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SHORT COMMUNICATION

Profile of Mutation on Drug Resistance *Mycobacterium leprae* Isolates in Indonesia Collected During 2003-2011

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Multidrug therapy (MDT) regiment has been used for leprosy all over the world for more than 20 years. Drug resistances of *Mycobacterium leprae* have been reported from many areas. The resistance mostly occurred due to mutation on the gene coding protein targeted by anti-leprosy drugs. Two hundreds and seventy *M. leprae* isolates from some area in Indonesia were examined for studying the profile of mutation among isolates collected during 2003-2011. Drug resistance determining region of the *folP1* gene and the *rpoB* gene was sequenced. The results showed 5 isolates of *M. leprae* harboured mutation only in the *folP1* gene and another isolate harbored mutation in both the *folP1* and *rpoB* gene. The point mutation in the *folP1* gene that was found in 2 isolates occurred in codon 53 (ACC →GCC; Thr →Ala). Double point mutations on codon 53 that was found in two isolates were ACC→AGA (Thr → Arg) and ACC →AGG (Thr →Arg). The point mutation in the *folP1* gene occurred in codon 55 were found in two isolates were CCC → CTC (Pro →Leu) and CCC → CGC (Pro → Arg). Whereas mutation in the *rpoB* gene in one isolate occurred in codon 410 was GAT → TAT (Asp →Tyr). These mutations that altered the amino acids of the protein revealed that isolates of *M. leprae* were resistant to drug with variable profiles.

Key words: dapsone, drug resistance, mutation, *Mycobacterium leprae*, rifampicin

Terapi kombinasi (*multidrug therapy*) telah digunakan untuk pengobatan penyakit kusta di seluruh dunia selama lebih dari 20 tahun dan kasus-kasus resistensi *Mycobacterium leprae* terhadap obat anti kusta telah dilaporkan dari beberapa daerah. Resistensi terjadi karena adanya mutasi pada gen yang mengkode protein target dari obat-obat anti kusta. Dua ratus tujuh puluh isolat *M. leprae* dari beberapa daerah di Indonesia diperiksa untuk mengetahui profil mutasi isolat-isolat yang dikoleksi selama tahun 2003-2011. Daerah gen yang bertanggung jawab pada resistensi terhadap obat yakni gen *folP1* dan *rpoB* telah disekuensing. Hasil penelitian menunjukkan 5 isolat *M. leprae* mengalami mutasi hanya pada gen *folP1*, sementara satu isolat mengalami mutasi pada gen *folP1* dan *rpoB*. Mutasi titik pada gen *folP1* yang ditemukan pada dua isolat terjadi di kodon 53, yaitu ACC→GCC (Thr →Ala). Mutasi titik ganda di kodon 53 ditemukan juga pada dua isolat, yaitu ACC → AGA (Thr →Arg) dan ACC →AGG (Thr →Arg). Mutasi titik pada gen *folP1* yang terjadi di kodon 55 ditemukan pada dua isolat, yaitu CCC → CTC (Pro →Leu) dan CCC → CGC (Pro →Arg). Sementara mutasi pada gen *rpoB* ditemukan hanya pada satu isolat, yakni pada kodon 410 (GAT → TAT; Asp → Tyr). Mutasi-mutasi yang ditemukan mengubah asam amino dari protein, menunjukkan bahwa isolat-isolat *M. leprae* yang mengalami mutasi tersebut resisten terhadap obat dengan profil mutasi yang bervariasi.

Kata kunci: dapson, mutasi, *Mycobacterium leprae,* resisten obat, rifampisin

Mycobacterium leprae is the causal agent of leprosy, a chronic infectious disease that still becomes major problem especially in Indonesia. Since multidrug therapy (MDT) for leprosy has been introduced in 1982, the prevalence of leprosy worldwide significantly decreased (Lockwood and Suneetha 2005). Indonesia has reached elimination program in year 2000 with prevalence rate less than 1/10.000 inhabitant (Departemen Kesehatan RI 2008), but new cases still remain high (World Health Organization (WHO) 2011). There are some endemic pocket areas in Indonesia with high prevalence and high number of patient (WHO 2010).

As an effective treatment for leprosy, MDT regiment has effectively reduced prevalence of the disease, but the drug resistance to the MDT component still emerged. Drug resistance cases have been reported from many areas (Dela Cruz *et al*. 1996; Kai *et al*. 1999; Cambau *et al*. 2002; Matsuoka *et al*. 2007), and

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some are multidrug resistance (Cambau *et al*. 1997; Matsuoka *et al*. 2000; Maeda *et al*. 2001; You *et al*. 2005).

