 DNA marker (PHARMACIA). Electrophoresis was carried out using an electric voltage of 100 volts for 60 minutes.

2.5. ripA gene sequencing and bioinformatic analysis

The amplified PCR products of ripA fragments were purified and sequenced at Macrogen Korea Laboratory sequencing through PT. Biotek Prima Indoplus, Indonesia. The sequencing data in the form of ABI files from the ripA gene were edited manually to ensure sequence accuracy and quality. The results of sequence editing were entered in the nucleotide BLAST (Basic Local Alignment Search Tool) web ((http://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI to see homology with the closest species. The sequences were aligned with Clustal Oemga (https://www.ebi.ac.uk/Tools/msa/clustalo/) using as a reference the genome of the strain \( M. \) \( \text{tuberculosis} \) H37Rv (GenBank, Access number NC_000962.3). Finally, we report the sequences of the ripA for isolates from clinical samples. In order to evaluate the mutation in the ripA gene, we analyzed the sequence based ripA gene alignment and position in the ripA structure.
3. Results and Discussion

3.1. Results

Figure 2. Multiple sequence alignment of ripA gene for N-terminal domain peptidoglycan hydrolase for clinical sample. Changes in the amino acid at several positions (Ala701Gly) are shown in the green square.

During the study period, four sputum specimens from TB patients were prepared in the national referral laboratory (Balai Besar Laboratorium Kesehatan Surabaya, BBLK). We found that the two sputa
only have grown in LJ medium. The other sputum could not observe. Probably this can cause by the poor quality of sputum. Budayanti et al. [14] reported that sputum's bad quality could lead to missed *M. tuberculosis*. A total of one sample was obtained from the patient and clinically categorized according to WHO guidelines [15] as new cases.

The PCR products for ripA genes produces targeted at 912 bp. The sequence data were confirmed for their identity using BLAST. The multiple sequence alignments for evaluated the mutation showed in Figure 2. The ripA gene sequences for the sample were compared to the established reference ripA gene of *M. tuberculosis* H37Rv. The ripA sample sequences covered of N-terminal domain in the ripA gene (Figure 1). ripA gene was found to have variation at codon 701 (Ala701Gly) in the clinical sample.

3.2. Discussion
The molecular biology studies carried out to investigated genetic basis in almost all clinical laboratories and research. It has been obtained several results related to mutation present in the ripA gene of *M. tuberculosis*. In this study, we have observed ripA gene mutation related to N-terminal dominant of ripA peptidoglycan hydrolase. This domain plays an important role in positioning the catalytic domain for peptidoglycan degradation [16]. A bioinformatics approach was used in this study to elucidate the effect of mutation in ripA gene. As we showed in Figure 2, sequencing was used to evaluation of mutation in *M. tuberculosis*.

The clinical sample showed a mutation in a defined region of Ala701Gly. Martinelli and Pavelka [17] reported that mutations in ripA gene generally result in increased susceptibility to a limited number of antibiotics and detergent. However, a specific mutation in codon 701 can result in a mutation of Alanin to Glycine and lead to sensitivity to existing β-lactam antibiotic. These results were further proved by Bhuwan et al. [18] that observed the deletion within N-terminal dominant of ripA peptidoglycan hydrolase lead to inhibition of export system in *M. tuberculosis*. This is caused by the localization of peptidoglycan hydrolase and results in sensitivity to existing β-lactam antibiotics.

In addition, the role of the ripA gene in *M. tuberculosis* is synthesis and cleavage of the cell wall polymer peptidoglycan (PG) [19]. Most bacteria possess a PG layers to the external permeability barrier. Figure 3. shows the schematic diagram of PG biosynthesis. Our analysis supported the hypothesis that genetic diversity in *M. tuberculosis* is important to understanding their pathogenicity, especially antibiotic resistance.

