Mutation Of Gyra Gene Found In Mycobacterium Leprae From Leprosy Patient In West Papua And Papua, Indonesia

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ABSTRACT

Leprosy is still a public health problem in Indonesia especially in Eastern Part of Indonesia. In the midst of our effort to combat leprosy, drugs resistance was reported in some endemic areas of Indonesia. Drug resistance surveillance and typing strains of Mycobacterium leprae are necessities to magnitude our effort reaching leprosy elimination. gyrA gene is associated with ofloxacin antibiotic, the second line of leprosy treatment after standard Multi Drugs Therapy (MDT). In this study we aim to reveal our finding of mutations in the gyrA gene M. leprae from leprosy patients in West Papua and Papua provinces.

Bacterial samples were collected from slit skin smear and extracted using Qiaamp mini DNA extraction kit. gyrA amplification was carried out using GoTaq Green Mastermix. Sanger sequencing was done using BigDye Terminator v3.1. Alignment analysis was performed with M. leprae TN as the referral strain. The phylogenetic tree was constructed using Mega 7 to get the M. leprae gyrA cluster. The RNAalifold server was employed to generate the conserved 2D structure for the gyrA multiple sequence alignment (MSAs). Six variants were found in the gyrA M. leprae obtained from the provinces of West Papua and Papua provinces. Bacterial samples were collected from slit skin smear and extracted using Qiaamp mini DNA extraction kit. gyrA amplification was carried out using GoTaq Green Mastermix. Sanger sequencing was done using BigDye Terminator v3.1. Alignment analysis was performed with M. leprae TN as the referral strain. The phylogenetic tree was constructed using Mega 7 to get the M. leprae gyrA cluster. The RNAalifold server was employed to generate the conserved 2D structure for the gyrA multiple sequence alignment (MSAs). Six variants were found in the gyrA M. leprae obtained from the provinces of West Papua and Papua provinces. The six variants are H71R, K73R, D95G, A101T, R107W, A127V. The existence of mutations in the gyrA M. leprae gene found in this study can be information in the treatment of leprosy in Papua if using Ofloxacin as an alternative treatment. Based on phylogenetic analysis found there are three distinct clusters of gyrA gene. The five variants are H71R, K73R, A101T, R107W, A127V are new variant of gyrA M. leprae. The D95G variant has been confirmed to cause resistance to Fluoroquinolone by in vitro methods, while the H71R, K73R, A101T, R107W, A127V variants are new variants. Based on the impact mutation analysis, the H71R mutants had moderate impact, while the K73R, A101T, R107W, A127V mutants had moderate impact. However, an in vivo test is required to validate these results.

Key words: Leprosy, gyrA, ofloxacin, Mycobacterium leprae

INTRODUCTION

Leprosy is an ancient disease that still becomes a public health problem in many countries including Indonesia. Many constraints in combating leprosy in Indonesia. Beside the late case finding that causes delay therapy and disability, drug resistance is also the major issue of leprosy program (WHO, 2018). Complete treatment using multidrug therapy (MDT) is the key to reducing the leprosy case burden. MDT consists of rifampicin, dapsone and clofazimine (WHO, 2018). MDT has decreased the number
of cases after treatment but has had little impact on the emergence of new cases (WHO, 2020).

Increases in relapse case and mutation in *M. leprae* were reported (Cambau et al., 2018). *M. leprae* is one of the bacteria that cannot grow in vitro (Cambau, Chauffour-Nevejans, Tejmar-Kolar, Matsuoka, & Jarlier, 2012). Resistance to anti-leprosy such as Rifampicin, Dapsone and fluoroquinolones in vivo has been explained since 1967. However, this method requires a long time and expensive cost (Cambau et al., 2012; Shepard, 1960). Since 2011, several countries have begun to detect anti-leprosy drug resistance in patients with both new and relapsed cases. Antimicrobial resistance (AMR) is one of the keys in the intervention of leprosy globally. The availability of resistance data makes it possible to monitor trends of drug resistance from time to time in new or cured cases of leprosy (WHO, 2017a).

