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Case Report

DAPSONE RESISTANCE IN A *Mycobacterium leprae* ISOLATE WITH TWO POINT MUTATIONS IN *folP* GENE FROM A LEPROSY PATIENT

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ABSTRACT

Drug resistance in leprosy is important for Leprosy Control Program, since the WHO-Multidrug regiment (MDT) has been used for global treatment of leprosy for more than two decades already. A Dapsone resistance case in a Multibacillary (MB) leprosy case is reported. The patient was diagnosed and treated in Tajuddin Chalid Hospital Makassar, South Sulawesi. Previously he was treated in a health center at South Sulawesi and was given a treatment for one year, before referred to the hospital. The leprosy skin lesions are still active with erythematous skin lesions and thickened ear lobe. Bacteriological examination was positive for Acid Fast Bacilli, the Bacterial Index was 3+ and the Morphological Index was 1%. The specimens of M.leprae isolation was sent to the Institute of Tropical Disease Surabaya for drug resistance study. Using the Lp1-2 and Lp3-4 nested primers, PCR test was positive for M.leprae. Sequencing result for folP gene showed a double mutation at codon 53 (ACC / Threonin) which become (AGG / Arginine). Simultaneous mutation at two nucleotides at one codon has never been reported in Indonesia before and this phenomenon is important for leprosy control policy.

Keywords: *leprosy, M.leprae, dapsone resistance, folP gene, mutation*

ABSTRAK

Latar Belakang: *Resistensi obat pada pengobatan kusta adalah faktor penting untuk Program Pengendalian Kusta, Sejak WHO mencanangkan penggunaan Multi Drug Regiment (MDT) untuk pengobatan kusta secara global, dalam kurun waktu lebih dari dua dekade resistensi Dapson dalam kasus (MB) kusta Multibasiler di Indonesia dilaporkan. Pasien didiagnosis dan dirawat di Rumah Sakit Tajuddin Chalid Makassar, Sulawesi Selatan. Sebelumnya ia dirawat di sebuah pusat kesehatan di Sulawesi Selatan dan mendapat perawatan selama satu tahun, sebelum dirujuk ke rumah sakit. Kulit kusta lesi masih aktif dengan kulit eritematosa lesi cuping telinga menebal.* ***Metode:*** *Pemeriksaan Bakteriologis adalah positif untuk Basil Tahan Asam, Indeks bakteri adalah 3 + dan Indeks Morfologis 1%. Spesimen dari pasien dikirim ke Institute of Tropical Disease Surabaya untuk studi resistensi obat. Spesimen dideteksi terlebih dahulu adanya basil M.leprae dengan analisa PCR menggunakan primer LP1-2 dan LP3-4.* ***Hasil:*** *Hasil Sequencing untuk gen folP menunjukkan mutasi pada kodon 53 (ACC / Threonin) menjadi (AGG / Arginine). Mutasi simultan di dua nukleotida pada satu kodon belum pernah dilaporkan di Indonesia sebelumnya dan fenomena ini penting untuk kebijakan pengendalian kusta.*

Kata Kunci: *leprosy, M.leprae, dapsone resistance, folP gene, mutation*

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, primarily attacks peripheral nerves and secondarily affects skin and other organs. The disease can cause disabilities and often creates social problems. Approximately 17.000 new leprosy cases are detected every year in Indonesia, and most of them grow in the Eastern part of the country.[1] The WHO Multi-drug Therapy (MDT) has been implemented for leprosy all over the world since 1980s, using a combination of 3 drugs: Rifampicine, Dapsone and Clofazimine.[2] Dapsone is the

oldest remedy for leprosy which has been used since 1940s as a monotherapy. Dapsone resistance in leprosy was firstly reported from Malaysia in 1964[3] and it was proven by mouse foot pad inoculation technique, which is difficult and time consuming.[4] Due to the difficulties in cultivating *M.leprae*, the molecular biology of the bacilli become very important. Development of molecular biology techniques has made some improvements in *M.leprae* studies,[5,6,7] including the detection of drug resistance study of leprosy, which is more rapid and accurate.[8] DNA sequencing of *folP gene* of the bacilli will give some information if there is a change or mutation which is related to Dapsone resistance. A case of leprosy with Dapsone resistance, proved by molecular biology study is reported. Some aspects related to drug resistance in leprosy are also discussed.

