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Original Article

Development of an Assay System for Genotyping *Mycobacterium leprae* Resistant to Dapsone, Rifampicin, and Ofloxacin

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Even after the availability of effective anti-leprosy drugs, leprosy treatment is facing the challenge of emergence of *Mycobacterium leprae* strains resistant to dapsone, rifampicin and ofloxacin. As the conventional mouse foot pad (MFP) assay is time consuming and requires sufficient bacterial load, it is important to adapt, develop and use molecular assay(s) that can detect *M. leprae* strains resistant to the drugs. Real-time PCR (rPCR) assay targeting RLEP sequences was used for detection of *M.leprae* DNA. Further a nested PCR reaction technique was optimized using sets of primers targeting *folP*, *rpoB* and *gyrA* gene target. Amplicons were sequenced to detect mutations. The optimal reactions were applied to slit skin smear samples from 20 leprosy patients and was found to be applicable for slit skin smear samples. The optimized assay could genotype 17 *M. leprae* strains for drug resistance, all (100%) of these strains were found to be sensitive for dapsone and rifampicin and 5.9% (1/17) resistant for fluoroquinolones (ofloxacin). This genotyping test could be used to detect leprosy drug resistance and may be useful for patient care. It will be also be important to validate the assay developed in this study with mouse foot technique and compare it with other molecular assays developed by investigators from different countries and then choose the best for patient care/surveillance purposes.

Keywords : Genotyping Assay, Drug Resistance, *Mycobacterium leprae*

Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. This disease remains a significant health problem in certain areas of the world. Further despite the existence of effective drugs to treat leprosy occurrence of certain *M. leprae* strains that are resistant to these existing medications poses challenge (Renault and Ernst 2015, Walsh and Meyers 2011). The number of new leprosy cases worldwide in 2016, as shown in the Global Leprosy Update data published by the World Health Organization (WHO), was 214.783, with the highest case load being present in Southeast Asia, followed by America and Africa, respectively (WHO 2017a,b). In 2016, a total of 18.200 leprosy cases, with 16.826 being new

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cases, was reported by the Ministry of Health of Republic of Indonesia (Kementerian Kesehatan 2017).

Globally, the treatment of leprosy including in Indonesia is based on WHO guidelines and employs a multidrug therapy (MDT) regimen composed of rifampicin, dapsone, and clofazimine. Other anti-leprosy medications such as ofloxacin, clarithromycin, or minocycline also can be used if there is no clinical response visible after application of the first-line drugs (Kementrian Kesehatan 2012, WHO 2017, Williams and Gillis 2012).

Notably, however, cases of *M. leprae* resistance to dapsone (*folP*), rifampicin (*rpoB*), and ofloxacin (*gyrA*) have been reported in many regions, especially in those with high prevalence of leprosy such as in Southeast Asia (Guerrero et al 2014, Kai et al 2011, Maeda et al 2001, Matsuoka et al 2007, Mori et al 2012, Rocha et al 2012, Wahyuni et al 2012, Williams et al 2014). Data from the Global Sentinel Surveillance for Drug Resistance in Leprosy project, coordinated by the WHO (2010), reported nine cases (10%) of dapsone-resistant and one case (1.1%) of rifampicin-resistant leprosy, respectively, out of 88 multibacillary leprosy (MB)–relapse cases (WHO 2011). Research performed in Indonesia by Adriaty et al (2009) and Wahyuni et al (2012) showed the frequency of drug resistance to be approximately 4.3% and 2.22%, respectively, with dapsone being the most drug most impacted. Meanwhile, a separate study conducted at Cipto Mangunkusumo Hospital in Jakarta identified a drug resistance frequency for rifampicin of as much as 15.8% (Siskawati 2013). Resistance to ofloxacin, despite its second-line drug status, has also been reported in some countries, allegedly because of the increasing use of this drug to treat infection (Mejía et al 2014, Lavania et al 2018, Matsuoka et al 2010, WHO 2017a,b,c).

