Indonesian Journal of Pharmacy

VOL 32 (1) 2021: 35–42 | RESEARCH ARTICLE

Mutations of *gyrA* Gene in *Mycobacterium leprae* from Leprosy Patients in West Papua and Papua, Indonesia

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Info Article

Submitted: 16-10-2020
Revised: 22-12-2020
Accepted: 26-01-2021

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ABSTRACT

Leprosy is still a public health problem in Indonesia especially in the Eastern part of Indonesia. In the midst of our effort to combat leprosy, drug resistance was reported in some endemic areas of Indonesia. Drug resistance surveillance and strain typing of *Mycobacterium leprae* are necessary to magnitude our effort to eliminate leprosy. *gyrA* gene is associated with ofloxacin antibiotic, the second line of leprosy treatment after the standard Multi Drugs Therapy (MDT). In this study, we aimed to reveal our findings on the mutations on the *gyrA* gene of *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 of *gyrA M. leprae* were found from West Papua and Papua provinces. The six variants are H71R, K73R, D95G, A101T, R107W, A127V. The existence of mutations in the *gyrA* gene of *M. leprae* found in this study can be information for the treatment of leprosy in Papua if ofloxacin is used as an alternative treatment. Based on the phylogenetic analysis, there are three distinct clusters of the *gyrA* gene. The D95G variant has been confirmed to cause resistance to fluoroquinolone *in vitro*, while the H71R, K73R, A101T, R107W, A127V variants are new variants. Based on the analysis of the mutation impact, the H71R, K73R, A101T, R107W, A127V mutants had moderate impacts. However, an *in vivo* test is required to validate these results.

Keywords: 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. Besides the late case finding that causes delayed therapy and disability, drug resistance is also a major issue in the 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 cases and mutation in *M. leprae* were reported (Cambau *et al*., 2018). *M. leprae* is one of the bacteria that cannot grow *in vitro* (Cambau, *et al*., 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 to 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 the global disease survey on drug resistance by *M. leprae* using PCR sequencing. The guidelines include 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 is related to mutations in the *gyrA* gene encoding the subunit A of DNA gyrase (Cambau *et al*., 1997) (Willby, *et al*., 2015). In order to assess the severity of the drug resistance-associated mutation, bioinformatic tools can be employed to determine the clustering of the mutation, and its impact on the integrity of the mRNA structure. The phylogenetic tree-based clustering has been employed in various localities of infection sites and provides insight into 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 direct structural changes in the protein (Hofacker & Stadler, 1999; Hofacker, *et al*., 2002). In this study, we reveal our finding of the mutation in the *gyrA* gene to the molecular modeling of the 2D structure of the *gyrA* mRNA from *M. leprae* samples of the 2 biggest leprosy pockets in Indonesia, Papua and West Papua. It is expected that the structural annotation will provide insight into the biochemical repertoire of the infection severity.

MATERIAL AND METHODS

Study design

This is a descriptive study using a molecular approach to analyze gene mutation of *gyrA* that is associated with ofloxacin susceptibility of leprosy.

Population and sample collection

The population of this study is leprosy patients that are actively and passively recruited from our study sites in South Manokwari (West Papua Province) and Asmat (Papua Province). The inclusion criteria were: leprosy-diagnosed patient and voluntary involvement in the study by signing the informed consent. The exclusion criteria were patients having other severe diseases.

Specimen collection

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

Deoxyribonucleic (DNA) extraction

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

Amplification of *gyrA M. leprae* gene

gyrA amplification was performed according to the procedure of Kai et.al (Kai *et al*., 2011) using GoTaq Green MasterMix 2X. The polymerase chain reaction master mix consisted of 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. The amplification reaction was done using a Thermocycling machine with the cycles and temperature set up as follows: pre-denaturation at 94°C for 5min, denaturation at 95°C for 1min, annealing at 60°C for 30s, extension at 72°C for 1min, and a final extension at 74°C for 10min. The cycle was repeated for 40 cycles. Visualization of DNA was performed in 5% agarose gel. Furtherly, 38 samples among 200 showed adequate results for sequencing.

Sequencing

The PCR result was purified by ExoSap IT with a ratio of 2:5. The purified PCR product was the subjected to the sequencing process. The sequencing cycle used BigDye Terminator v3.1 5X, BigDye Terminator buffer 5X, 1µL molded DNA, and primer 0,8pmol. DNA pGEM -3Zf was used as a positive control and primary control -21 M13 was used as a positive control primer. The reaction of the sequencing cycle was carried out under conditions: 96°C 1min, 96°C 10s, 50°C 5s, 60°C 4min.

Table I. 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
28	28A	Papua	8	1	Papuan	PB
29	29A	Papua	11	2	Papuan	MB
30	30A	Papua	12	1	Papuan	PB
31	31A	Papua	69	2	Papuan	MB
32	32A	Papua	30	2	Papuan	MB

The cycle was repeated 25 times and the result of the sequencing cycle was purified by XTerminator Solution and SAM solution 5:22,5. The sample volume used was 5µL. The tube containing the premix solution was vortexed for 30min, then was centrifuged for 2min. The supernatant was inserted into a 20µL wellbore slab and read 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 with 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 a phylogenetic tree with the following parameters adjusted: Trees Clustering Algorithm → Neighbour Joining (Thompson, *et al*., 2002). The Neighbour Joining (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, *et al*., 2016). 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.