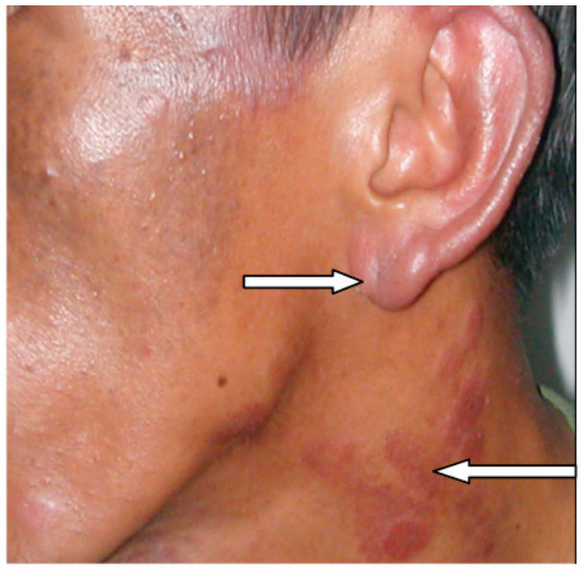

Figure 1. Borderline Lepromatous (BL type) of Leprosy

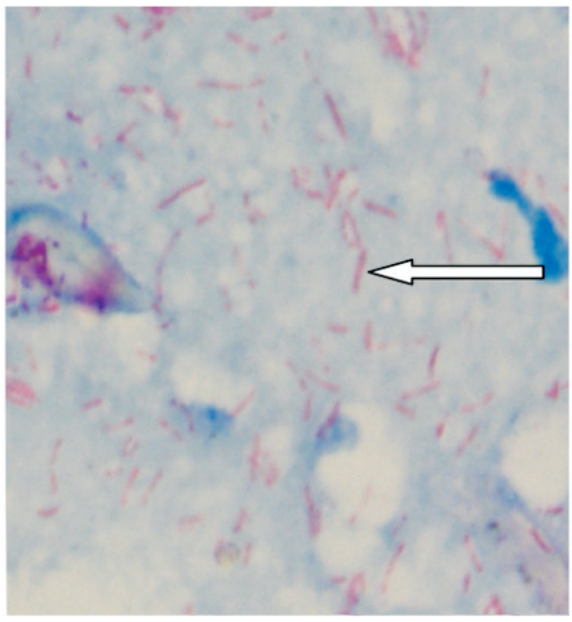

Figure 2. Acid Fast Bacili (AFB) Ziehl Neelsen staining

CASE

A thirty years old man from Makassar, South Sulawesi, was refered to Tajuddin Chalid Hospital Makassar due to a persistent skin lesion after 1 year treated for leprosy in the peripheral health center in South Sulawesi. This patient was given MDT drugs irregularly since he was a sailor. Clinical examination in the hospital revealed a Borderline Lepromatous (BL) Leprosy, with positive bacteriological examination. The Bacteriological Index (BI) was 3+, with the Morphological Index (MI) of 1%.

Skin slit smear specimen from this patient was sent to the Institute of Tropical Disease Universitas Airlangga Surabaya for drug resistance study. The study included Dapsone, Rifampicin and Quinolone resistance.

Poymerase Chain Reaction

Detection of *M.leprae* was perfomed by PCR study. DNA extraction was conducted by mixing the specimen with Qiagen kit. All samples identified the existence of *Mycobacterium leprae* by detection of the 18 kDa antigen *M.leprae* in region RLEP3 *repetitive element* (X17153) using nested PCR. Amplification will produce about a 129 bp for external (*outer*) and a 99 bp for internal (*inner*) product. PCR was carried out using a G mixture of FailSafe PCR System (EPICENTRE, Madison, WI, USA, Cat. No. FSP995G) in a 20 µl volume of reaction mixture containing at least 0.1 pg of genomic DNA in 2 µL of template *Taq* polymerase (Failsafe Cat. No. FS99250) and 2 µL of 5 µM primers. Primers Lp1 5' TGCATGTCATGGCCTTGAGG 3' and Lp2 5' CACCGATACCAGCGGCAGAA 3' and the amplification was conducted in a thermal-cycler-machine (*BioRad i-cycler*) under the conditions of 2 min at 98º C for preheating, 20 sec at 98º C for denaturation, 30 sec at 56º C for annealing and 30 sec at