Resistant *M. leprae* strains can be detected with a few assays, such as an in vivo assay or molecular assay (genotyping). In vivo assay is typically performed in experimental animals for example, mice by cultivating *M. leprae* in the hind footpads of the mice. This assay is currently a gold standard for drug resistance testing of leprosy and is commonly called the mouse footpad assay. However, despite the definitive results that are often obtained by use of this method, this technique is cumbersome, very expensive, and time-consuming to perform. Therefore, to overcome disadvantages, researchers have developed an alternative method, which is genotyping assay. Polymerase chain reaction (PCR) DNA direct sequencing, one of many molecular methods that have been developed, is the most definitive assay available at this time for this purpose and has been used by the WHO in their drug resistance of leprosy surveillance program to detect leprosy drug resistance. This molecular assay simplifies susceptibility testing because the technique is easier and quicker to perform as compared with conventional testing by mouse footpad assay (Matsuoka 2015, Scollard et al 2006, WHO 2017c).

In Indonesia, there is only one laboratory available for drug resistance testing for leprosy. This fact has thus become an obstacle for the management of leprosy cases, especially of relapsed patients located in areas far from the facility, because of the lengthier time required for testing and delivery of the test samples and results as well as the increased costs. It is, therefore, very crucial to adapt or develop a genotypic assay(s) capable of detecting leprosy drug resistance in a more timely and cost-efficient manner. With this goal in mind, in the present study, we have attempted to develop an assay system for genotyping *M. leprae* for detection of resistance to dapsone, rifampicin, and ofloxacin.

Materials and Methods

Clinical Specimens

A descriptive, cross-sectional study of MB patients was designed for the detection of drug resistance among visitors to the Dermatology clinic at Cipto Mangunkusumo Hospital. Slit skin smear samples of MB patients were collected from March 2017 to March 2018. All patients were examined and diagnosed by experienced dermatologists. Sample collection was performed by experienced laboratory technicians in all cases under the supervision of dermatologists. The inclusion criteria in this study were MB patients with a bacteriological index (BI) of 3+ and above on Ridley scale (Ridley 1964) at one location/involving two skin lesions and the provision of written consent for enrollment in the study. Patients with blood coagulation diseases and/or those taking anticoagulant drugs were excluded from participation in this study. For PCR assay, the samples were collected into 10-mL tubes containing 1.5 mL of 70% alcohol and subsequently transported to laboratory at room-temperature conditions. This study was approved by the Ethical Committee of Medicine Research of the Faculty of Medicine of Universitas Indonesia (no. 131/UN2.F1/ETIK/2017).

DNA Extraction

About 750 µL of samples was centrifuged at 14000g for five minutes. The supernatant was discarded and pellet was extracted by using the Qiamp DNA minikit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions with 60 µL of final elution.

Real-time PCR

Real-time PCR (rPCR) was first performed for feasibility and/or to meet the requirements of genotyping assay. Primers (forward: 5'-GCA GTA TCG TGT TAG TGA A-3' and reverse: 5'-CGC TAG AAG GTT GCC GTA TG-3') and probe (FAM-TCG ATG ATC CGG CCG TCG GCG-3') were specific for common region of RLEP (family of dispersed repeats) (70 bp) and performed as reported previously (Truman et al 2008) and direct microscopic enumeration of the bacilli is complex, labor intensive, and suffers from limited sensitivity and specificity. We have developed a real-time PCR assay for quantifying *M. leprae* DNA in biological samples. Primers were identified to amplify a shared region of the multicopy repeat sequence (RLEP. rPCR conditions were employed as reported previously (Devita et al 2016), with compositions (20 µL): 1 × KAPA PROBE FAST PCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), 0.2 µM of forward and reverse primers, 0.2 µM of the probe, and 6 µL of DNA template. Thermal cycling (IQ5; Bio-Rad Laboratories, Hercules, CA, USA) was performed according to the following conditions: 95°C for three minutes; 45 cycles of 95°C for 15 seconds, and 55°C for one minute.