Table II. Mutations in the *gyrA M. leprae* gene. There are six variants in the *gyrA M. leprae* gene derived from cases in 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),	*nonsense mutation*	Papua
	nucleotide 380 (GCG→GTG)	A127V	Papua
13A	nucleotide 288 (ACG→ACA)	*nonsense mutation*	Papua
14A	nucleotide 181 (TTA→CTA)	*nonsense mutation*	Papua
22	nucleotide 319 (CGG→TGG)	R107W	Papua
24	nucleotide 212 (CAC→CGC),	H71R	Papua
	nucleotide 276 (TCG→TCA),	*nonsense mutation*	Papua
	nucleotide 301 (GCG→ACG)	A101T	Papua
31	nucleotide 192 (GGT→GGC),	*nonsense mutation*	Papua
	nucleotide 285 (GAC→GGC)	D95G	Papua

Result: The RefSeq structure was 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 Indonesia.

RESULT AND DISCUSSION

A total of 32 among 200 samples were successfully amplified for the *gyrA* gene and adequate for further molecular analysis. The characteristics of samples (Table I).

Drugs Resistance Surveillance is one of WHO recommendations to high endemic countries as one of the efforts towards leprosy elimination. Multi Drugs Therapy (MDT) consisting 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, *et al*., 2010). Single-dose 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 burdens 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 in 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 co-existence of leprosy with active TB disease (Hargrave *et al*., 2010) and 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 II). 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 I). Changes in nucleotides and amino acids were carried out by aligning the results of sequencing with the reference sequences of *M. leprae* TN.

The result of sequence alignment shows that there are 7 samples containing mutations (Figure 1). Sample 8A contained two mutation points namely nucleotide 351 (TTC→TTT) and nucleotide 380 (GCG→GTG). Sample 24 contained three mutation points namely nucleotide 212 (CAC→CGC), nucleotide 276 (TCG→TCA), nucleotide 301 (GCG→ACG) nucleotide 192 (GGT→GGC).