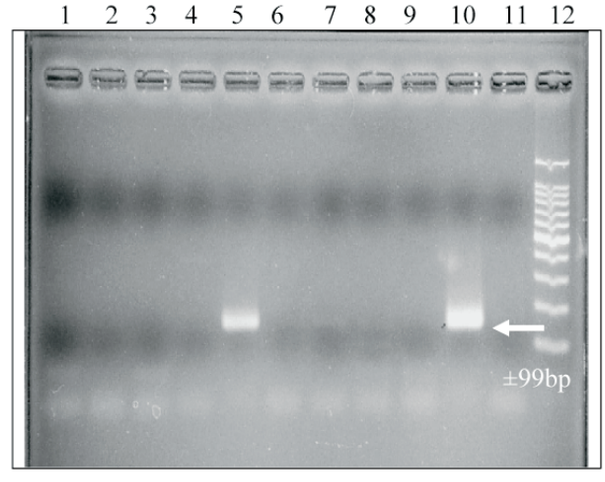

Figure 4. PCR Product of *M.leprae* Detection.
Lane 1-4, 6-9 : negative results
lane 5 : isolate from Makassar: positive result;
lane 10: PC, positive control (*M.leprae* strain *Thai-53*);
lane 11: NC, negative control;
lane 12: DNA size marker of 100bp DNA ladder

72º C for elongation/extension (repeated for 35 cycles) followed by prolonged extension of 5 min at 72º C and then inactivation at 4º C. Amplicon was then nested with primers Lp3 5' TGAGGTGTCGGCGTGGTC 3' and Lp4 5' CAGAAATGGTGCAAGGGA 3' under the conditions of 2 min at 98º C for preheating, 20 sec at 98º C for denaturation, 30 sec at 56º C for annealing and 30 sec at 72º C for elongation/extension repeated for 30 cycles followed by prolonged extension of 5 min at 72º C and then inactivation at 4º C. The full length of this amplicon was separated by electrophoresis in 3% (w/v) HS agarose gel (Cambrex Bioscience, Rockland, ME, USA) using TBE (Tris/Boric/EDTA, pH 8.0) buffer at 100 V. After amplification, the amplicon was distributed in agarose gel for electropheresis process and the PCR results were examined using UV light and recorded. (figure 3).

Amplification of *folP, rpoB* gene of *M.leprae* using their specific primers, all gave positive bands.

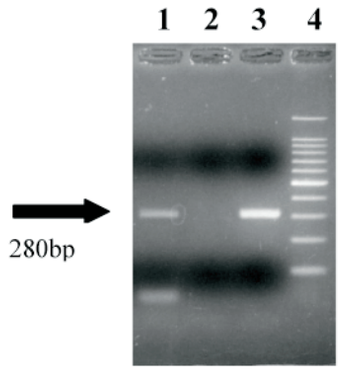

Figure 5. PCR Product of *folP* gene.
lane 1: isolate from Makassar
lane 2: NC, negative control
lane 3: PC, positive control (*M.leprae* strain *Thai*-53)
lane 4: DNA size marker 100bp DNA ladder.

DNA Sequencing Study

Using the Long Reed Tower machine, the results of DNA sequencing study of *rpoB* and *gyrA* gene revealed no mutations, but the sequence of the *folP* showed a mutation at codon 53 (figure 6a). The nucleotides changed from normally *ACC / Threonin* to *(AGG / Arginine* (figure 6b).

The other drug resistance study for Rifampicine (*rpoB gene*) and Quinolone (*gyrA gene*) revealed no mutation regarding this *M.leprae* isolation.