Resistance Genotyping (Nested PCR and DNA Sequencing)

Six pairs of primers were designed to target the three genes, *rpoB*, *folP* and *gyrA* (Table 1). The primers covered the mutations that are responsible for resistances to rifampicin (*rpoB* at positions 438, 441, 451, 456, and 458), dapsone (*folP* at positions 53 and 55), and ofloxacin (*gyrA* at positions 89 and 91).

Several PCR parameters were optimized including primer annealing, DNA template, and cycle numbers. The optimal compositions of PCR I (50 µL) were 1 × PCR Buffer (Qiagen, Hilden, Germany), 2.5 µM of $MgCl_2$, 1 × Q Solution, 200 µM of dNTP, 0.3 µM of primer, 1.5 U of HotStar Taq DNA Polymerase, and 15 µL of DNA template. For PCR II (40 µL), the compositions were 2 µL of DNA template of PCR I in 40 µL reaction, with the same concentrations of the other reaction components as in the case of PCR I, except for HotStar Taq DNA Polymerase (1.2 U). Thermal cycling for PCR I was 95°C for 15 minutes, 94°C

for 30 seconds, 56°C for 30 seconds, and 72°C for 40 seconds for 40 cycles, followed by 72°C for 10 minutes. PCR II employed the same thermal cycling temperature, but for 35 cycles. The optimization of primer annealing temperature was done with a temperature gradient between 54°C and 64°C for PCR I and between 56°C and 66°C for PCR II. The PCR product was then ran in electrophoresis gel. The agarose gel concentration that we used was 2%.

Purification before DNA sequencing was completed using a DNA purification kit (Qiagen, Hilden, Germany). Sequencing was done by Sanger method, using the BigDye Terminator v3.1 cycle sequencing kit chemistry. Sequencing results (quality of electropherogram charts) were analyzed using the Sequence Scanner v1.0 (Applied Biosystems).

Results

Characteristics of Patients

Fourteen of 20 patients included in the study were male and six patients were female. Of the included patients, the majority of patients were between 20 years and 69 years of age (median: 38 years). Sixteen patients were still being treated and four patients were finished with their treatment. The four patients which was finished the treatment had BI 3+. All of the patients had been diagnosed with MB leprosy. The borderline lepromatous type was present in the highest number of infected patients (16 patients), followed by lepromatous leprosy (three patients) and borderline tuberculoid (one patient).

Real-time PCR

All 20 samples showed positive rPCR results (Fig. 1) with the range of Ct values being from 15 to 37 (data not shown).