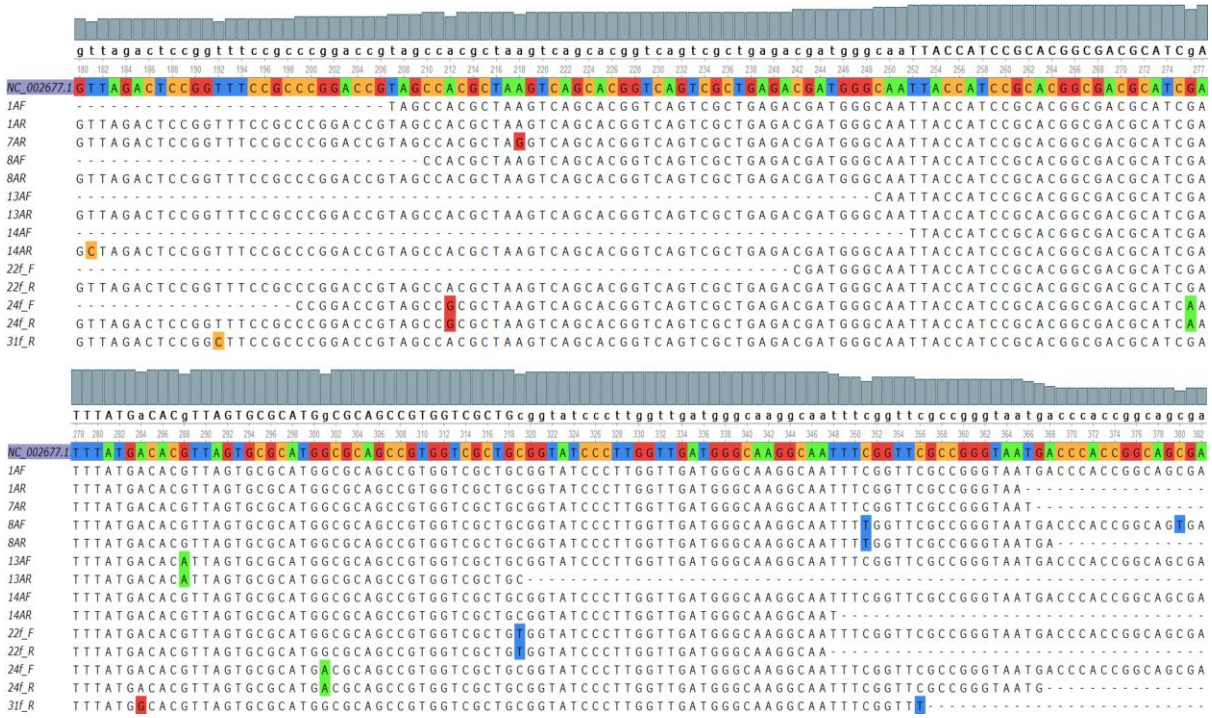

Figure 1. Alignment results of *gyrA M. leprae* sequencing with reference sequences (*M. leprae TN*) using the UgeneUnipro Software (Aligment using MUSCLE). The position of the nucleotides undergoing change are *nucleotide* 218 (AAGAGG), *nucleotide* 351 (TTCTTT), *nucleotide* 380 (GCGGTG), *nucleotide* 288 (ACGACA), *nucleotide* 181 (TTACTA), *nucleotide* 319 (CGGTGG), *nucleotide*212 (CACCGC), *nucleotide* 276 (TCGTCA) *nucleotide* 301 (GCGACG), *nucleotide*192 (GGTGGC), *nucleotide* 285 (GACGGC).

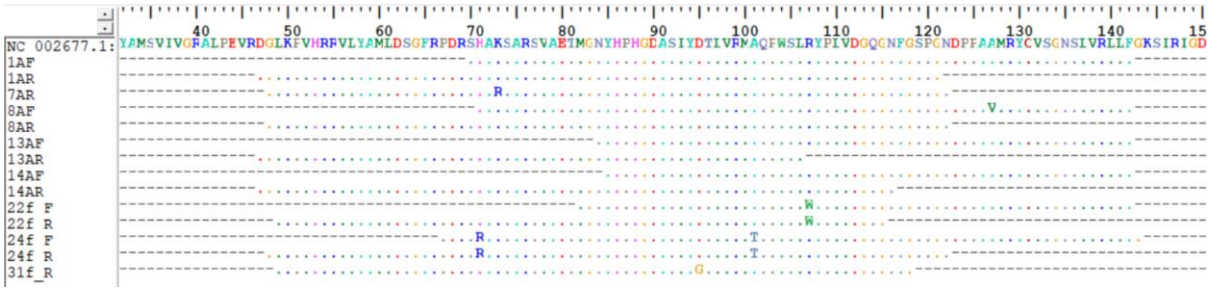

Figure 2. Alignment of gyrA *M. leprae* sequencing results with reference sequences (*M.leprae* TN) using Bioedit software. There are six mutations in the nucleotide causing changes in the amino acid arrangement of gyrA *M. leprae* gene and five mutations that did not cause changes in amino acids (nonsense 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), GACGGC (D95G). There is one sample containing two mutations in gyrA (H71R and A101T), i,e, sample no. 24.

Seventh samples containing mutations were obtained from the 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.

Some of the most common mutations in the *gyrA M. leprae* found previously are A91V (Shepard, 1960; Williams and 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).

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. An amino acid substitution from Asp to Gly or Asn at position 95 adds a higher resistance to DNA gyrase compared to the change 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 case 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. Bioinformatic approach is one of the solutions to predict the effects of these mutations. Another plausible solution is the development of siRNA-based drugs, but the wet experiment to support this solution is limited and expensive. RNA molecule tends to be unstable as well in the experimental assay.

Epidemiological data is necessary to determine the clustering of the localities (Supplementary File (Figure 1a)). It was found 3 clusters. Standard and time cohort trees were elucidated. The infection or the molecular clock (Supplementary File (Figure 1b)). The sequences of the *gyrA* genes were found to elicit 3 clusters and show a tendency to developing novel strains.

The structure is bizarre because it formed a very strained bulge that in accordance with the thermodynamic law will require huge activation energy to exist, and a strong steric effect observed (Supplementary File (Figure 2)). The structure is not plausible. In comparison with 4B, the structure in 4A is definitely not plausible. It advises that the mutations have created a much 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 to why antibiotic resistance strains of the *leprae* occurred.

Moreover, it is also found that the structural diversity of the *gyrA* mRNA shows 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 or not, 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 (Supplementary File (Figure 3)). A91V-resistant mutants 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 (Supplementary File (Figure 3a)) at a distance of 3.5 Å (Supplementary File (Table I)). These mutations cause destabilizing of the protein and have a high impact overall. The H71R and D95G mutants also have high impact to proteins (Supplementary File (Table I)), thus, they may cause resistance to ofloxacin. The K73R, A127V, R107W, and A101T mutants have long distances from the binding cavity of ofloxacin (Supplementary File (Figure 3) 18.1 Å, 12.3 Å, 23.6 Å, 17.3 Å respectively (Table I)). Overall, the K73R, A127V, R107W, and A101T has a moderate impact on protein, possibly leading to decreased effectiveness of ofloxacin.

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 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, K73R, A101T, R107W, A127V mutants had moderate impacts. However, an *in vivo* test is required to validate these results.

ACKNOWLEDGEMENT

The authors thank the National Institute of Health Research and Development for funding this research. We also thank the Head of Papua Institute of Research and Development Center for Biomedicine who allowed researchers to conduct research in their laboratory. Thanks also go to the Institute of Research and Community Empowerment (LPPM) of the Indonesia and the International Institute for Life Sciences for supporting the clustering and molecular modeling efforts.

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