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Molecular detection of primary dapsone resistant *Mycobacterium leprae* in Myanmar

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Currently recommended control measures for treating leprosy with multi-drug therapy (MDT) should control the spread of drug resistant strains; however, dapsone (DDS) resistance continues to be reported. Comprehensive estimates of drug-resistant leprosy are difficult to obtain due to the cumbersome nature of the conventional drug susceptibility testing method using mouse footpad inoculation, which requires at least 6 months to obtain results. Recently, it has been determined that DDS resistant strain contains missing mutations in codon 53 and 55 of the *fol* P1 gene of *Mycobacterium leprae*, and definitive evidence linking these mutations with DDS resistance in *M. leprae* has been obtained. Based on these mutations, *fol* P1 gene contained these mutation points (hot spots) was amplified by polymerase chain reaction (PCR) and followed by sequencing to detect mutations. A total of 50 multibacillry leprosy cases before MDT treatment attending Central Special Skin Clinic, Yangon General Hospital were determined *fol* P1 gene mutations. DDS resistance was detected in four cases (8%). This study is part of the detection of multi-drug resistant leprosy in Myanmar.

INTRODUCTION

Primary dapsone (DDS) resistance arises by the infection of new persons with resistant bacteria often shed by a person with acquired resistance to DDS. Primary DDS resistance is very prevalent in many leprosy endemic areas of the world and represents a serious potential threat to the leprosy control programs in these regions [1, 2].

During the late 1960 and 1970, there were alarming reports of increasing secondary and primary DDS resistance at the end of DDS mono-therapy era, which prompted the World Health Organization (WHO) to recommend the introduction of MDT, using DDS, rifampicin and clofazimine [3], which should control the spread of drug resistant strains. DDS remains the first line drug for the treatment of leprosy as well as key component in the chemotherapeutic regimens of MDT. However, DDS resistant strains of *M. leprae* continue to be reported even in areas of world with successful implementation of MDT [4, 5, 6]. Although DDS has a weaker bactericidal action than rifampicin, we need to prevent the development of resistance to any component drug in MDT schedules. One could image a dangerous scenario developing in which high level primary DDS resistance and poor clofazimine compliance leads effectively to rifampicin monotherapy, a condition in which it is already known that rifampicin resistance in leprosy can develop [7].

In Myanmar, DDS mono-therapy was introduced in 1952 and MDT was introduced in 1986, although the coverage of MDT reached 100% a decade later (1996). Since 1980, Mar Mar Nyein *et al.* have been testing DDS resistance using the mouse footpad model [8]. DDS resistance

prevalence survey was done in Myingyan area in 1980 and 1983. Out of 779 lepromatous patients who had been treated with DDS mono-therapy for more than 5 years (90% of them had been treated for more than 10 years), 301 out of 771 (38.6%) were found to have DDS resistant leprosy and annual incidence in the 2 subsequent years was 40 to 45 per 1000 lepromatous patients or 3.8% per year [8].

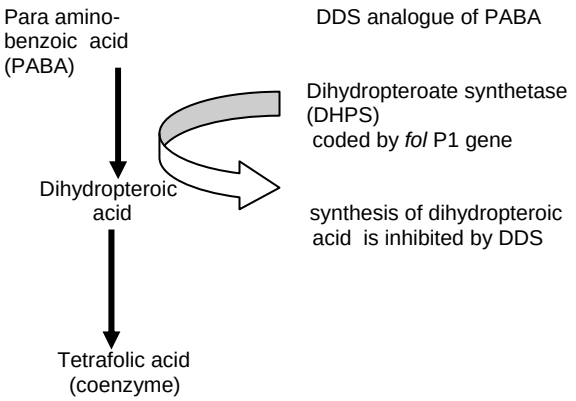
The genetic basis of DDS resistance has been described [9]. Although genetic detection of drug resistance is technically sophisticated, the application of technology in selected leprosy endemic areas will allow a more rapid and widespread assessment of the problem. The gene method was first validated by paralleled mouse footpad experiments in order to be sure what level of DDS resistance was being detected. The results of recent studies on the molecular mechanism of DDS resistance in *M. leprae* demonstrated that the target of DDS is the enzyme dihydropteroate synthetase (DHPS). DDS resistance appears to be the results of mutations in the *fol* P1 gene encoding for DHPS in *M. leprae* [6]. Although many DDS resistant cases have been detected in Myanmar [8], no study has examined the molecular characteristics of the DDS resistant strains or the status of DDS resistance in the country. The aim of the present study, based on PCR and a direct DNA sequencing assay, was to determine the prevalence of *fol*P1 mutations in *M. leprae* isolates from clinical cases of DDS resistant leprosy in Myanmar.