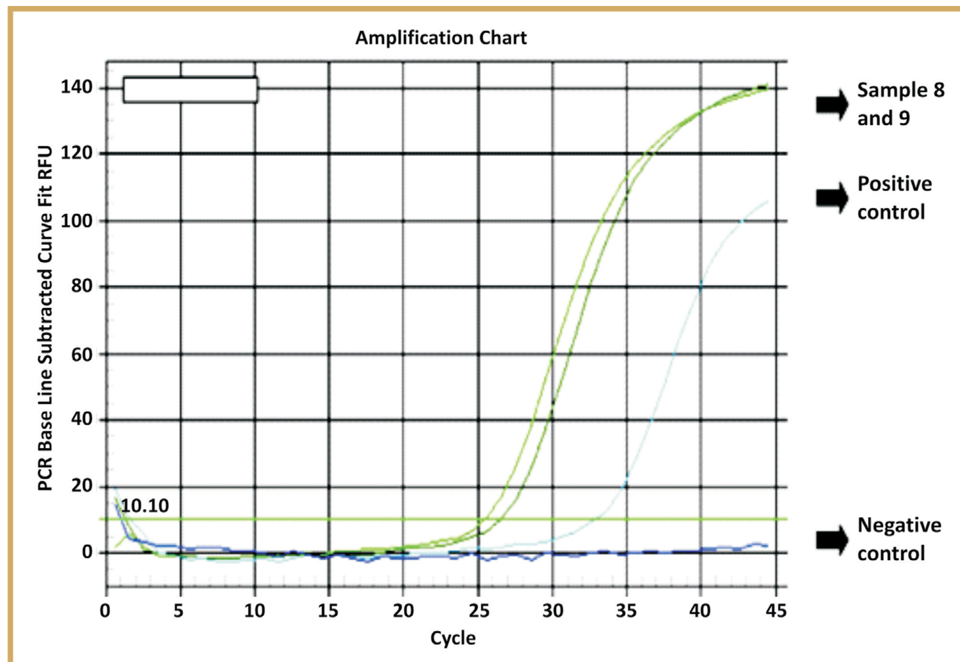

Fig 1 : Examples of sigmoid rPCR curves specific for common region of RLEP (family of dispersed repeats) of *Mycobacterium leprae*.

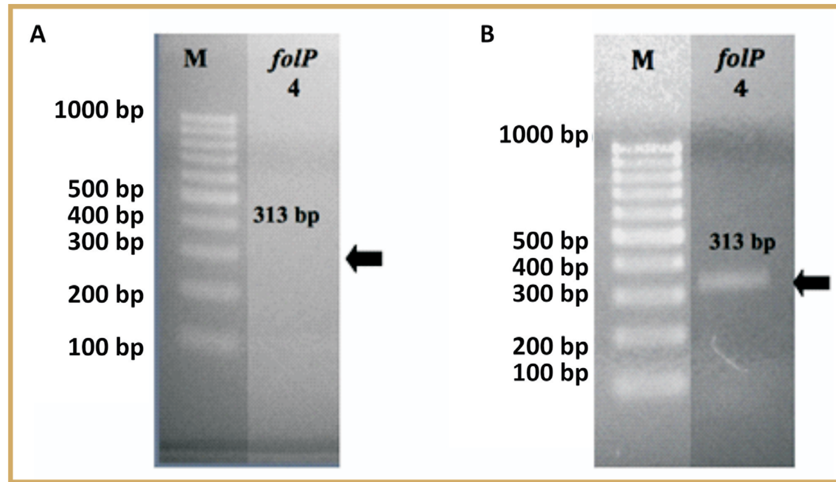

Fig 2 : (A) 10 µL of DNA template in 40 µL of reaction (sample number 4 for *folP* was not showing a DNA band as pointed out by the arrow).

(B) 15 µL of DNA template in 50 µL of reaction (sample number 4 for *folP* was showing a DNA band as pointed out by the arrow); M: *marker.*

Table 1 : Primers for nested PCR and DNA sequencing

Genes	Tests	Primers	Sequences (5' – 3')	Position
folP	PCR I	FolP-F01	TGCAGGTTATTGGGGTTTTGAA	6
		FolP-R02	CCACCAGACACATCGTTGAC	110
	PCR II	FolP-F02	CTTGATCCTGACGATGCTGT	26
		FolP-R02	CCACCAGACACATCGTTGAC	110
	DNA sequencing	FolP-R01	GCGTAGTATCGATACTTACTG	89
rpoB	PCR I	Rpo-F01	AGCGGATGACCACCCAGGA	402
		Rpo-R02	TCGTCGCTGACCACACCGT	527
	PCR II	Rpo-F02*/ Rpo-F01**	AGGCGATCACGCCGCAGA/ AGCGGATGACCACCCAGGA	410 402
		Rpo-R02*/ Rpo-R01**	TCGTCGCTGACCACACCGT/ CGACAATGAACCGATCAGACCT	527 501
	DNA sequencing	Rpo-R01*	CGACAATGAACCGATCAGACCT	501
		Rpo-F02**	AGGCGATCACGCCGCAGA	410
gyrA	PCR I	Gyr-F01	TGACTGATATCACGCTGCCA	1
		Gyr-R01	TAACGCATCGCTGCCGGT	130
	PCR II	Gyr-F01	TGACTGATATCACGCTGCCA	1
		Gyr-R02	TACCCGGCGAACCGAAATTG	122
	DNA sequencing	Gyr-F02	GGTCTCAAACCGGTACATCG	48

