Molecular Detection Mutation of rpoB Gene Mycobacterium leprae in Relapse and Default of Leprosy Patient in Jayapura City, Papua

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Abstract

Background: Leprosy remains a prominent health problem in Papua especially in Jayapura City. Numerous cases of relapse and default are also challenges in leprosy elimination in Jayapura. Studies in Relapse cases and history of defaults revealed some resistance related to multi-drug treatment (MDT). The purpose of this study was to detect the presence of mutation in rpoB M. leprae gene in patient relapse, default and patients who are less sensitive to MDT therapy in Jayapura City.

Method: Samples were obtained from patients diagnosed with leprosy with criteria of relapse, default and symptomatic patients after receiving MDT therapy. A total of 34 samples were taken in the form of skin incision (skin silt) of the earlobe. DNA was extracted using Qiagen kit. rpoB gene from extracted DNA was amplified through PCR method followed by nucleotide sequences. Analysis of mutation was elaborated using BLAST according to GenBank database.

Result: 34 samples were examined, and 9 were positive for Ziehl-Neelsen (ZN) staining, while the 25 were negative. In the PCR results, the samples that successfully amplified were 31 samples, and 3 samples were not amplified. The results of BLAST indicated that no mutations in the rpoB gene found in which able to initiate resistance to rifampicin.

Conclusion: The conclusion of this study is the rpoB Mycobacterium leprae gene from Jayapura did not contain any mutations that could trigger resistance to rifampicin. Thus rifampicin is still sensitive for leprosy treatment in Jayapura City. (Health Science Journal of Indonesia 2018;9(1):31-6)

Keywords: Leprosy, rpoB gene, rifampicin, Mycobacterium leprae
Mycobacterium leprae is a bacterium that causes Hansen disease (leprosy). Leprosy is a skin infection disease, mucus membranes, and peripheral nerves. This disease is slowly changing skin lesions into lesions that damage the face causing social stigma and isolation for leprosy patients. The prevalence of this disease has declined dramatically after the use of MDT, but new incidental cases continue to occur.

The World Health Organization (WHO) decreed year 2000 as a milestone in achieving leprosy elimination as an effort to control leprosy in the world. Indonesia achieved this target in the same year, but the development of the last 10 years has shown a static trend in the discovery of new cases. In the last 12 years (2000-2011), the situation of leprosy disease in Indonesia has not changed and the disease remains a serious health problem.

Leprosy is one of the dominant diseases in Jayapura City, Papua. Papua is still a province with high burden. Based on the number of second-level defects and these are the provinces with the highest level of disability per 1 million people in Indonesia in 2013 were Papua (26.88), Aceh (18.62), and West Papua (17.72). According to data from the Directorate General of Disease Control and Environmental Health, MoH RI 2014, the number of new cases of leprosy and case detection rate (CDR) for Papua Province by sex was 35.64 case detection rate (CDR) per 100,000 population for 2013, while for the year 2014 experienced a slight decrease of 30,43 case detection rate (CDR) per 100,000 population.

Rifampicin is a major component in chemotherapy used to treat leprosy and tuberculosis. On the recommendation of WHO, one type of treatment in a multi-drug therapy (MDT) program is rifampicin, although some leprosy patients are less susceptible to rifampicin and suspected of resistant M. leprae strains. Research conducted by Grosset et al reported that of 39 strains of M. leprae isolated from relapse patients, there were 22 strains that were resistant to rifampicin. Drug resistance characterized by initial treatment did not show progress or worsened, new lesions appeared and patient condition decreased. Based on reports of cases of Jayapura City Health Office 2016, there were 34 patients with a history of relapse, defaults and some of which showed no significant improvement after taking MDT.

Common identification methods for M. leprae are using microscopic observation and Ziehl-Neelsen Staining. These methods are generally used for the early screening of M. leprae although this method is less sensitive compared to PCR. The most important problem in drug-resistant detection of leprosy is M. leprae remains unable to be cultured by conventional method. The availability of genomic sequences from M. leprae and increased understanding of the genetic basis of drug resistance in mycobacteria led to the development of molecular methods for the detection of mutations associated with dapson, rifampicin, and fluoroquinolone. 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 sequence reference of DRDR M. leprae strain TN (NC_002677.1 GenBank) to determine the presence of drug resistance mutations. The DRDR area for rifampicin is at about 1225 bp to 1503 bp in the rpoB gene.

Rapid information about susceptibility to a drug is crucial treatment effort for an individual especially the diseases caused by Mycobacterium by using rifampicin. Thus, it is necessary to do research to see the mutation in rpoB M. leprae gene. Research is expected to be used as information by the program that will be able to accelerate the decrease of incidence rate and cure of leprosy patient especially in Jayapura City.