MDT prescribed by WHO, containing dapsone, rifampicin, and clofazimine, is the major regiment for leprosy treatment, and it is important to detect the resistant cases for evaluating efficacy of MDT and supporting leprosy elimination program. The resistance mostly occurred due to mutation on the gene coding protein targeted by anti-leprosy drugs (Kai *et al*. 1999; Cambau *et al*. 1997; Matsuoka *et al*. 2000; Maeda *et al*. 2001). Clofazimine resistance has not been described, and its mechanism and molecular methods to detect this, are unknown WHO Regional Office for South-East Asia. 2009; Matsuoka 2010).

Some *M. leprae* isolates from some areas in Indonesia were examined for studying the profile of mutation in the *folP1* and *rpoB* genes among isolates collected during 2003-2011. The study was established for exploring mutation on *M. leprae* isolates as information of drug resistance cases in Indonesia.

Two hundreds and seventy *M. leprae* isolates were obtained from multibacillary (MB) leprosy patients by taking skin slit specimens from the lesion or ear lobe. Samples were taken from Jawa Timur, Nusa Tenggara Barat, Nusa Tenggara Timur, South Sulawesi, Maluku, and Papua Barat. One sample from Kalimantan were taken in Surabaya. Samples were taken from 144 under MDT treatment patients, 63 Release From Treatment (RFT) patients, 59 new case patients, 3 ROK (Rifampicin, Ofloxacin, Clarithromycin) treatment patients, and 1 single (dapsone) treatment patient. Specimens were put in 1.5 mL eppendorf tube contain phosphate buffered saline (PBS) solution.

DNA isolation from the specimens was done by using QIAprep Spin Miniprep Kit (Qiagen) with procedures as described in manual book. Amplification of DNA by PCR was done in BIORAD I-cycler machine using folP1-folPR2 primers (folP1 5'-GCTTCTCGTGCCGAAGCGCTC-3'; folPR2 5'-GCGCGTAGTATCGATACTTAC-3'; PCR product length 305 bp) and rpoBF-rpoBR primers (rpoBF 5'-CAGGACGTCGAGGCGATCAC-3'; rpoBR 5'-CAGCGGTCAAGTATTCGATC-3'; PCR product length 374 bp) to obtain DNA sequence target of the *folP1* and *rpoB* genes. The condition of both PCR was as follows : 1) 98 °C for 2 min; 2) 98 °C for 30 sec, 63 °C -59°C down 1 degree percycle for 30 sec, 72 °C for 30 sec; for 5 cycles; 3) 98 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec, for 40 cycles; 4) 72 °C for 5 min. The mixture for all PCR condition was 12.4 µL of distilled water, 20 µl 2X Premix G, 0.4 µ+l *Taq* polymerase, 1.6 µL primer each (stock solution 5M), DNA template was added for 4 µL, total volume was 40 µL. PCR kit (Premix G, *Taq* polymerase) was from Failsafe™ PCR system (EPICENTRE Biotechnologies). PCR product was purified using GFX™ PCR DNA and gel band purification kit (GE Healthcare).

Dual CyDye™ Terminator Sequencing Kits (GE Healthcare) was used in the preparation of sequencing reaction. The reaction was performed according to the manufacture's manual. The sequencing reaction was done in BIORAD I-cycler machine under the following condition: 95 °C for 20 sec, TM of sense primer +3 °C for 15 sec, 70 °C for 1 min. The condition was done for 35 cycles. The sequencing product was then purified by ethanol precipitation and dried followed by dissolving in 2 µL of loading dye and was loaded into prepared acrylamide gel in Long-Read Tower™ System (Amersham Biosciences). Sequence analysis was done using Long-Read Tower™ System (Amersham Biosciences) with the temperature was set on 60 °C as described as in the protocol.

Study showed that all isolates for dapsone and rifampicin resistances were positive in PCR examination. Sequencing result showed, there were six out of two hundred and seventy *M. leprae* isolates (2.22%) harbour mutation in the *folP1* gene. Four isolates had mutation on codon 53 and two isolates on codon 55. Different five profiles of mutation in the *folP1* gene have been found. Only one out of two hundreds and seventy *M. leprae* isolates (0.37%) had mutation in the *rpoB* gene on codon 410 (Table 1). The partial DNA sequence of the *M. leprae* isolates showed that all profiles of mutation found in this study were varied (Fig 1) and were same to the types of mutation that have been previously reported (Matsuoka *et al*. 2008).