* first choice of primers, **second choice of primers

Genotyping for detection of resistance

Prior to performing nested PCR and DNA sequencing for clinical samples, the assay parameters (i.e., primer annealing, DNA template, and reaction volumes of PCR I) were optimized. Optimal primer annealing conditions of nested PCR were obtained by gradient temperatures from 54°C to 64°C. The optimal primer annealing temperature for the three genes for PCR I and II (*folP*, *rpoB*, and *gyrA*) was 56°C (data not shown).

For template and reaction volumes of PCR I, the test was only performed for one gene (*folP*). Based on the test, we detected a specific band in the 50 µL PCR reaction with 15 µl of DNA template, as shown in Fig. 2.

In this study, we also analyzed the appropriate sequencing primers to obtain sequencing results with lower associated noise. The sequencing primers that we used in each gene have been shown in Table 1. The primers for *folP* and *gyrA* used for DNA sequencing included FolP-R01 and Gyr-F02. Both primers resulted in a good electropherogram chart with little noise (data not shown). Meanwhile, for *rpoB*, there were two choices of DNA sequencing primer, Rpo-R01 and Rpo-F02. It was decided that, if the first primer (Rpo-R01) did not show a good electropherogram chart (Fig. 3A), the test could be repeated by using Rpo-F02 (Fig. 3B).

