A novel method for simple detection of mutations conferring drug resistance in *Mycobacterium leprae*, based on a DNA microarray, and its applicability in developing countries

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A simple method to detect mutations in the genome of *Mycobacterium leprae* that confer resistance to key drugs for leprosy was exploited on the basis of a reverse hybridization system. A series of oligonucleotide probes corresponding to each mutation in the *folP1*, *rpoB* and *gyrA* genes for dapsone, rifampicin and ofloxacin resistance, respectively, were selected and fixed on a glass slide as capture probes, to develop a DNA microarray termed the leprosy drug susceptibility-DNA microarray (LDS-DA). Mutations in clinical isolates of *M. leprae* were successfully identified by the LDS-DA. Feasibility studies were conducted to evaluate the performance of the LDS-DA in two developing countries, Myanmar and the Philippines. The high concordance of the results obtained by this method with the results of nucleotide sequencing strongly supports the applicability of the LDS-DA as a drug susceptibility test in place of sequencing, a time-consuming and costly procedure. This is a rapid and simple method for the simultaneous susceptibility testing of three front-line drugs for leprosy, and solves the problems of previously reported methods.

Received 14 April 2008
Accepted 18 June 2008

INTRODUCTION

The current strategy for leprosy control relies mainly on multidrug therapy (MDT) (WHO, 1998). However, cases of leprosy caused by drug-resistant *Mycobacterium leprae* have been documented as the result of therapeutic failure (Cambau *et al.*, 2001; Maeda *et al.*, 2001; Matsuoka *et al.*, 2000, 2003). Although information on the drug susceptibility of clinical isolates contributes to the better outcome of treatment, susceptibility testing has rarely been done because of its difficulty. Antibiotic susceptibility testing of *M. leprae* still relies on a time-consuming method based on the growth of bacteria in mouse footpads (Shepard, 1960),

which takes up to 12 months to give a result. This has hindered the comprehensive surveillance that would offer useful information to evaluate the efficacy of MDT and to prevent the spread of drug-resistant strains. Recent advances in the molecular biology of drug-resistant *M. leprae* have enabled the development of drug susceptibility tests for key component drugs of MDT, by the detection of relevant gene mutations that confer resistance (Williams & Gillis, 2004). The molecular mechanism of rifampicin resistance was first demonstrated in *Escherichia coli* and thereafter in *M. leprae* (Honoré & Cole, 1993). Rifampicin resistance is strongly correlated with mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase (Honoré & Cole, 1993; Honoré *et al.*, 1993; Williams *et al.*, 1994, 2001; Matsuoka *et al.*, 2000, 2003; Cambau *et al.*, 2001; Maeda *et al.*, 2001; Zhang *et al.*, 2004). Resistance to fluoroquinolones has been proved to correlate with

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Abbreviations: BI, bacterial index; DRDR, drug-resistance-determining region; LDS-DA, leprosy drug susceptibility-DNA microarray; MDT, multidrug therapy.

mutations in the *gyrA* gene, encoding the A subunit of DNA gyrase in *M. leprae* (Cambau *et al.*, 1997; Matsuoka *et al.*, 2000; Cambau *et al.*, 2001), as in many other bacteria. In addition, mutations in the *folP1* gene, encoding dihydrofolate synthetase, have been shown to be responsible for dapsone resistance (Kai *et al.*, 1999; Matsuoka *et al.*, 2000, 2003, 2007; Williams *et al.*, 2000; Lee *et al.*, 2001; Maeda *et al.*, 2001; Cambau *et al.*, 2006). The prevalence of drug resistance in selected areas was surveyed through the application of molecular analysis to detect mutations conferring drug resistance (Matsuoka *et al.*, 2007). Analysis of mutations is generally performed by sequencing the target genomic region, amplified by PCR, although the implementation of sequencing is not easy in many developing countries. Therefore, a simple and rapid method to detect mutations conferring drug resistance has been long awaited. In the current study, a DNA microarray method was developed and the applicability of this system was evaluated in Myanmar and the Philippines.

METHODS

Design of capture probes. Mutant nucleotide sequences conferring resistance to dapsone, rifampicin and ofloxacin and their corresponding wild-type sequences in *Mycobacterium leprae* (Table 1) were employed in this study. Nearly all the drug-resistant strains of *M. leprae* reported so far are covered by the mutations selected. Capture oligonucleotide probes (14- to 18-mer) for the detection of the

mutations were designed according to these data. Optimal sequences of oligonucleotides corresponding to each missense mutation were designed empirically as shown in Fig. 1(a). The array of capture oligonucleotide probes was covalently bound to the surface of a glass slide coated with polycarbodiimide and the resulting DNA microarray was designated the leprosy drug susceptibility-DNA microarray (LDS-DA), as shown in Fig. 1(b).