DISCUSSION

Our leprosy case showed a typically Borderline Leprosy (BL), and still no complication or disability. There were some hyperpigmented patches over the patient's body and earlobe thickness which indicated a Multibacillary leprosy.[9] There was peripheral nerve thickening over his both ulnar nerves, but no signs of neuritis. Since there is no data of previous bacteriological examination, it is difficult to categorize the patient as a relapse case or a back-to-

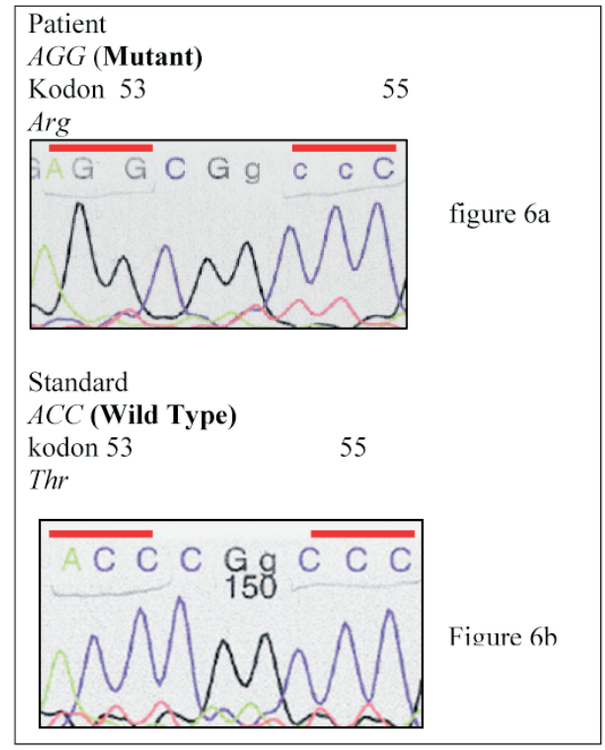

Figure 6. Nucleotides sequencing at codon 53, comparison of mutant type (figure 6a) and normal / wild type (figure 6b).

treatment case. As a sailor who had to sail for weeks or months, the patient could not take the drugs continuously and the MDT treatment became irregular. This situation resulted in persistent skin lesions and also the positive bacteriological examination.

Mycobacterium leprae still can not be cultivated in culture media until today. Detection of Dapsone resistance in the past was performed by injecting the bacilli to the mouse footpad (*in vivo* method), which needed about six months before the result could be established.[10,11] Using the molecular biology technique is possible now to detect the resistance in a few days. The Dihydropteroate synthase enzyme has been known as a target of Dapsone, which is an important enzyme for growth and metabolism of *M.leprae*.[12] The *folP* gene is responsible in the synthesis of this enzyme by coding the formation of amino acids which arrange the protein structure.[13] If a mutation occurs in this gene, the protein arrangement will change and the enzyme will also changed. The end result of this is the failure of Dapsone to inhibit the new enzyme, which means that the bacilli remains active or resistant to this drug.

Using this molecular biological techniques, many Dapsone resistance cases were reported from some Asian countries,[14,15,16] including Indonesia,[17,18] but the incidence was relatively low.

The normal sequence of nucleotides of the *folP* gene has been mapped completely and could be retrieved from GeneBank.[19] The mutation sites usually occurred at codon 53 (normally ACC/Threonine) and only involved one

nucleotide change (i.e. GCC/Alanine or AGA/Arginine). But, in our case we found double mutations, from ACC/ Threonine into AGG/Arginine.

Missense mutations associated with Dapsone resistant in *M.leprae* has been documented and could be outlined as follows.

Table 1. Dapsone resistant in *M.leprae*

drug	*gene*	*Codon no.*	*susceptible*		*resistant*	
Dapsone	folP	53	ACC	(Thr)	GCC	(Ala)
					GTC	(Val)
					ATC	(Ile)
					AGG	(Arg)
					AGA	(Arg)
		55	CCC	(Pro)	TCC	(Ser)
					CGC	(Arg)
					CTC	(Leu)

Adapted from: Leprosy, Science working towards dignity. p 63[21]

From a molecular biology point of view, this type of mutation is relatively rare and should be paid more attention for preventing the spread of primary resistance to Dapsone.[20] Since the WHO-MDT regiment contains Dapsone for daily treatment, an alternative regiment should be anticipated for Dapsone resistant case.

Our case had been treated with Dapsone irregularly, which probably induces the mutation of the bacilli. Although resistance to other drugs (Rifampicine and Quinolone) was not found, the irregular treatment of this patient could induce another drug resistance. This resistance will be solved by changing the Dapsone with other anti leprosy drugs while the patient should take the medicine regularly. It needs patient education and monitoring of regularity of treatment.

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