![Figure 3](image)

**Figure 3.** Model for ripA peptidoglycan hydrolase of *Mycobacterium tuberculosis* activation to cleave the peptidoglycan in daughter cell (mycobacterial cytokinesis).
Nowadays, many genetic techniques are conducted and reported to help identify *M. tuberculosis*. This is due to *M. tuberculosis* has spread worldwide, and the mutation rate has led to an increase during this decade. Therefore, the assay could be detecting a mutation is connected to antibiotic resistance. However, the utility of molecular diagnosis techniques related to the appropriate information about the unprecedented rate of any given mutation in the antibiotic target.

### 4. Conclusion

The ripA primer produces a well-amplified ripA gene on N-terminal domain peptidoglycan hydrolase. The nucleotide sequence obtained for the isolate was approximately 912 bp, sufficient for NCBI-BLAST searches and multiple sequence alignment evaluation. The least mutation was detected among several dozen amino acid mutations identified in the ripA region. Only a few mutations are directly conscientious for *M. tuberculosis's* resistance to rifampicin. Further evaluation of the ripA domain is required to evaluate the antibiotic-resistant. Furthermore, this information implies local diagnostic and treatment guidelines to cell-wall targeting antibiotics.

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### References

[1] Patterson B, Koch A, Gessner S, Dinkele R, Gqada M, Bryden W, Cobelens F, Little F, Warner D F and Wood R 2020 *BMC Inf. Dis.* **20** 587

[2] World Health Organization 2019 *Global Tuberculosis Rep*. WHO/CDS/TB/2019.15.

[3] Dewi D N S S, Mertaniasih N M and Soedarsono 2020 *Afr. J. Infec. Dis.* **14** 8

[4] Nahid P, Mase SR, Migliori GB, Sotgiu G, Bothamley GH, Brozek J L, Cegielski J P, Chen L, Daley C L and Dalton T L 2019 *ATS J.* **200** e93

[5] Soeroto A Y, Lestari B W, Santoso P, Andriyoko L C, Alisjahbana B, Crevel R and Hill P C 2019 *PLoS ONE* **14** e0213017

[6] Zhu J H, Wang B W, Pan M, Zeng Y N, Rego H and Javid B 2018 *Nat. Commun.* **9** 4218

[7] Botella H, Vaubourgeix J, Lee M H, Song N, Xu W, Makinoshila H, Glickman M S and Ehrt S 2017 *EMBO J.* **36** 536

[8] Vincent A T, Nyongesa S, Morneau I, Reed M B, Tocheva E I and Veyrier F J 2018 *Front. Microbiol.* **9** 2341

[9] Chao M C, Kieser K J, Minami S, Mavrici D, Aldridge BB, Fortune S M, Alber T and Rubin E J 2013 *PloS Pathogen* **9** e1003197

[10] Martinelli D J and Pavelka M S 2016 *J. Bacteriol* **198** 1464

[11] Palange P, Narang R and Kandi V 2016 *Cureus* **8** e757

[12] Koentjoro M P and Prasetyo E N 2019 Pros. Sem. Nas. Tek. Sains. ISBN:978–623–91277–6–3 41

[13] Sambrook E F, Fritsch E F and Maniatis T 1989 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

[14] Budayanti N S, Suryawan K, Iswari I S and Sukrama D M 2020 *Front. Med.* **6** 64

[15] World Health Organization 2017 *TB Manual National Tuberculosis Programme Guideline* (Warsaw: WHO Europe)

[16] Steiner E M, Lyngso J, Guy J E, Bourenkov G, Lindqvist Y, Scheider T R, Pedersen J S, Schneider G and Schnell R 2018 *Proteins* **86** 912

[17] Martinelli D J and Pavelka M S 2016 *J. Bacterio.* **198** 1464

[18] Bhuwan M, Arora N, Sharma A, Khubaib M, Pandey S, Chaudhuri T K, Hasnain S E and Ehtesham N Z 2016 *mBio.* **7** e02259

[19] Healy C, Gouzy A, and Ehrt S 2020 *mBio.* **11** e03315