**METHODS**

This study was a cross section. This research has received approval from the Ethics Committee of National Institute of Health Research and Development number LB.02.01/5.2/KE.048/2017. The study sample was ear incision taken from patients; relapsed, defaults, and patients who were less sensitive to treatment. The ear incisions that have been taken are then examined microscopically and molecularly. Microscopic examination was done by Ziehl-Neelsen (ZN) staining method. Assessment of microscopic examination results to determine the bacterial index performed according to the logarithm of Ridley. Molecular examination begins with DNA extraction process using Qiagen Kit, followed by Polymerase Chain Reaction (PCR) process. The PCR reagent mix composition consisted of Go Taq Green Master Mix 2X, set primers (forward and reverse) 10 pmol, DNA template 0,5 µg and nuclease free water. The primers used to amplify the rpoB gene in this study were WHOR1 5’-CAGACGCTGATCAATATCCGT-
3’s as a forward primer and WHOR2 5’-CA-GCGGTCAAGTATTGCATC-3’s as reverse primers. The first PCR results were followed by a second PCR using rpoB forward primers in CAATATCCGTCCGGTGTTGTC and reverse rpoB sequences in GTATTCCGATCTCGCGTGA sequence. PCR was adjusted with pre-denaturation temperature at 95 °C for 5 minutes, denaturation at 94 °C for 1 minute, annealing at 60 °C for 30 seconds, extension at 72 °C for 1 minute and final extension at 72 °C for 10 minutes. The sequencing process uses ACGCTGATCAATATCCGTCC forward sequencing primers and CGACAATGAACCGATCAGAC reverse sequencing primer. The PCR conditions were arranged in sequence, 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. The PCR result was purified by ExoSap IT with a ratio of 1:5. The purified PCR product was followed by a sequencing process. The sequencing cycle uses BigDye Terminator v3.1 5X, BigDye Terminator buffer 5X, 100 ng 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 homogenised with samples by using vortex, for 30 minutes then in the centrifuge for 1 minute. The supernatant was inserted into a 20 μl wellbore slab and read by using 3500 Genetic Analyser. The sequencing results were then processed in the gene bank to identify the presence of mutations in the rpoB gene.

RESULTS

A microscopic examination was performed to observe at M. leprae bacilli in samples obtained from ear incision of leprosy patients. BTA staining was performed using the Ziehl-Neelsen method. Total numbers of samples examined were 34, and 9 of which were positive for Ziehl-Neelsen while the other twenty (25) were negative (Table 3). In staining Ziehl-Neelsen M. leprae gave red colour and looked straight or slightly bent (Figure 1).

Figure 1. Microscopic examination results at 100x magnification

There were three characteristics of patients in this study; the default, less sensitive to treatment and relapse. A default is a condition in which a PB patient does not take / take medication for more than 3 months and patients with MB over 6 months are cumulative. (4) Less sensitive patients were the administered patients with MDT but keep producing new lesion.

According to WHO, relapse patients were patient who successfully completes an adequate course of MDT, but who subsequently develops new signs and symptoms of the disease either during the surveillance period (2 years for PB and 5 years for MB leprosy) or thereafter. Based on the data obtained, most of the relapsed patients were as much as 59%, then default 32% and less sensitive to treatment 9% (Figure 2).

Figure 2. Patient characteristics

Results of rpoB Mycobacterium leprae gene amplification derived from a clinical sample of leprosy patients using the Polymerase Chain Reaction (PCR) method. PCR product had of approximately 300 bp (Figure 3). 31 samples were amplified, and three samples were not amplified (Tabel 1). Inadequate cell of M. leprae within the sample perhaps was the main issue why the PCR amplification led to failure, compared to the sample which examined with microscopic observation method.
The alignment of *M. leprae* sequencing obtained from leprosy patients in Jayapura showed that all was 100% homology after performing BLAST process in the gene bank (Table 1). The reference strain used as a comparison was *M. leprae* TN strain (accession number NC_002677.1). These results indicated no mutations detected in our observed the *M. leprae* *rpoB* gene. In another study, several mutations were successfully detected in the *rpoB* *M. leprae* gene that triggered resistance to rifampicin.16,19 resistance to first-line (dapsone, rifampin

Table 1. Comparison between patient status, PCR result and *rpoB* *M. leprae* gene sequencing result from Jayapura

<table>
<thead>
<tr>
<th>Status</th>
<th>Microscopic (ZN)</th>
<th>PCR/sequencing</th>
<th>NCBI BLAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Default</td>
<td>4/11</td>
<td>11/11</td>
<td>100% <em>M. leprae</em></td>
</tr>
<tr>
<td>Relapse</td>
<td>4/20</td>
<td>18/20</td>
<td>100% <em>M. leprae</em></td>
</tr>
<tr>
<td>Less sensitive</td>
<td>1/3</td>
<td>2/3</td>
<td>100% <em>M. leprae</em></td>
</tr>
<tr>
<td>Total</td>
<td>9/34</td>
<td>31/34</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSIONS**

Detection of mutations in the *rpoB* *M. leprae* gene associated with rifampicin resistance is crucial. It is useful to comprehend the effectiveness of rifampicin as one of the components of MDT in the fight against leprosy. Papua is one of the provinces with high leprosy burden so that information about mutations, especially ropB gene that can be used to accelerate leprosy elimination in the Papua Province. Microscopic examination results showed there were 9 positive samples containing leprosy bacilli. In most samples did not show the presence of *M. leprae* bacilli; however these results remained requiring further confirmation by molecular examination.