Amplification of three target gene fragments. Target regions of *folP1* (accession no. AL 583917, gene ML583917), *rpoB* (accession no. AL583923, gene ML1891) and *gyrA* (accession no. AL583917, gene ML0006) were simultaneously amplified with three primer pairs in one PCR. The sequences of the primers are listed in Table 2. PCR was carried out using the G mixture of the FailSafe PCR System (EPICENTRE) in a volume of 25 µl with 1 µM of each primer. Cycling conditions began with an initial incubation at 94 °C for 4 min, followed by 40 cycles of annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and denaturation at 94 °C for 30 s. Finally, incomplete PCR products were extended for 5 min at 72 °C. The amplified DNA fragments were confirmed by gel electrophoresis through 4.0 % Metaphor Agarose (FMC Corp.) in TBE (Tris/borate/EDTA, pH 8.0) buffer.

LDS-DA assay. A 2 µl aliquot of the resulting PCR mixture was mixed with 38 µl UniHyb Hybridization Solution (TeleChem International), heat denatured at 98 °C for 5 min and quickly chilled. The solution was then applied to the LDS-DA and incubated at 42 °C for 60 min followed by stringent washing with 50 µl washing solution (3 M tetramethylammonium chloride; Sigma-Aldrich) at 47 °C for 60 min. The biotin-labelled DNA fragments hybridizing to the capture probes on the LDS-DA were detected by avidin-biotin-horseradish peroxidase complex (VECTASTAIN Elite ABC kit, Vector

Table 1. Missense mutations associated with drug resistance in *M. leprae*

Drug	Gene	Codon no.	Susceptible		Resistant		References†
			Codon	AA*	Codon	AA*	
Dapsone	<i>folP1</i>	53	ACC	Thr	GCC	Ala	1, 2, 3
					GTC	Val	4
					ATC	Ile	2, 3, 5, 6
					AGG	Arg	3
		55	CCC	Pro	AGA	Arg	4
					TCC	Ser	1, 7
					CGC	Arg	1, 3
					CTC	Leu	2, 3, 8
Rifampicin	<i>rpoB</i>	407	CAG	Gln	GTG	Val	9
		410	GAT	Asp	AAT	Asn	2
					TAT	Tyr	8
		420	CAC	His	TAC	Tyr	2
					GAC	Asp	9, 10
		425	TCG	Ser	ATG	Met	9, 10
					TTG	Leu	2, 9
					TTC	Phe	11, 2, 6, 7, 3
Ofloxacin	<i>gyrA</i>	427	CTG	Leu	CCG	Pro	2
		89	GGC	Gly	TGC	Cys	2
		91	GCA	Ala	GTA	Val	12, 2, 6

*AA, amino acid.

†1, Lee *et al.* (2001); 2, Maeda *et al.* (2001); 3, Williams *et al.* (1994); 4, Matsuoka *et al.* (2007); 5, Kai *et al.* (1999); 6, Matsuoka *et al.* (2000); 7, Matsuoka *et al.* (2003); 8, Zhang *et al.* (2004); 9, Cambau *et al.* (2001); 10, Honoré & Cole (1993); 11, Honoré *et al.* (1993); 12, Cambau *et al.* (1997).

(a)