The optimized PCR and DNA sequencing conditions were applied to 20 skin-scraping

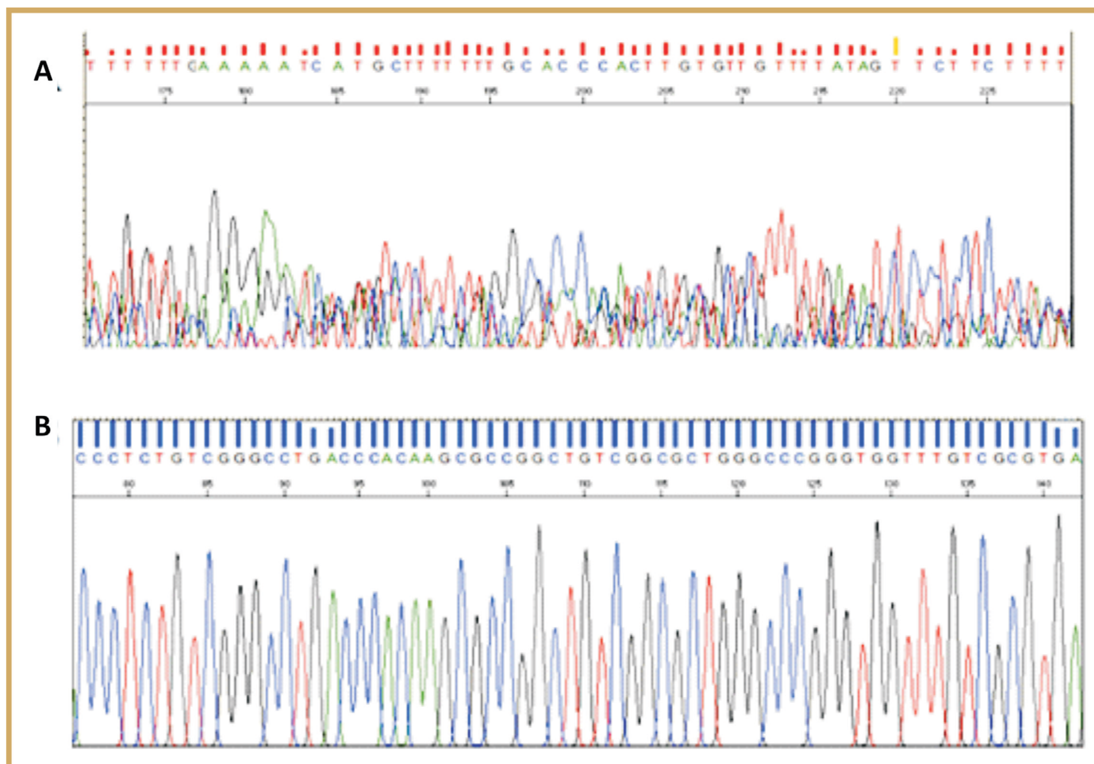

Fig 3 : (A) The electropherogram chart was inadequate with the Rpo-R01 primer, so (B) an electropherogram chart of *rpoB* using the Rpo-F02 primer was generated.