In the three criteria of the patients used in this study were defaults, and relapsed did not show significant improvement after having treatment with rifampicin. This rose concern since suspects for mutations in *M. leprae* that infected these patients. Most of the patients obtained in this study were relapse patients, and around 59% from total. Patients revealed being relapsed as if after RFT there found new lesions on the skin. Determination of patient relapse done by Kusta Wasor at Public Health Centre where patient got treatment. In addition to relapse patients, also found 32% of patients who had default. In this study also found patients who were less sensitive to MDT treatment by 9% which was marked by the emergence of new lesions during the treatment period. Detection of mutations in the *rpoB* *M. leprae* gene of the patient is essential to see the presence of mutations that can cause resistance. There are two possible patients who had been expressed RFT with negative skin smear returning symptom that was the possibility of relapse or re-infection.20

Lepr**o**sy treatment in Indonesia follows is in accordance with the treatment program by WHO, actually with Multi Drug Therapy (MDT). MDT consists of rifampicin, dapsone and clofazimine (lamprene).4 Resistance to anti-leprosy drugs, such as dapsone, rifampicin and fluoroquinolones, has been described since 1967 using in vivo models.21 Molecular testing for leprosy drug resistance facilitates susceptibility testing and provides means for monitoring resistance globally.22 Simple PCR-based approach to rapid
screening of strains that have resistant alleles can be performed since the areas involved in rifampicin resistance are very limited. The alignment of *M. leprae* sequencing obtained from leprosy patients in Jayapura showed that all showed 100% homology after performing BLAST process in the gene bank (Table 1). The reference strain used as a comparison was *M. leprae* TN strain (Figure 4).

Rifampicin normally binds to RNA polymerase, which inhibits the transcription process in bacteria. A change in the order of nucleotides (mutations) in the gene causes rifampicin not to bind to RNA polymerase so that the transcription process in bacteria persists leaves the effects of rifampicin in leprosy patients to be ineffective. According to alignment results, *M. leprae* from Jayapura remained sensitive to rifampicin. This can be observed in the results of *rpoB* gene sequencing that did not contain mutations that might trigger resistance to rifampicin. In such study conducted by Maladan, 2017, there was found a mutation at codon 477 ACT→ACC on *M. leprae* from Jayapura. Such mutations have not been reported in comparison with the existing publications. These mutations occur within the drug resistance determining region (DRDR) region. This region is an area that encodes rifampicin resistance in the event of mutations in it. Rifampicin resistance case reports have been widely reported that are associated with mutations at a particular point in the DRDR *M. leprae* region. However, the case reports of rifampicin resistance in Indonesia are very limited. Mutations reported previously were mutations at codon 410. Meanwhile, reports of cases of resistance from Papua have not been reported.

Case reports from several countries have also been reported. Some of these are mutations in H420 and S425. In addition, reports of cases of rifampicin resistance in leprosy patients in China have been reported. The resistant *M. leprae* strain of the patient contains 441 codon mutations in the *rpoB* gene. The report is the fourth mutation in China after codon 456, 451, 481. Based on the results of this study it was known that *M. leprae* obtained from leprosy patients with a history of defaults remained susceptible to rifampicin. Similarly, *M. leprae* obtained from patients who showed no improvement after treatment was still susceptible to rifampicin. The possible cause of this lack of progress is due to a default. The existence of defaults indicates non-compliance with leprosy treatment. In *M. leprae* obtained from relapsed patients there was also no mutation in the *rpoB* gene. Relapse in leprosy patients other than due to drug resistance, can be caused by several such bacterial persistence, immunosuppression of the host, pregnancy, the presence of advanced leprosy, reinfection, and factors associated with failures in operational health care, such as late diagnosis, inadequate or irregular treatment of the disease, and misclassification of earlier disease. In accordance to this study, it seemed that patients presumed to be relapsed due to these factors.

In conclusion, there was no mutation found in *rpoB* gene of *M. leprae* from Jayapura that might trigger resistance to rifampicin. According to the research findings, rifampicin remained sensetive for leprosy in Jayapura. Thus the relapse and default patients used as the subject in research were not associated with rifampicin resistance. It is necessary to detect mutations in other genes that can lead to resistance towards dapsonc and clofazimine (lamprene) drugs, which are the rifampicin component of the MDT component in leprosy treatment. In addition, to support the success of treatment it definitely is expected leprosy patients should take medication regularly and intensive assistance from health workers and family.

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