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Dot blot hybridization method for rapid detection of drug resistant *Mycobacterium leprae* in Myanmar

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Information of susceptibility for the *Mycobacterium leprae* isolates is beneficial for the proven multi-drug treatment and for verification of the efficacy of current leprosy control program. The simple genotypic method to detect mutations conferring resistance to dapsone (DDS), rifampin, and quinolone was exploited on the basis of hybridization with capture probe fixed to the glass slide. Mutations were discriminated by a series of oligonucleotide probes corresponding to each mutation in the *fol*P, *rpo*B, and *gyr*A genes of *Mycobacterium leprae*. Based on these mutations, gene contained these mutation points (hot spots) was amplified by polymerase chain reaction (PCR) and followed by dot blot hybridization to detect mutations. This method is simple, rapid and not very expensive. The result can be obtained within 8 hours. A total of 100 mutibacillary leprosy cases attending the Central Special Skin Clinic, Yangon General Hospital were determined gene mutations. Ninety-three cases (93%) were susceptible to three drugs and seven cases (7%) were resistant. Among resistant cases, 3 were DDS resistance, 1 was rifampicin resistance, 2 were quinolone resistance and 1 was both DSS and rifampicin resistance (MDR).

INTRODUCTION

Current strategy of leprosy control relies mainly on multi-drug treatment (MDT). Although information of susceptibility for the isolates contributes to better treatment, susceptibility test is not done in almost all areas with high prevalence of leprosy. Comprehensive surveillance for the spreading of resistant cases offers useful information to evaluate efficacy of MDT and to prevent spreading of resistant strains, but a little is known about that. On the other hand, susceptibility test for anti leprosy drugs has been done by cumbersome and time-consuming mouse foot-pad method for over three decades, however, recent advances in molecular biology for drug resistance enabled to test susceptibility to dapsone, rifampin, and quinolone by the detection of relevant mutations which confer resistance in each drug. Analysis of mutations is done by sequencing of target genes so far. Implementation of sequencing is not easy in some developing countries. In this context, a simple and rapid method to detect mutations was exploited and transferred to developing countries from Leprosy Research Center, National Institute of Infectious Diseases, Japan.

The data obtained by the method were compared with the results of sequencing to evaluate the reliability of the method. Prevalence of the drug resistant cases in a few Asian countries was also analyzed by the sequencing of the *fol*P, *rpo*B, and *gyr*A genes.

MATERIALS AND METHODS

Sample collection

After getting informed consent, skin scrap samples were collected from leprosy patients attending the Central Special Skin Clinic (CSSC), Yangon General Hospital from 2005 to 2007 and 100 multibacillary (MB) patients with clinically and bacteriologically documented cases agreed to participate in the study. Leprosy patients were classified clinically and microscopically according to World Health Organization classification. 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 0.5ml of 70% ethanol and stored at room temperature until test and prepared for DNA template by Klatser's method [1].

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 is 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 50µl of lysis buffer containing proteinase K 10mg/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.

Exploitation of dot blot hybridization method (DBH) for detecting mutation

A series of oligonucleotide probes corresponding to each mutation for the *fol*P, *rpo*B, and *gyr*A genes which reveled to confer drug resistance in previous studies [2, 3, 4 & 5) was designed and tested empirically for the use of capture probe. Capture probes consist of 8 kinds of oligonucleotide for the *fol*P gene, 9 kinds of oligonucleotide for the *rpo*B gene, and 2 kinds of oligonucleotide for the *gyr*A gene respectively. Probes were absorbed on the glass slide. PCR to amplify simultaneously drug resistance determining regions (DMDRs) of the *fol*P, *rpo*B, and *gyr*A genes was performed in a tube using primers (Table 1) where reverse primers are biotinylated. Two microlitre of PCR products was mixed with 38µl of hybridization buffer followed by the incubation for 80 min at 42°C. After discarding hybridization buffer, glass slide was washed for 60 min at 47°C. PCR products hybridized with probes, were detected by the color developing with oxidization of 3',3',5',5',-tetra methyl benzidine (TMB) for 80 min at room temperature and then read by computer scanner.

Table 1. Primers used for the amplification target region in the *fol*P, *rpo*B and *gyr*A genes

Target gene	Forward primer	Reverse primer biotinylated
*fol*P	5'-ATGGTCGCGGAAGGCGCGGC-3'	5'-CAAGTTCTTTTACGACAGG-3'
*rpo*B	5'-TCGCCGCTATCAAGGAATTC-3'	5'-TCACGCGACAAACCACCCGG-3'
*gyr*A	5'-GAGACTCCGGTTTCCGCCC-3'	5'-GCAGCGACCACGGCTGCGC-3'

RESULTS

Prevalence of drug resistance deduced by the dot blot hybidization is shown in Table 2. Many clinical samples showed negative PCR results because of inadequate sampling and preservation. Together with mutations revealed in this study, oligonucleotide capture probes for wild type and mutant type shown in Table 3 were spotted on the glass slide. Mutation detection using DBH method was successfully conducted at both places (Fig.1). Coincidence between results of

DBH and sequencing was 99% respectively. Discordant results were obtained when the intensity of color developing was not good in DBH.