Drug resistance surveillance and strain typing of *M. leprae* are useful molecular tools for leprosy control (Li et al., 2012). In 2008 the WHO recommended guidelines for a global disease survey on drug resistance by *M. leprae* using PCR sequencing. The guidelines contain DNA isolation from skin biopsies of multi-bacillary relapse patients (MB), PCR amplification of targeted DNA fragments containing drug resistance determining regions (DRDRs) from *M. leprae* using specific primers, sequencing, and alignment with DRDR *M. leprae* TN strain sequence references (NC_002677.1 GenBank) to determine the presence of drug resistance mutations (Williams and Gillis, 2012b). The genes that were targeted in the detection of *M. leprae* drug resistance were *folP1* for dapsone resistance, *rpoB* for rifampicin and *gyrA* for ofloxacin (Cambau et al., 2018; WHO, 2017a). Fluoroquinolone resistance to mutations in the *gyrA* gene encoding the subunit A of DNA gyrase (Cambau et al., 1997) (Willby, Sikes, Malik, Metchock, & Posey, 2015).

In order to assess the severity of the drug resistance mutation, bioinformatics tools will be employed to determine the clustering of the mutation, and its impact to the integrity of the mRNA structure. The phylogenetic tree-based clustering has been employed in various localities of infection sites, and provides insight on the diagnostics tool’s developments (Akand & Downard, 2017; Chen et al., 2013). Moreover, the observation on how the mutation will affect the 2D structure of the mRNA should be observed because any structural changes in the mRNA will possibly directed structural changes in the protein (Hofacker & Stadler, 1999; Hofacker, Fekete, & Stadler, 2002).

In this study we reveal our finding of the mutation in the gene *gyrA* to the molecular modeling of the 2D structure of the *gyrA* mRNA from *M. leprae* samples of 2 biggest leprosy pockets in Indonesia, Papua and West Papua. It is expected that the structural annotation will provide insight on the biochemical repertoire of the infection severity.

**MATERIAL AND METHODS**

**Study design**

This is descriptive study using molecular approach to analyse gene mutation of *gyrA* that associated with Ofloxacin susceptibility of leprosy.

**Population and sample collection**

Population of this study is leprosy patients that are actively and passively recruited from our study sites, South Manokwari (West Papua Province) and Asmat (Papua Province). The inclusion criteria’s were: diagnosed leprosy patient and voluntary involved in study by signing the informed consent. Exclusion criteria was patients have other severe diseases.
Specimen collection

Bacterial DNA was collected from slit skin smear from ear loop incision specimens that preserve in a 500 µL PBS buffer.

Deoxyribonucleic (DNA) extraction

Bacterial DNA was extracted using the DNA Qiaamp mini column (Qiagen-Germany). Bacterial DNA extraction was performed using a manual instruction from the kit. Two hundred µL of slit skin smear specimens in PBS buffer was used and eluted in 50 µL of DNA yield.

Amplification of gyrA M. leprae gene

gyrA amplification was performed referring to the procedure of Kai et.al (Kai et al., 2011) using GoTaq Green MasterMix 2X. Polymerase Chain Reaction master mix were GoTaq Green MasterMix 2X, set primers (f G3 5’-GATGGTCTCAAACCGGTACA-3’ as a forward primer and G4 5’-CCCAAATAGCAACCTCACCA-3’ as reverse primers) 10 pmol, DNA template 5 µL and nuclease free water. Amplification reaction was done using Thermo machine with the cycles and temperature set up as follow: pre-denaturation at 94°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 74°C during 10 minutes. The cycle was repeated for 40 cycles. Visualization of DNA was perform in 5% agarose gel. Furtherly, 38 samples among 200 show adequate results for sequencing.