Drug	Gene	Codon no.	Capture probes for wild-type	Capture probes for mutants
Dapsone	<i>folP1</i>	53	FW1 : GTGGCGAATCG <u>ACCC</u> GG	FM1 : TGGCGAATCGG <u>CC</u> CGG FM2 : TGGCGAATCGG <u>TC</u> CGG FM3 : TGGCGAATCGA <u>TC</u> CGG FM4 : GGCGAATCGA <u>GG</u> CGG FM5 : TGGCGAATCGA <u>G</u> CGG FM6 : GACCGGT <u>CC</u> GGTGCC FM7 : GACCGGCGCGGTGCC FM8 : 8GACCGGCTCGGTGCC
		55	FW2 : CGG <u>CCC</u> GGTGCCATTA	
Rifampicin	<i>rpoB</i>	407	RW1 : AGCTGT <u>CGCAG</u> TTCATG	RM1 : AGCTGT <u>CGG</u> TGTTCAT
		410	RW2 : TTCATGGA <u>T</u> CAGAA	RM2 : TTCATGA <u>AT</u> CAGAACAA RM3 : TTCATGTATCAGAACAA
		420	RW3 : CCTGACCC <u>ACA</u> AGCGC	RM4 : GCCTGACCT <u>ACA</u> AGCGC RM5 : GCCTGACCGACAAGCGC
		425	RW4 : CGCCGACTGT <u>CGG</u> CGCTG	RM6 : 6CCGACTGAT <u>G</u> CGCGC RM7 : GCCGACTGT <u>TGG</u> CG RM8 : GCCGACTGT <u>TCG</u> CG RM9 : GGCGCGGGGCCCGGTG
		427	RW5 : GCGCTGGGGCCCGGGTG	
Ofloxacin	<i>gyrA</i>	89	GW1 : ATCCGCACGG <u>G</u> CACGCA	GM1 : ATCCGCAC <u>TGC</u> GACGCA
		91	GW2 : CGGCGACG <u>CAT</u> CGATT	GM2 : CGGCGACG <u>TAT</u> CGATT
Positive hybridization control in <i>gyrA</i>			GP : GGACCGTAGCCACGCTAA	
Negative hybridization control in <i>gyrA</i>			GN : GGACCGTCATCAGCTAA	

(b)

Fig. 1. Development of the LDS-DA. (a) The oligonucleotide sequences used in the test. Codons relating to drug resistance are underlined. Oligonucleotide GP, positive control for PCR amplification and hybridization; GN, negative control for hybridization. (b) Schematic representation of the array of oligonucleotides on the LDS-DA. Black circles represent spots with biotin as landmarks for conjugate reaction control; grey circles are the wild-type spots; white circles are the mutant spots. The region with oligonucleotides designated FW- and FM- is for dapsone resistance detection (the dapsone field); the region designated GW- and GM- is the ofloxacin field; the region designated GP- and GN- is the control field; and the region designated RW- and RM- is the rifampicin field.

Laboratories) and then visualized by TMB peroxidase substrate (Vector Laboratories). The resulting spot patterns were recorded by a conventional scanner and a computer. Only the results of the LDS-DA with proper signals on both positive and negative control spots (GP and GN in Fig. 1) were used for further analysis. The colour intensity of each spot in a row (covering the same region of each gene) was examined. The spot with the highest colour intensity was considered to reflect the sequence of the gene fragment in the sample.

Evaluation of the LDS-DA with clinical specimens. The LDS-DA system was transferred to laboratories in the Department of Medical

Research in Yangon, Myanmar, and in the Leonard Wood Memorial in Cebu, the Philippines, and was evaluated on 63 and 73 clinical specimens, respectively, in these laboratories. A majority of the samples in this study had been examined previously (Matsuoka *et al.*, 2007). Of the 63 samples in Myanmar, 44 were from new cases and 19 were from patients with relapse. Of the 19 relapsed patients, one patient had received monotherapy with dapsone for 4 years followed by monotherapy with rifampicin for 4 years, while the other 18 patients had been treated with the standard MDT regimen for multibacillary leprosy. Samples from the Philippines included 64 from new cases and nine from relapsed cases. Of the nine relapsed

Table 2. Sequences of oligonucleotide primers for *M. leprae*

Gene	Primer	Sequence (5'–3')	PCR products (bp)
<i>folP1</i>	MLfolP1DA-Ft;	GTGAGTTTGGCGCCAGTGCA	119
	MLfolP1DA-RB;	Biotin-GCAAGTTCTTTTACGACAGG	
<i>rpoB</i>	MLrpoBDA-F;	TCGCCGCTATCAAGGAATTC	127
	MLrpoBDA-RB;	Biotin-TCACGCGACAAACCACCCGG	
<i>gyrA</i>	MLgyrADA-F;	TGAGACTCCGGTTTCCGCCC	139
	MLgyrADA-RB;	Biotin-CAGCGACCACGGCTGCGCC	

cases, three patients had been treated with the WHO MDT regimen for 2 years and the other six patients had received dapsone monotherapy or combined treatment with clofazimine and rifampicin. All cases had a positive bacterial index (BI) and were therefore, by definition, multibacillary. Genomic DNA templates were prepared as described previously (Matsuoka *et al.* 2005, 2007). Briefly, slit-skin smear specimens were collected from the skin lesions of patients in the same manner as the routine procedure for BI determination. The bacilli were washed out from the blade into 70% ethanol and collected as a pellet by centrifugation at 10 000 *g* for 20 min. Genomic DNA templates for PCR were prepared by treatment with a lysis buffer as described elsewhere (de Wit *et al.*, 1991). The LDS-DA assays were performed as described above and the results were translated into nucleotide sequences according to the positions of the spots for comparison with the sequence data.