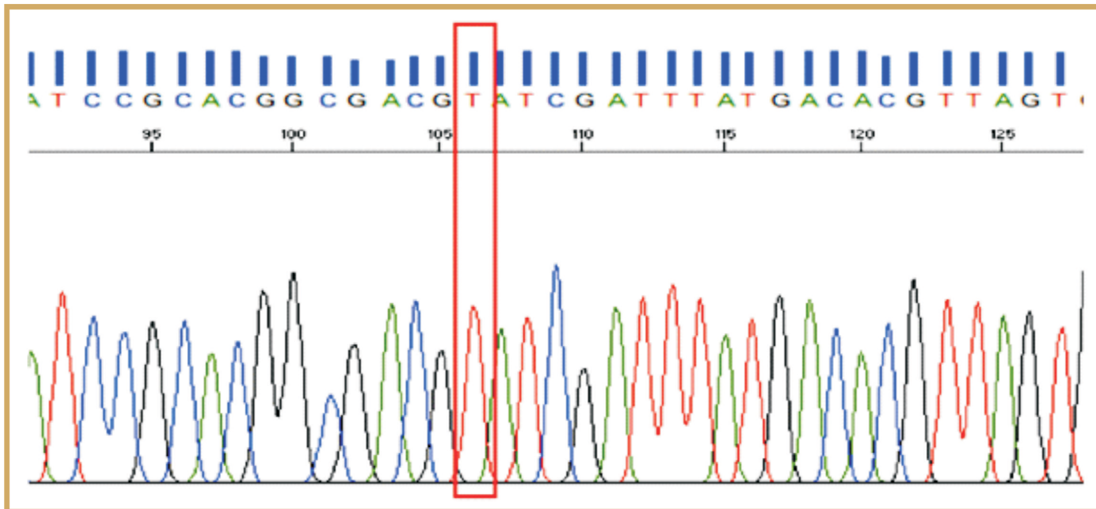

Fig 4 : Single mutation in *gyrA* gene

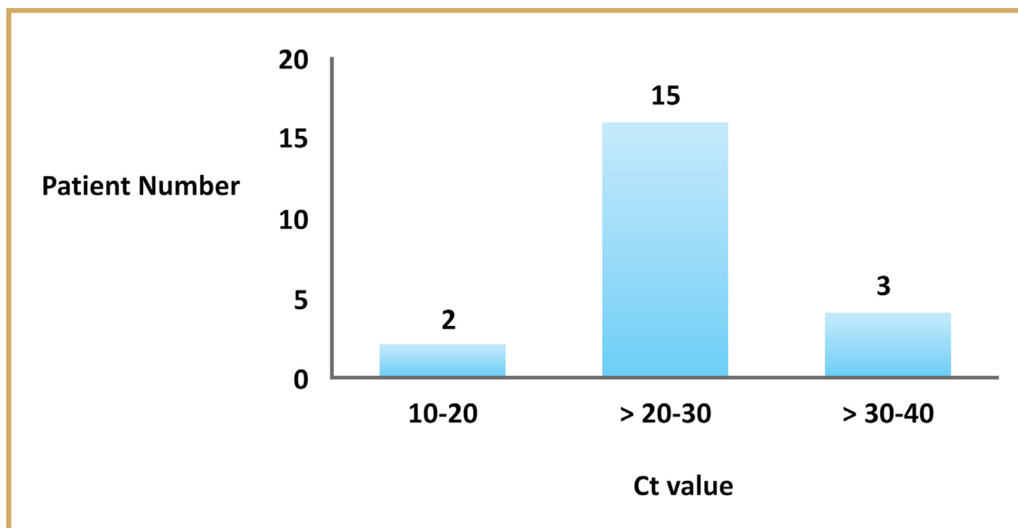

Fig 4 : Single mutation in *gyrA* gene

samples. Among 20 skin slit smear samples that were tested with nested PCR, 17 samples (85%) showed positive results for all three genes. One sample did not yield an amplification of *folP*, but was positive for *rpoB* and *gyrA*. The Ct value of this sample was 32.95. Additionally, one other sample positive for *gyrA* was negative for *rpoB* and *folP,* with a Ct value of 37.55. There was also one sample that did not yield an amplification of any of the three genes, with a Ct value of 31.02.

A total of 17 samples with PCR positivity were sequenced and 16 samples showed good sequencing results for all three genes, while the remaining one sample showed good sequencing

for *folP* and *gyrA* but not *rpoB* (could not be analyzed). For this one sample that showed a bad result, we repeated the test using a different *rpoB* sequencing primer and this result offered a better electropherogram chart (Fig. 3).

Of the 17 samples with good electropherogram charts, 16 samples showed no mutation in all three genes (*folP*, *rpoB*, and *gyrA*). One sample showed a single mutation in *gyrA* at position 91 (GCA → GTA) (Fig. 4) but no mutation in either *folP* or *rpoB*.

Cutoff of Genotyping Test

To determine the feasibility of genotyping testing, we analyzed the Ct(s) values of rPCR results that were associated with genotyping processes. Fig. 5 shows the range of cycle threshold (Ct) values of all samples in this study. There were 17 samples that showed Ct values of less than 30, whereas three samples showed Ct values of more than 30. All of the samples with Ct values of less than 30 exhibited positive genotyping tests, while the other three samples exhibited negative results. Based on this study, the cutoff for genotyping assay was rPCR results with the Ct being not more than 30 (Ct ≤ 30).

Discussion

One of the problems being increasingly faced in leprosy patients at this time is the emergence of drug resistance, particularly regarding rifampicin, dapsone, and ofloxacin. Therefore, we developed a system assay for the genotyping of resistant *M. leprae*. The major factor that determined the success of PCR was primer design. The design of a primer can be performed based on a DNA sequence that is already known or from an intended protein sequence (Handoyo and Rudiretna 2001). From the primer that we designed in this study, we obtained the amplification of the three genes and determined it was viable to use our created primer for this study. The primer sequence refers to the genome sequence of *M. leprae* that was published by the WHO (2017). In this study, nested PCR assay was performed to amplify the target DNA. Nested PCR assay has been previously performed in the studies by Matsuoka et al (2011) and Kai et al (2007) and is the method used in WHO (2017c) surveillance efforts. However, there was a difference between the primer that was used in this study versus the primer that was used in the studies by Matsuoka et al (2007) and Kai et al (2011).

An optimal annealing temperature is required for the primer to successfully attach to the target in PCR (Handoyo & Rudiretna 2001). In the present study, we determined the optimal annealing temperature for PCR I and II, which was 56°C. Notably, Matsuoka et al (2007) used the same annealing temperature in their research. There was, however, a difference in comparison with the annealing temperature used by Kai et al (2011) which was 55°C, while the WHO (2017) uses an annealing temperature of 57°C in its surveillance efforts.