Sequencing

The PCR result was purified by ExoSap IT with a ratio of 2:5. The purified PCR product was followed by a sequencing process. The sequencing cycle uses BigDye Terminator v3.1 5X, BigDye Terminator buffer 5X, 1µL molded DNA, and primer 0.8 pmol. DNA pGEM -3Zf was used as a positive control and primary control -21 M13 as a positive control primer. The reaction of the sequencing cycle was carried out under conditions: 96°C 1 min, 96°C 10 sec, 50°C 5 sec, 60 °C 4 min. The cycle was repeated 25 times later the result of the sequencing cycle is purified by XTerminator Solution and SAM solution 5:22.5. The sample volume used is 5 µL. The tube contained of premix got vortexed sample for 30 minutes then in the centrifuge for 2 minutes. The supernatant was inserted into a 20 µl wellbore slab and read by using 3500 Genetic Analyzer. The sequencing results were then processed in the gene bank to identify the presence of mutations in the gyrA gene.

Sequenced analysis and protein modeling

The bioinformatics pipeline is processed in accordance to the established pipelines (Parikesit, 2018; Parikesit & Nurdiansyah, 2020). The refseq sequence of the gyrA gene was downloaded from the genbank (Accession ID: X87124.1)The ClustalX software was employed to generate multiple sequence alignment and phylogenetic tree with the following parameters adjusted: Trees Clustering Algorithm → Neighbour Joining (Thompson, Gibson, & Higgins, 2002). The NJ was picked because it is more accurate in predicting molecular clock than UPGMA. Bootstrap trials: 1000. Random number generator seed: 111. Iteration: Iterate final alignment. The output formats are CLUSTAL, PHYLIP, and NEXUS. The tree was visualized with MEGAX software (Kumar, Stecher, & Tamura, 2016). Thus, the RNAalifold server was employed to generate the conserved 2D structure for the gyrA MSAs (Bernhart et al., 2008). The adjusted parameters are
RNAalifold version: new RNAalifold with RIBOSUM scoring. Fold algorithms and basic options: minimum free energy (MFE) and partition function, avoid isolated base pairs. Lastly, the RNA fold was employed to annotate the 2D structures for gyrA refseq (Gruber et al., 2008; Lorenz et al., 2011). Parameters: Fold algorithms and basic options: minimum free energy (MFE) and partition function, avoid isolated base pairs. Result: The refseq structure is radically different from the RNAalifold result.

**Effect of mutations on ofloxacin**

The impact of each mutation was analyzed using Hansen’s Disease Antimicrobial Resistance Profiles (HARP) (Vedithi et al., 2020).

**Ethical approvement**

This research has received approval from the Ethics Committee of Research and Development of Health Ministry of Health RI.

**RESULT AND DISCUSSION**

Total of 32 among 200 samples were successfully amplified for gyrA gene and adequate for further molecular analysis. The characteristics of samples were performed in table 1.

Table. 1 The characteristics of samples

| No | Code | Site     | Age | Sexes | Ethnic | Classification |
|----|------|----------|-----|-------|--------|----------------|
| 1  | 1A   | West Papua | 42  | 1     | Papuan | MB             |
| 2  | 2A   | West Papua | 43  | 1     | Papuan | MB             |
| 3  | 3A   | West Papua | 12  | 2     | Papuan | PB             |
| 4  | 4A   | West Papua | 18  | 1     | Papuan | MB             |
| 5  | 5A   | West Papua | 21  | 1     | Papuan | MB             |
| 6  | 6A   | Papua     | 6   | 1     | Papuan | MB             |
| 7  | 7A   | Papua     | 8   | 1     | Papuan | MB             |
| 8  | 8A   | Papua     | 6   | 2     | Papuan | MB             |
| 9  | 9A   | Papua     | 10  | 1     | Papuan | MB             |
| 10 | 10A  | Papua     | 8   | 1     | Papuan | MB             |
| 11 | 11A  | Papua     | 5   | 1     | Papuan | PB             |
| 12 | 12A  | Papua     | 25  | 2     | Papuan | PB             |
| 13 | 13A  | Papua     | 40  | 1     | Papuan | PB             |
| 14 | 14A  | Papua     | 40  | 1     | Papuan | MB             |
| 15 | 15A  | Papua     | 21  | 1     | Papuan | MB             |
| 16 | 16A  | Papua     | 50  | 1     | Papuan | MB             |
| 17 | 17A  | Papua     | 7   | 2     | Papuan | PB             |
| 18 | 18A  | Papua     | 8   | 2     | Papuan | PB             |
| 19 | 19A  | Papua     | 10  | 2     | Papuan | PB             |
| 20 | 20A  | Papua     | 7   | 2     | Papuan | MB             |
| 21 | 21A  | Papua     | 7   | 2     | Papuan | PB             |
| 22 | 22A  | Papua     | 8   | 1     | Papuan | MB             |
| 23 | 23A  | Papua     | 8   | 1     | Papuan | PB             |
| 24 | 24A  | Papua     | 8   | 1     | Papuan | PB             |
| 25 | 25A  | Papua     | 9   | 2     | Papuan | MB             |
| 26 | 26A  | Papua     | 8   | 1     | Papuan | MB             |
| 27 | 27A  | Papua     | 10  | 1     | Papuan | MB             |
Drugs Resistance Surveillance is one of WHO recommendations to high endemic countries as one of the efforts towards leprosy elimination. Multi Drugs Therapy (MDT) const of rifampicin, clofazimine and dapsone were supplied by WHO to all of the countries that reported leprosy cases. It is very effective in releasing many countries from leprosy endemicity. In this elimination era, an increase of relapse cases and mutation are reported (WHO, 2017b).