Molecular biological detection of dapsone resistant Mycobacterium leprae

M. leprae has not been cultivated on artificial media, therefore, to identify drug susceptibility pattern, bacteria are tested using mouse footpad technique. This *in vivo* method requires long time and relatively large number of bacteria. Nowadays, molecular biological techniques for DDS resistant *M. leprae* can be done by detection of mutations in the *fol* P1 gene.

The *fol*P1 gene of *M. leprae*, which encodes dihydropteroate synthetase (DHPS) was studied for the presence of mutations associated with resistance to dapsone. When the *fol*P1 genes of several DDS resistant clinical isolates of *M. leprae* were sequenced, two missense mutations were identified. One mutation occurred at codon 53, substituting isolucine for threonine in DHPS-1, and a second mutation occurred at codon 55 substituting arginine for proline [9]. DHPS is a key enzyme involved in de novo synthesis of folate catalyzing synthesis of 7,8 dihydropteroate from 7,8 dihydroprotein-pyrophosphate and para-aminobenzoic acid (PABA). The mode of action of the DDS is to inhibit the synthesis of 7,8 dihydropteroate by the competitive incorporation of sulphonamide as the structural analogue of PABA.

Bacteriostatic mechanism of DDS



Dihydropteroate synthetase of DDS resistant bacteria does not bind DDS.

MATERIALS AND METHODS

Sample collection

After taking informed consent, skin scrap samples were collected from leprosy patients attending the Central Special Skin Center (CSSC), Yangon General Hospital. Fifty MB patients with clinically and bacteriologically documented disease agreed to participate in the study. Leprosy patients were classified clinically and microscopically according to WHO classification [10] which consists of two

categories, paucibacillary (PB) and multi-bacillary (MB). PB leprosy is defined as five or fewer skin lesions with no bacilli in skin smears, and MB leprosy cases have six or more lesions and may be skin smear positive. The project was approved by Institutional Ethical Review Committee, Department of Medical Research (LM).

Specimen preparation

The collected skin scrapings were dipped in 1.5 ml tubes containing 0.5 ml of 70% ethanol and stored at room temperature until prepared for DNA template by Klatser's Method [11].

DNA template preparation (DNA extraction)

DNA was prepared from skin scraps according to the method of Klatser *et al*. Briefly, under sterile condition, the blade was scratched by wood prick and centrifuged at 14,000 rpm for 10 minutes. After discarding the supernatant, the precipitate was washed with PBS and centrifuged again at 14,000 rpm for 10 minutes to remove remaining alcohol. The washed precipitate was suspended in 50 µl of lysis buffer containing proteinase K 10 mg/ml in 1M Tris-HCL, pH 8.5 and 0.5% Tween 20 and incubated at 60°C for 18 hours. Five microlitre of mineral oil was over-layed to prevent evaporation of water from the mixture. After heating at 97°C for 10 minutes, the suspended solution was treated with freezing and thawing twice to inactivate proteinase K, which inhibits Taq polymerase during PCR.