The first profile of mutation in the *folP1* gene was point mutation occurred in codon 53 from ACC (threonine) to GCC (alanine). It was occurred in two isolates. The second profile was double point mutation on codon 53 from ACC (threonine) to AGA (arginine). The third was also double point mutation on codon 53 which was changed to AGG (arginine). The fourth was point mutation occurred on codon 55 which was from CCC (proline) changed to CTC (leucine). The last profile was point mutation on codon 55 which was changed to CGC (arginine). All these profiles of mutation that changed amino acid of the protein indicated that isolates were resistance to dapsone. Only one profile of mutation found in the *rpo*B gene on

Table 1 Mutation profile of six drug resistance case-*Mycobacterium leprae* isolates in Indonesia

No	Isolate	Treatment status	Drug	Gene	No.	Wild Type	Mutant
1.	Surabaya1	Under MDT treatment	Dapsone	*folP1*	53	ACC (Thr)	GCC (Ala)
2.	Surabaya2	Under MDT treatment			53	ACC (Thr)	GCC (Ala)
3.	Surabaya3	New case			53	ACC (Thr)	AGA (Arg)
4.	Makassar1	Under MDT treatment with dapsone monotherapy before			53	ACC (Thr)	AGG (Arg)
5.	Kalimantan1	New case			55	CCC (Pro)	CTC (Leu)
6.	Pasuruan1	Relapse case			55	CCC (Pro)	CGC (Arg)
			Rifampicin	*rpoB*	410	GAT (Asp)	TAT (Tyr)

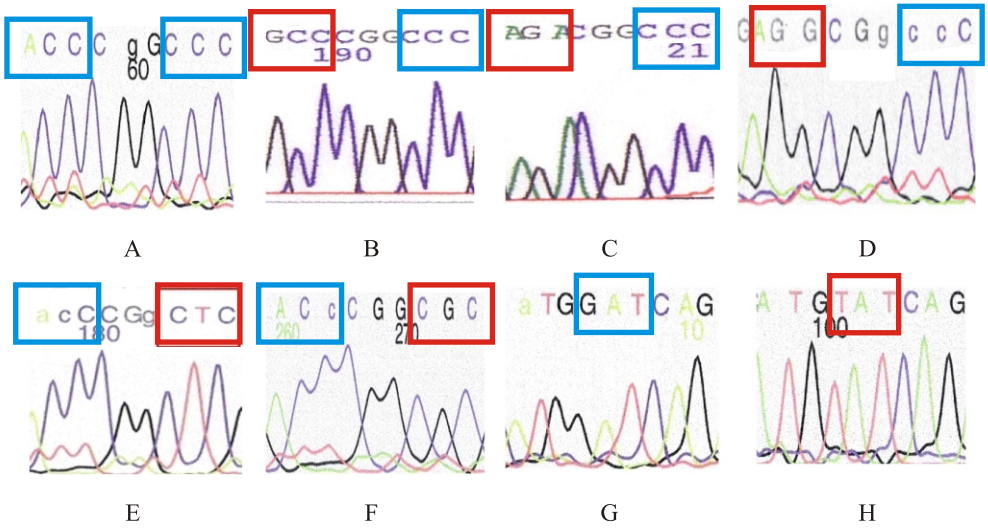

Fig 1 The partial DNA sequence of the *Mycobacterium leprae* isolates; A. Wild Type of *folP1* gene on codon 53 and 55, B-D. Mutant of *folP1* gene on codon 53, E-F. Mutant of *folP1* gene on codon 55, G. Wild Type of *rpoB* gene on codon 410, H. Mutant of *rpoB* gene on codon 410 (blue square represent no mutation, red square represent mutation).

codon 410 that was alteration from GAT (aspartic acid) to TAT (tyrosine) and again indicated that the isolate was resistant to rifampicin.

While treatment status of six drug resistance case-*M. leprae* isolates are known, it can be concluded that two isolates were primary resistant to dapsone, since both were new cases. Other isolates were regarded to be secondary resistant to dapsone. One isolate (Pasuruan1) harboured mutation on both gene (*folP1* and *rpoB*). The isolate was regarded to be secondary resistant to dapsone and rifampicin as the isolate was taken from relapse case assumed to be resistant.

Treatment with inappropriate regiments is one main reason of drug-resistant strains development and the occurrence of relapses with a resistant strain (Matsuoka 2010). It was found in one isolate (Makassar1) that had dapsone monotherapy. Beside that inconsistent prolonged treatment can also raises the drug-resistant strains (Robert 1976) unfortunately there is no data on medication compliance among isolates available in this study, so it could not be described further.

Here, we are describing a method for detecting the mutation that responsible for drug resistances by PCR followed by direct sequencing which is simpler and faster than mouse footpads culture method. It is also very convenience to conduct but the implementation in developing country such as Indonesia is still not easy. It is also not suitable and expensive for high number of sample. Development of simple detection methods as a leprosy drug susceptibility-DNA microarray (LDS-DA) and a reverse hybridization DNA strip test will be very much helpful for maintaining drug resistance study in developing country where the prevalence of leprosy is still high (Matsuoka 2010; Matsuoka *et al.* 2008; Cambau *et al.* 2012). This study supports and

complements on the information about mutation profile of drug resistance *M. leprae* isolates in Indonesia.

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