Nucleotide sequencing. To confirm and verify the results obtained by the LDS-DA method, nucleotide sequences of PCR products were determined with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) using the same primers for PCR amplification with an ABI3130 genetic analyser.

Ethical approval and consent. The study was approved by the institutional ethics committee of the National Institute of Infectious Diseases, Japan, and two local institutional review boards. Bacterial samples were collected after informed consent was obtained.

RESULTS

Development of a DNA microarray for drug susceptibility testing of *M. leprae*

The target regions of the genes with expected length, 119 bp for *folP1*, 127 bp for *rpoB* and 139 bp for *gyrA*, were amplified simultaneously by multiplex PCR as shown in Fig. 2. Several oligonucleotides corresponding to each of the wild-type and mutant sequences of *folP1*, *rpoB* and *gyrA*

Fig. 2. Electrophoretic pattern obtained by multiplex PCR for *folP1*, *rpoB* and *gyrA*. Lane 1, 20 bp ladder size markers; lane 2, PCR products.

were synthesized, spotted on a glass slide and examined for hybridization with amplicons from the multiplex PCR. The best oligonucleotides, which hybridized with corresponding PCR products without reacting with others, were selected. A DNA microarray with selected oligonucleotides was established as presented in Fig. 1 and designated LDS-DA. The performance of the LDS-DA was examined using PCR products from *M. leprae* isolates and artificially produced DNA fragments with known mutations. PCR products containing the drug-resistance-determining region (DRDR) for each gene were obtained by multiplex PCR (Fig. 2). Fig. 3 shows the hybridization patterns obtained from isolates grown in nude mice footpads, Thai-

Fig. 3. Signals obtained by the LDS-DA with: a susceptible strain, Thai-53; strain Zensho-4, with mutation from ACC to ATC at codon 53 in the *folP1* gene, from TCG to TTG at codon 425 in the *rpoB* gene and from GCA to GTA at codon 91 in the *gyrA* gene; strain Kusatsu-6, with mutation from CCC to CTC at codon 55 in the *folP1* gene and from GAT to TAT at codon 410 in the *rpoB* gene; and strain Zensho-9, with mutation from CCC to CTC at codon 55 in the *folP1* gene and from CAC to TAC at codon 420 in the *rpoB* gene.

53, Zensho-4 (Matsuoka *et al.*, 2000) and two other strains with known nucleotide mutations (Zhang *et al.*, 2004; Maeda *et al.*, 2001). In Thai-53, which is susceptible to dapsone, rifampicin and ofloxacin, positive signals were observed on all of the wild-type spots. In contrast, the highest colour intensity was seen on the spot with the mutant oligonucleotide in drug-resistant *M. leprae*. In Zensho-4, with a three-drug-resistant phenotype, three positive signals shifted from the wild-type to the mutant spots. In the dapsone field, a mutation at codon 53 was identified by a positive signal on spot FM3 instead of FW1. In the ofloxacin and rifampicin fields, similar events were observed. Spots corresponding to mutant-type and wild-type sequences were also found in the other two isolates. Likewise, all the spots with mutant oligonucleotides were verified as to their proper reactivity with the PCR products carrying corresponding known mutations (data not shown).

Evaluation of the LDS-DA system in two countries with high leprosy prevalence

The LDS-DA system was successfully transferred to a laboratory in Yangon, Myanmar, and a laboratory in Cebu, the Philippines. The BI values of the samples from Myanmar varied from 1 to 6. Most were more than 3. Almost all samples from the Philippines showed a BI of more than 4, with a few samples of BI 2. Positive PCR results were obtained even from samples with a BI of 1, although it was usually hard to obtain good results from PCR and colouring from samples with a BI of less than 3. One of the relapsed cases from Myanmar harboured *M. leprae* with mutations CCC to CGC at position 55 in the *folP1* gene and TCG to ATG at position 425 in the *rpoB* gene. One resistant isolate with the mutation ACC to GCC at position 53 in the *folP1* and another isolate with the mutation GAT to TAT at position 410 in the *rpoB* gene