The DNA polymerase chain reaction (PCR)

A set of primers (*fol*P F and *fol*P RS) was used for amplification of the specific region of *M. leprae fol*P1 gene. Chromosomal *M. leprae* DNA was kindly supplied by Dr. M. Matsuoka, Leprosy Research Center, NIID, Japan. This DNA served as a positive control in all PCR experiments. The 50 µl reaction mixture contained 10 µl of template solution, 0.2 µl of *Ex Taq* DNA polymerase (Takara Shuzo Co., Shiga, Japan), 1 µM of each primer, 5 µl of 10x DNA PCR buffer, 8 µl of dNTP solution and 25.8 µl of water. The reaction mixture was overlayed with 5 µl of mineral oil. The reaction was performed with programmable thermal Mastercycler (eppendorf USA).

The reaction mixture was heated 94°C for 1 min, rounds of amplification consisted of a 30 second denaturation step at 94°C, a 2 min annealing step at 55°C and a 3 min elongation step at 72°C for 45 cycles. The amplified DNA fragments were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels in Tris-Borate EDTA (TBE) buffer.

Following primers were used in PCR

Genes	Primers	Sequence
fol P 1	*Fol* P F	GCTTCTCGTGCCGAAGCGCTC
	Fol P RS	GCAAGTTCTTTTACGACAGG

DNA sequencing

First PCR products were electrophoresed in 1.5% agarose gel. The gel was cut and DNA recovery was done by Easy Trap Kit (TaKaRa Shuzo). DNA sequencing was done by BigDye Terminator Cycle Sequencing, FS Ready Reaction kit (Perkin-Elmer applied Bio system, Norwalk, Conn.) and ABI Prism 310 genetic analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed by the DNASIS computer program (Hitachi Software Engineering, Yokohama, Japan).

RESULTS

A total of 50 new MB leprosy patients attending the Central Special Skin Center (CSSC), YGH were studied to detect *M. leprae* from skin scrap by PCR using the *fol*P1 gene amplification and following sequencing to detect mutations. In 50 MB patients including both BI positive and negative cases, 4 patients (8%) showed mutations. Three cases revealed mutations

Homology region [All regions]

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                20                30                40                50                60
M-12. Seq       GAGAGTTTGGCGCCAGTGCAGGTTTTGGGGTTTTGAACGTACTGACAATTCGTTCTCA
Fol P -5'       GAGAGTTTGGCGCCAGTGCAGGTTTTGGGGTTTTGAACGTACTGACAATTCGTTCTCA
                10                20                30                40                50

                70                80                90               100               110
M-12.Seq        GATTGGCGGACGTTGACTCCTGACGATGCTGCCCAGCGGCCTGGCAATGGTCGCG
Fol P-5'        GATTGGCGGACGTTGACTCCTGACGATGCTGCCCAGCGGCCTGGCAATGGTCGCG
                60                70                80                90               100

               120               130               140               150               160
M-12.Seq        GAAGGCGCGGCGATTGTCGACGTCGGTGGCGAATCGGCCCGGCCCGGTGCCATTAG
Fol P- 5'       GAAGGCGCGGCGATTGTCGACGTCGGTGGCGAATCGACCCGGCCCGGTGCCATTAG
                110               120               130               140               150

                170               180               190               200
M-12.Seq        GATCCTCGAGTTGAACTCTCTCGTATCGTTCCTGTCGTAAAAGAACTTGC
Fol P-5'        GATCCTCGAGTTGAACTCTCTCGTATCGTTCCTGTCGTAAAAGAACTTGC
                160               170               180               190
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Fig. 1. Nucleotide sequence of the target region of the *fol*P1 gene from Myanmar sample (resistant strain) and Thai 53 (sensitive strain)
Mutation occurred at codon 53, substituting alanine (GCC) for threonine (ACC) and no mutation at codon 55 (CCC) in Myanmar sample No. 12 (M-12).