Ofloxacin is a second-line anti-leprosy drug (Hargrave, Wallace, & Lush, 2010). A single dose of ofloxacin has been used in several countries such as India, Bangladesh and Brazil (Hargrave et al., 2010). The limitation of surveillance coverage in countries with high leprosy burden such as India and Brazil has become a concern because of the potential risk of resistance to MD (Cambau et al., 2018). This should also be a concern in Indonesia, especially provinces with a high burden of leprosy such as West Papua and West Papua. This research has an important role in ensuring the effectiveness of the use of ofloxacin if it becomes an alternative treatment for leprosy in Papua and West Papua Provinces. There are several considerations for the use of rifampicin and dapsone as the first line for certain cases such as the presence of co-existent leprosy with active TB disease (Hargrave et al., 2010), Dapsone Hypersensitivity Syndrome (DHS). In addition, there were genetic variations in the genes responsible for Rifampin and Dapsone resistance. We have found SNPs in the rpoB M. leprae gene in strains from Papua (unpublished data). In the folP1 gene, several variations were found, such as V39I and V48A (Maladan et al., 2019).

The PCR-positive samples were then sequenced by the nucleotides to identify mutations. Sequencing results showed that the gyrA M. leprae gene obtained from West Papua and Papua contained several mutation points (Table 2). In this study, we found several mutations in the gyrA M. leprae gene. There are 6 mutation points found, namely H71R, K73R, D95G, A101T, R107W, A127V. There were also several nonsense mutations (Table 1). Changes in nucleotides and amino acids were carried out by aligning the results of sequencing with the reference sequences of M. leprae TN.

Table 2. Mutations in the gyrA M. leprae gene. There are six variants in the gyrA M. leprae gene derived from West Papua and Papua. The six variants are H71R, K73R, D95G, A101T, R107W, A127V.

| Sample Code | Result of DNA sequencing | Substituted Amino Acid | Origin |
|-------------|--------------------------|------------------------|--------|
| 7A          | nucleotide 218 (AAG→AGG) | K73R                   | Papua  |
| 8A          | nucleotide 351 (TTC→TTT), nucleotide 380 (GGG→GTG) | nonsense mutation, nonsense mutation | Papua  |
| 13A         | nucleotide 288 (ACG→ACA) | nonsense mutation       | Papua  |
| 14A         | nucleotide 181 (TTA→CTA) | nonsense mutation       | Papua  |
| 22          | nucleotide 319 (CGG→TGG), nucleotide 212 (CAC→CGC), nucleotide 276 (TCG→TCA) | R107W, H71R, nonsense mutation | Papua  |
| 24          |                          |                        |        |
The result of sequence alignment shows that there are 7 samples containing mutations (Figure 1). In sample 8A it contained two mutation points namely nucleotide 351 (TTC→TTT) and nucleotide 380 (GCG→GTG). Sample 24 contains three mutation points namely nucleotide 212 (CAC→CGC), nucleotide 276 (TCG→TCA), nucleotide 301 (GCG→ACG) nucleotide 192 (GGT→GGC). Seventh samples containing mutations were obtained from Asmat district, Papua. Asmat Regency is one of the leprosy pockets in Papua. In the meantime, we did not find any mutations in samples originating from West Papua.