were new cases. In the samples from the Philippines, three *M. leprae* with mutations in the *folP1* gene, CCC to CTC and CCC to TCC at position 55, were from relapsed cases. Two resistant isolates with mutation CCC to CGT at position 55 in the *folP1* were from new cases. The results obtained by the LDS-DA system in these laboratories were compared with the nucleotide sequences of the corresponding genes, as shown in Table 3. All the samples possessing wild-type sequences were judged to be wild-type by both the LDS-DA and sequencing. Concordant results were also observed with seven specimens carrying mutations, five in Myanmar and two in the Philippines. Two unclear results were obtained in *folP1* in Myanmar and one in *rpoB* in the Philippines. In these three samples, the signals were not strong enough to be judged. In the row of codon 55 in *folP1*, no signal was observed with two specimens in the Philippines. Overall, the concordance between the LDS-DA and sequencing results on *folP1* in Myanmar and the Philippines is 96.8 % (61/63) and 97.3 % (71/73), respectively. The LDS-DA results on *rpoB* exhibited good agreement with sequencing results, 100 % (63/63) and 98.6 % (72/73) in Myanmar and the Philippines, respectively. No discordance was found between the LDS-DA and sequencing results on *gyrA* in either country (Table 4).

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 the DRDR in the *folP1*, *rpoB* and *gyrA* genes respectively (Williams & Gillis, 2004). A total of

Table 3. Comparison of results obtained by the LDS-DA and sequencing on clinical specimens in Myanmar and the Philippines

Study site	Status	<i>folP1</i>			<i>rpoB</i>			<i>gyrA</i>		
		No. of specimens	LDS-DA	Sequence	No. of specimens	LDS-DA	Sequence	No. of specimens	LDS-DA	Sequence
Myanmar	Concordant	59	WT*	WT	61	WT	WT	62	WT	WT
		1	53:GCC†	53:GCC	1	410:TAT	410:TAT	1	91:GTA	91:GTA
		1	55:CGC	55:CGC	1	425:ATG	425:ATG			
Philippines	Discordant	2	Unclear‡	WT						
	Concordant	68	WT	WT	72	WT	WT	73	WT	WT
		2	55:CTC	55:CTC						
	Discordant	1	55:TCC	55:TCC						
		2	55:null§	55:CGT	1	Unclear	WT			

*Wild-type sequence.

†Codon number:codon sequence.

‡Data could not be translated because of weak signals.

§No signal was observed on the spots in raw *folP1* 55.

Table 4. Concordance of LDS-DA results with sequencing in clinical specimens in Myanmar and the Philippines

Study site	Target gene		
	<i>folP1</i>	<i>rpoB</i>	<i>gyrA</i>
Myanmar	61/63 (96.8 %)	63/63 (100 %)	63/63 (100 %)
The Philippines	71/73 (97.3 %)	72/73 (98.6 %)	73/73 (100 %)
Total	132/136 (97.1 %)	135/136 (99.3 %)	136/136 (100 %)

106 isolates without mutation in the *rpoB* gene and 63 isolates without mutation the *gyrA* gene were susceptible to rifampicin and ofloxacin, respectively. All isolates resistant to rifampicin or ofloxacin harboured mutations in the DRDR of *rpoB* or *gyrA*, respectively (Honore & Cole, 1993; Honore *et al.*, 1993; Williams *et al.*, 1994, 2001; Cambau *et al.*, 1997, 2001; Matsuoka *et al.*, 2000, 2003; Maeda *et al.*, 2001; Zhang *et al.*, 2004). Resistance of *M. leprae* to dapsone in the mouse footpad is classified into three degrees, namely, low, intermediate and high. A total of 84 isolates without mutation in the *folP1* gene were susceptible to dapsone, but one isolate was resistant with intermediate degree and five isolates were resistant with low degree (Cambau *et al.*, 2006). On the other hand, a total of 24 isolates resistant to dapsone with high or intermediate degree revealed amino acid substitution at the DRDR of the *folP1* gene (Kai *et al.*, 1999; Matsuoka *et al.*, 2000, 2003, 2007; Williams *et al.*, 2000; Lee *et al.*, 2001; Maeda *et al.*, 2001; Cambau *et al.*, 2006). An isolate with mutation ACC to GCC at codon 53 was demonstrated to be resistant with low degree (Cambau *et al.*, 2006), though it is not clear whether dapsone resistance with low degree is true resistance (Matsuoka *et al.*, 2007). Other isolates with this mutation were found to be resistant to dapsone with intermediate degree. Therefore contradiction between mutation in the *folP1* gene and the results obtained by the mouse footpad drug susceptibility test has been encountered for only one case so far.