Homology region [All regions]

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                20                30                40                50                60
M-11. Seq       GTGAGTTTGGCGCCAGTGCAGGTTATTGGGGTTTTGAACGTCACTGACAATTCGTTCT
Fol P -5'       GTGAGTTTGGCGCCAGTGCAGGTTATTGGGGTTTTGAACGTCACTGACAATTCGTTCT
                10                20                30                40                50

                70                80                90               100               110
M-11.Seq        GATGGCGGACGTTACCTTGATCCTGACGATGCTGTCCAGCACGGCCTGGAATGGTCG
Fol P-5'        GATGGCGGACGTTACCTTGATCCTGACGATGCTGTCCAGCACGGCCTGGAATGGTCG
                60                70                80                90               100

               120               130               140               150               160
M-11.Seq        GAAGGCGCGGCGATTGTCGACGTCGGTGGCGAATCGACCCGGCCCGGTGCCATTAG
Fol P- 5'       GAAGGCGCGGCGATTGTCGACGTCGGTGGCGAATCGACCCGGCCCGGTGCCATTAG
                110               120               130               140               150

                170               180               190               200
M-11.Seq        GATCCTCGAGTTGAACTCTCTCGTATCGTTCCTGTCGTAAAAGAACTTGC
Fol P-5'        GATCCTCGAGTTGAACTCTCTCGTATCGTTCCTGTCGTAAAAGAACTTGC
                160               170               180               190
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Fig. 2. Nucleotide sequence of the target region of the *fol*P1 gene from Myanmar sample (sensitive strain) and Thai 53 (sensitive strain)
No mutation occurred at codon 53 and codon 55 from Myanmar sample No.11 (M-11).

Table 1. Mutations in the *fol*P1 genes of *M..leprae* from leprosy patients

Case No.	Age	Sex	Diagnosis & treatment	*fol* P1 condon	Mutations	Mutant amino/a in DHPS
4	17	F	MB-MDT	53	ACC - ATC	Isoleucine
9	52	M	MB-MDT	53	ACC - AGA	Arginine
12	47	M	MB-MDT	53	ACC - GCC	Alanine
34	60	F	MB-MDT	55	CCC - CAC	Histidine

DHPS = Dihydropteroate synthetase
MB-MDT = Multibacillary – Multidrug Treatment

at condon 53 and one case detected mutation at codon 55 (Table 1). Fig.1 shows mutation at codon 53 substituting alanine (GCC) for theonine (ACC). Fig. 2 shows no mutation at codon 53 and 55.

DISCUSSION

Dapsone is still one of the most important of anti-leprosy drug used today. It is stable, inexpensive, relatively nontoxic and one of the mainstays of the WHO-MDT for both MB and PB leprosy. Unfortunately, development of acquired or secondary resistance in patients treated with DDS mono-therapy for many years has provided a source of infection resulting in the appearance of primary DDS resistance among new patients diagnosed with the disease.

This study is part of the research on detection of multi-drug resistant leprosy in Myanmar. Multidrug resistant leprosy means resistance to both DDS and rifampicin. We performed detection of *fol*P1 mutation for DDS resistance and *rpo*B gene mutation for rifampicin resistance simultaneously. Fortunately, these 4 cases of DDS resistance were susceptible to rifampicin, which is an exceptionally potent bactericidal agent against *M. leprae*. A single dose of 600 mg is capable of killing more than 99.9% of viable organisms [3]. It is a key drug in MDT. These 4 primary DDS resistant cases were given anti-leprosy drugs of rifampicin and clofazimine for 12 months as WHO regime and will be taken regular follow-up for clinical examination and slit skin smear to check *M. leprae*.

In the present study, relatively simple and rapid molecular techniques (PCR and direct sequencing) were applied in an effort to determine *fol*P1 mutations in *M. leprae* isolates. The mutations indicate that mutations in codons 53 or 55 of *M. leprae fol*P1 are responsible for DDS resistance in Myanmar. This information should lead to a better understanding of the status of DDS resistant leprosy in Myanmar and assist in the diagnosis of DDS resistant *M. leprae*.

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