| Nucleotide | Mutation          | Location |
|------------|-------------------|----------|
| 301        | (GCG→ACG)        | A101T    |
| 192        | (GGT→GGC)        | D95G     |
| 285        | (GAC→GGC)        |          |
| 351        | (TTC→TTT)        |          |
| 380        | (GCG→GTG)        |          |

Some of the most common mutations in the gyrase M. leprae found previously are A91V (Shepard, 1960; Williams & Gillis, 2012; You et al., 2005). In addition to the A91V mutation, the G389C mutation is a cause of resistance to ofloxacin (Maeda et al., 2001; Veziris et al., 2013). Changes in the composition of nucleotides due to mutations cause
changes in the composition of amino acids in the protein encoded by the *gyrA* *M. leprae* gene (Figure 2).

**Figure 2.** Alignment of *gyrA* *M. leprae* sequencing results with reference sequences (*M. leprae* TN) using Bioedit software. There are six mutations in the nucleotide causing changes in the amino acid arrangement of *gyrA* *M. leprae* gene and five mutations do not cause changes in amino acids (nonsense mutation). AAGAGG (K73R), TTCTTT (nonsense mutation), GCGGTG (A127V), ACGACA (nonsense mutation), TTACTA (nonsense mutation), CGGTGG (R107W), CACCGC (H71R), TCGTCA (nonsense mutation), GCGACG (A101T), GGTGGC (nonsense mutation), GACGCC (D95G). There is one sample containing two mutations in *gyrA* (H71R dan A101T) i.e sample no. 24.

Variants H71R, K73R, A101T, R107W, A127V found in this study have never been reported before. Whereas the D95G variant has been confirmed to cause resistance to Fluoroquinolone by *in vitro* methods. Amino acid substitution from Asp to Gly or Asn at position 95 adds a higher resistance to DNA gyrase compared to from Ala to Val at position 91 (Yokoyama et al., 2012). It is of concern as happened in India where a number of new patients with MDR-TB are also resistant to ofloxacin (Selvakumar et al., 2015). The discovery of mutations in *gyrA* *M. leprae* from a new casus casein was also found in China (Chokkakula et al., 2019).

Not all mutations in the *M. leprae* DRDR region cause resistance to ofloxacin (Matrat et al., 2008). Thus, variants H71R, K73R, A101T, R107W, A127V are necessary to study the effects of these mutations on ofloxacin. The bioinformation approach is one solution to predict the effects of these mutations. The plausible solution is the development of the siRNA-based drug, but the wet experiment to support that is limited and expensive. RNA molecule tends to be unstable as well in the experimental assay.

Based on the figure 3A, there are three distinct clusters of *gyrA* gene. Epidemiological data is necessary to determine the clustering of the localities. It was found 3 clusters. Standard and time cohort trees were elucidated. The figure 3B is necessary to show the origin of the infection or the molecular clock. The sequences of the *gyrA* genes were found to elicit 3 clusters, and show tendency of developing novel strains.
**Figure 3.** The phylogenetic trees of the gyrA gene sequences of the patients. A) the standard clustering tree, and the colored circles show the distinct variants of the gyrA gene. B) The time cohort trees

In the figure 4A, the structure is bizarre because it formed a very strained bulge that in accordance to the thermodynamic law will require huge activation energy to exist, and strong steric effect observed. The structure is not plausible. In comparison with 4B, the structure in the 4A is definitely not plausible. It advises that the mutations have created a very diverged structural variation that could affect the translation of the protein. **The translated proteins could be very diverse as well.** This structural diversity is one of the explanations why antibiotic resistance strains of the *M. leprae* occurred.
Figure 4. The 2D structures of the mRNA for the gyrA gene. A) The conserved structure of the gyrA from the patients. B) The refseq structure from the gene bank (Accession ID: X87124.1)