Table 2. Results of DBH on 2005-2006 & 2006-2007 in Myanmar

Total tested	2005-2006	2006-2007	Total
	50	50	100
Susceptible to 3 drugs	46 (92%)	47 (94%)	93 (93%)
Total resistant cases	4 (8%)	3 (6%)	7 (7%)
DDS resistance	2 (4%)	1 (2%)	3 (3%)
Rifampin resistance	1 (2%)	0 (0%)	1 (1%)
Quinolone resistance	0 (0%)	2 (4%)	2 (2%)
*MDR (DDS & Rifam)	1 (2%)	0 (0%)	1 (1%)

*MDR = Multi-drug Resistance

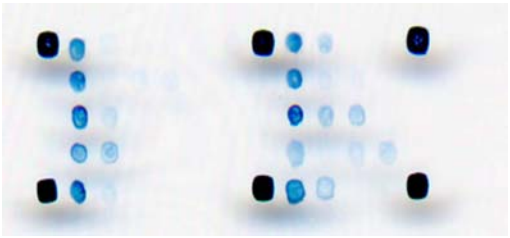

Fig. 1. DBH results for a susceptible case

Table 3. Sequence of capture probes

Gene	Position	Wild type	Resistant mutant
*fol*P	53	GTGGCGAATCG ACCCGG	TGGCGAATCGGCCCGG TGGCGAATCGGTCCGG TGGCGAATCGATCCGG GGCGAATCGAGGCGG TGGCGAATCGAGACGG
	55	CGGCCCGGTGC CATTA	GACCGGTCCGGTGCC GACCGGCGCGGTGCC GACCGGCTCGGTGCC
*rpo*B	513	AGCTGTCGCAGT TCATG	AGCTGTCGGTGTTCAT
	516	TTCATGGATCAG AA	TTCATGAATCAGAACAA TTCATGTATCAGAACAA
	526	CCTGACCCACAA GCGC	GCCTGACCTACAAGCGC GCCTGACCGACAAGCGC
	531	CGCCGACTGTC GGCGCTG	CCGACTGATGGCGC GCCGACTGTTGGCG GCCGACTGTTCGCG
	533	GCGCTGGGGCC CGGGTG	GGCGCCGGGGCCCGGG TG
*gyr*A	89	ATCCGCACGGC GACGCA	ATCCGCACTGCGACGCA
	91	CGGCGACGCAT CGATTT	CGGCGACGTATCGATTT
Positive hybridization control in the *gyr*A			GGACCGTAGCC ACGCTAA
Negative hybridization control in the *gyr*A			GGACCGTCAT CACGCTAA

DISCUSSION

Detection of drug resistance in *M. leprae* is crucial for the efficient treatment of leprosy and the prevention of the spread of drug resistant strains. The elucidation of the genetic background of resistance by molecular methods has enabled the prediction of drug susceptibility of *M. leprae*. Drug resistance to dapsone, rifampicin and ofloxacin has evolved by mutation in drug resistance determining regions in the *fol*P, *rpo*B, and *gyr*A genes respectively [6].

Although the direct sequencing of PCR products is definitive and allows rapid detection of resistant cases, it has the shortcomings of requiring expensive apparatus and high sequencing costs, so that it is not practical in many developing countries. The heteroduplex method (HAD) [7] and the PCR-single strand conformation polymerphism method (SSCP) [2] have been applied to the detection of mutants to overcome these disadvantages. The HAD method can identify mutations in the PCR amplified fragments by the electrophoretic mobility difference of heteroduplexes of wild-type products and test sample products, while the SSCP method analyzes that of single stranded products.

However, neither the HAD nor the SSCP method fully meets the required conditions in developing countries, since these methods demand complicated procedures and both methods detect silent mutations as resistant mutations. The recently developed Line Probe assay based on reverse hybridization can detect rifampicin resistant *M. leprae* simply and rapidly, but it cannot provide susceptibility information for other anti-leprosy drugs. The multiple-primer PCR amplification refractory mutation system is relatively simple but detects only nucleotide mutations and cannot distinguish silent mutations from missense mutations [8].

Our present study aimed to exploit a rapid, simple and simultaneous drug susceptibility test for three key anti-leprosy drugs to solve defects of each method previously reported,

based on DNA-DNA hybridization using a DNA microarray. The novel method, designated DBH, allows the simultaneous identification of mutations in three genes, responsible for resistance to dapsone, rifampicin and the quinolones. Easy accessibility and high reproducibility demonstrated by the studies with clinical materials in developing countries revealed the superior applicability of this method. The monitoring of drug resistant leprosy cases has been recommended in order to maintain the effectiveness of chemotherapy for leprosy [9, 10]. The DBH method developed in this study seems to be a simple and robust tool to assess the drug susceptibility of *M. leprae* in developing countries where susceptibility testing is rarely applied. Comprehensive data on the prevalence of resistant cases show that the level of drug resistance is low in some endemic countries [10].

It is, therefore, recommended to apply this method to samples from intractable cases and relapsed cases, to examine the susceptibility to anti-leprosy drugs and ensure effective treatment. Additionally, the capacity of the DBH method to identify the positions of mutations can be utilized for molecular epidemiological and geographical studies on the spread of drug resistant *M. leprae.*

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