In the first trial for PCR I, we used 10 µL of DNA template in 40 µL of reaction. With this composition, we ensured the amplification of the samples but, ultimately, there was a sample that showed no amplification. To increase the success of amplification, we changed the composition to 15 µL of DNA template in 50 µL of reaction. The sample that was not amplified previously subsequently becomes positive upon using this composition. The DNA target was expected to be more increased with the addition of DNA template and to show enhanced amplification. In PCR II, we did not change the concentration or composition of reaction, because the results obtained were fine at the time of the first trial.

Of 20 slit skin smear samples, there were 17 samples (86.3%) that showed positive results in nested PCR assay. Three samples could not

be amplified for one or all of the three genes. Amplification failure can caused by several reasons, including degradation of the DNA template, too little an amount of DNA template, the occurrence of an inhibitor in the sample, and a non-optimal reaction composition or PCR condition (Lorenz 2012, Promega 2018). In this study, the failure might have been caused by the limited number of bacterial DNA, leading to the lack of amplification in PCR I. The Ct value could be used to estimate the amount of bacteria in a sample, with a low Ct value indicating more bacteria in a sample and vice versa (Life Technologies Coorporation 2012). The Ct values in the three negative samples were quite high at 32.95, 31.02, and 37.55, so it can be assumed that the number of *M. leprae* DNA in these samples were not enough or conditions optimum for amplification in our PCR assay. Even though, in this study, we used a sample where the minimal bacteria index in one of the lesions was 3+, we did not know the exact number of bacteria in the samples for PCR because the samples that we used for AFB smear were different from the samples used for PCR. The positivity rate in the study by Kai et al (2011) was 69% (from 423 samples, only 290 samples showed a positive band). Negative results in the study by Kai et al (2011) were assumed to be the result of little or no bacteria in the sample or because the amount of bacteria in the sample was less than 100 (under the limit of detection). A separate study performed by Rocha et al (2012) using a biopsy sample showed an occurrence of PCR inhibitor in the sample that could not be amplified.

In the present study, 17 samples with positive PCR results for the three genes (*folP, rpoB, gyrA*) was sequenced to detect mutation(s). Of these, 16 samples presented good electropherogram charts for the three genes. Meanwhile, there was one sample that presented a good electropherogram chart for *folP* and *gyrA*, but its *rpoB* electropherogram chart could not initially be analyzed. This was because the priming site had many single nucleotide polymorphism (SNP) differences. After repeated the test using different primer, we obtained a good electropherogram chart for *rpoB* as well.

Evaluation of the 17 samples that were sequenced and analyzed showed no mutation in 16 samples for the three genes. One sample (5.9%) presented a mutation in *gyrA* at position 91, which changed GCA to GTA (AlaàVal), but had no mutation for either *folP* or *rpoB*. The mutation in this area was shown to be related with the occurrence of resistance to ofloxacin (Matsuoka 2015, Williams and Gillis 2012, WHO 2017c). The patient characteristics related to this mutation finding was female gender and treatment with an MDT regimen, where one of the drugs used was ofloxacin. However, we could not determine whether the resistance was classified as a primary or secondary mutation because we did not compare the sample between before and after treatment. Ofloxacin resistance has been noted in several countries (Mejía et al 2014, Matsuoka et al 2010), but till date no reports of quinolone resistance from Indonesia have been published (Karim et al 2015, Matsuoka et al 2007). It is believed that this ofloxacin resistance incident presented in the current study thus represents the first incident of such in Indonesia.

The main limitations of this study were that no positive controls were used to confirm the resistance and we did not perform external quality control. This study has also not addressed the correlation between PCR results and patient clinical characteristics. Samples furthermore were not taken before and after treatment, so we cannot know whether the resistance is primary or secondary.

Conclusion

The assay system discussed in the present research could be used to detect drug resistance to these three anti-leprosy drugs. Further investigations with larger / adequate study sample groups is necessary to know prevalence of the resistance in Indonesia. During the last 2-3 decades several molecular assays to detect resistance to various anti-leprosy drugs have been developed from different countries. It will be important to analyse the assay developed in this study with such assays and then choose the best for patient care/ surveillance purposes.

Acknowledgments

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