Moreover, it is also found that the structural diversity of the gyrA mRNA show that it is difficult to develop a silencing (si)RNA-based inhibitor to deter the bacterial genes. Whether this is a result of the antibiotic resistant gene expression, more experimental validation will be necessary, especially using the microarray assay.

gyrA encodes the formation of DNA gyrase which plays a role in the topological regulation of DNA. The presence of certain mutations in these genes can cause resistance to ofloxacin (Piton et al., 2010). The position comparison between wild type residues and mutants in the 3D structure was obtained from the HARP database (Figure 5). A91V resistant mutant were used as comparators in the analysis of the mutation effect of ofloxacin. The A91V mutation was close to the binding cavity of the DNA gyrase (Figure 5A) at a distance of 3.5 Å (Table 3). These mutations cause destabilizing of the protein and have a high impact overall. The H71R and D95G mutants also have high impact proteins (Table 3) so that they may cause resistance to ofloxacin. The K73R, A127V, R107W and A101T mutants have long distances from the binding cavity ofloxacin (Figure 5, 18.1 Å, 12.3 Å, 23.6 Å, 17.3 Å respectively (Table 3). Overall, the K73R, A127V, R107W and A101T has a moderate impact on protein, possibly leading to decreased effectiveness of ofloxacin.
Figure 5. Comparison of the positions of wild type and mutant residues in the 3D structure of *M. leprae* gyrase DNA. The A91V mutant was used as a comparison against the mutants obtained
The existence of mutations in the gyrA *M. leprae* gene found in this study can be information in the treatment of leprosy in Papua if using Ofloxacin as an alternative treatment. Apart from this the existence of mutations also provides information to increase alertness and increase patient compliance during the treatment process to avoid the emergence of strains that are resistant to anti-leprosy drugs.

**CONCLUSION**

We found six variants in the *M. leprae* gyrA gene obtained from Papua and West

| No | Mutation | Distance from Ofloxacin (Å) | mCSM (ΔΔG) Result (Kcal/mol) | Impact | Encom ΔΔS<sub> vib </sub> Predictions: Result (Kcal/mol/K) | Impact | Overall Impact of the Mutation | PUBMED Reference for the Mutation (PMID): |
|----|----------|---------------------------|-----------------------------|--------|-----------------------------------|--------|-------------------------------|-----------------------------------------|
| 1  | A91V     | 3.5                       | -0.322                      | Destabilizing | 0.068                             |        | Increase in Molecular Flexibility | High Impact                             | 23356028                                |
| 2  | K73R     | 18.1                      | -0.281                      | Destabilizing | -0.391                            |        | Decrease in Molecular Flexibility | Moderate Impact                         | No                                      |
| 3  | A127V    | 12.3                      | -0.288                      | Destabilizing | -0.006                            |        | Decrease in Molecular Flexibility | Moderate Impact                         | No                                      |
| 4  | R107W    | 23.6                      | -0.589                      | Destabilizing | -0.316                            |        | Decrease in Molecular Flexibility | Moderate Impact                         | No                                      |
| 5  | H71R     | 23.4                      | -0.717                      | Destabilizing | 0.18                              |        | Increase in Molecular Flexibility | High Impact                             | No                                      |
| 6  | A101T    | 17.3                      | -1.445                      | Destabilizing | -0.27                             |        | Decrease in Molecular Flexibility | Moderate Impact                         | No                                      |
| 7  | D95G     | 4.8                       | -0.993                      | Destabilizing | 0.146                             |        | Increase in Molecular Flexibility | High Impact                             | No                                      |
Papua. The six variants are H71R, K73R, D95G, A101T, R107W, A127V. The D95G variant has been confirmed to cause resistance to Fluoroquinolone by in vitro methods, while the H71R, K73R, A101T, R107W, A127V variants are new variants. Based on the impact mutation analysis, the H71R mutants had moderate impact, while the K73R, A101T, R107W, A127V mutants had moderate impact. However, an in vivo test is required to validate these results.

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