Although the direct sequencing of PCR products is definitive and allows rapid detection of resistant cases, it has the disadvantage of requiring expensive apparatus and high sequencing costs, so it is not practical in many developing countries. The heteroduplex method (HAD) (Williams *et al.*, 2001) and the PCR-single-strand conformation polymorphism method (SSCP) (Honore *et al.*, 1993) 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 analyses 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 detect silent mutations as resistant mutations. The

recently developed LineProbe 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 (Sapkota *et al.*, 2008).

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 LDS-DA, 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 two developing countries revealed the superior applicability of this method. Only five discordant results were found in 136 specimens examined. Three discordant results, two in Myanmar on *folP1* and one in the Philippines on *rpoB*, showed faint reactions on multiple spots probably caused by some technical errors. In the remaining two discordant results found in the Philippines, no signal was found at any position in row 55 of *folP1*. These samples were shown to carry a mutation at codon 55 in *folP1* from the wild-type CCC to CGT (Table 2), which was recently revealed to be associated with dapsone resistance (Cambau *et al.*, 2006) and was not covered by the oligonucleotide array on the LDS-DA. Although the signal was found neither at the wild-type nor at the mutant position in row 55 of *folP1*, this result can be taken as suggestive of dapsone resistance. The absence of a positive signal in the wild-type position implies the existence of base substitutions in the region covered by the oligonucleotide. Similar translation criteria have been applied to rifampicin-resistant *M. tuberculosis* by the commercially available INNO-LiPA Rif TB assay (Rossau *et al.*, 1997). Other possible mutation(s) related to drug resistance can also be distinguished under the same criteria.

The monitoring of drug-resistant leprosy cases has been recommended in order to maintain the effectiveness of chemotherapy for leprosy (Ji, 2002; Matsuoka *et al.*, 2007). The LDS-DA 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 shows that the level of drug resistance is low in some endemic countries (Matsuoka *et al.*, 2007). 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 LDS-DA method to identify the positions of mutations can be utilized for molecular epidemiological and geographical studies on the spread of drug-resistant *M. leprae*.

ACKNOWLEDGEMENTS

This study was supported by the following grants: a Health Research Grant for Emerging and Re-emerging Infectious Diseases, Ministry of Health, Labour and Welfare, Government of Japan; a grant from the International Medical Center, Ministry of Health, Labour and Welfare; a grant from the US-Japan Cooperative Medical Science Programs; and also by the Grants-in-Aid Program of the Founding Research Center for Emerging and Reemerging Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, to Y. S.

REFERENCES

- Cambau, E., Perani, E., Guillemin, I., Jamet, P. & Ji, B. (1997). Multidrug resistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. *Lancet* **349**, 103–104.
- Cambau, E., Bonnafous, P., Perani, E., Sougakoff, W., Ji, B. & Jarlier, V. (2001). Molecular detection of rifampicin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. *Clin Infect Dis* **34**, 39–45.
- Cambau, E., Carthagena, L., Chauffour, A., Ji, B. & Jarlier, V. (2006). Dihydropteroate synthase mutations in the *folP1* gene predict dapsone resistance in relapsed cases of leprosy. *Clin Infect Dis* **42**, 238–241.
- de Wit, M. Y. L., Faber, W. R. S., Krieg, R., Douglas, J. T., Lucas, S. B., Montreewasuwat, N., Pattyn, S. R. N., Hussain, R., Ponnighaus, J. M. & other authors (1991). Application of polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissue. *J Clin Microbiol* **29**, 906–910.
- Honoré, N. & Cole, S. T. (1993). Molecular basis of rifampicin resistance in *Mycobacterium leprae*. *Antimicrob Agents Chemother* **37**, 414–418.
- Honoré, N., Perani, E., Telenti, A., Grosset, J. & Cole, S. T. (1993). A simple and rapid technique for the detection of rifampicin resistance in *Mycobacterium leprae*. *Int J Lepr Other Mycobact Dis* **61**, 600–604.
- Ji, B. (2002). Rifampicin resistant leprosy: a review and research proposal of a pilot study. *Lepr Rev* **73**, 2–8.
- Kai, M., Matsuoka, M., Nakata, N., Maeda, S., Gidoh, M., Maeda, Y., Hashimoto, K., Kobayashi, K. & Kashiwabara, Y. (1999). Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. *FEMS Microbiol Lett* **177**, 231–235.
- Lee, S. B., Kim, S. K., Kang, T. J., Chae, G. T., Chun, J. H., Shin, H. K., Kim, J. P., Ko, Y. H. & Kim, N. H. (2001). The prevalence of *folP1* mutations associated with clinical resistance to dapsone, in *Mycobacterium leprae* isolates from South Korea. *Ann Trop Med Parasitol* **95**, 429–432.
- Maeda, S., Matsuoka, M., Nakata, N., Kai, M., Maeda, Y., Hashimoto, K., Kimura, H., Kobayashi, K. & Kashiwabara, Y. (2001). Multidrug resistant *Mycobacterium leprae* from patients with leprosy. *Antimicrob Agents Chemother* **45**, 3635–3639.
- Matsuoka, M., Kashiwabara, Y. & Namisato, Y. (2000). A *Mycobacterium leprae* isolate resistant to dapsone, rifampicin, ofloxacin and sparflaxacin. *Int J Lepr Other Mycobact Dis* **68**, 452–455.
- Matsuoka, M., Kashiwabara, Y., Zhang, L., Gotoh, M. & Kitajima, S. (2003). A second case of multidrug resistant *Mycobacterium leprae* isolated from a Japanese patient with relapsed lepromatous leprosy. *Int J Lepr Other Mycobact Dis* **71**, 240–243.
- Matsuoka, M., Zhang, L., Morris, M. F., Legua, P. & Wiens, C. (2005). Polymorphism in the *rpoT* gene in *Mycobacterium leprae* isolates obtained from Latin American countries and its possible correlation with the spread of leprosy. *FEMS Microbiol Lett* **243**, 311–315.
- Matsuoka, M., Teky, B., Khin, S. A., Kyaw, K., Tan, E. V., dela Cruz, E., Gelber, R., Saunderson, P., Balagon, M. A. & Pannikar, V. (2007). The frequency of drug resistance mutations in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from Myanmar, Indonesia and the Philippines. *Lepr Rev* **78**, 343–352.
- Rossau, R., Traore, H., de Beenhouwer, H., Mijs, W., Jannes, G., De Rijk, P. & Portaels, F. (1997). Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampicin. *Antimicrob Agents Chemother* **41**, 2093–2098.
- Sapkota, B. R., Ranjit, C., Neupane, K. D. & Macdonald, M. (2008). Developing and evaluation of a novel multiple-primer PCR amplification refractory mutation system for the rapid detection of mutations conferring rifampicin resistance in codon 425 of the *rpoB* gene of *Mycobacterium leprae*. *J Med Microbiol* **57**, 179–184.
- Shepard, C. (1960). The experimental disease that follows the injection of human leprosy bacillus into footpads of mice. *J Exp Med* **112**, 445–454.
- WHO (1998). WHO Expert Committee on Leprosy, seventh report. Technical report series 87. Geneva: World Health Organization.
- Williams, D. L. & Gillis, T. P. (2004). Molecular detection of resistance in *Mycobacterium leprae*. *Lepr Rev* **75**, 118–130.
- Williams, D. L., Waguespack, C., Eisenack, K., Crawford, J. T., Portaels, F., Salfinger, M., Nolan, M. C., Abe, C., Stich-Groh, V. & Gillis, T. P. (1994). Characterization of rifampicin resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother* **38**, 2380–2386.
- Williams, D. L., Spring, L., Harris, E., Roche, P. & Gillis, T. P. (2000). Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. *Antimicrob Agents Chemother* **44**, 1530–1537.
- Williams, D. L., Pittman, T. L., Gillis, T. P., Matsuoka, M. & Kashiwabara, Y. (2001). Simultaneous detection of *Mycobacterium leprae* and its susceptibility to dapsone using DNA heteroduplex analysis. *J Clin Microbiol* **39**, 2083–2088.
- Zhang, L., Namisato, M. & Matsuoka, M. (2004). A mutation at codon 516 in the *rpoB* gene of *Mycobacterium leprae* confers resistance to rifampicin. *Int J Lepr Other Mycobact Dis* **72**, 468–472.