Analysis of Drug-Resistant Strains of *Mycobacterium leprae* in an Endemic Area of Vietnam

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Background. Multidrug therapy has effectively reduced the number of leprosy cases in the world. However, the rate of reduction has decelerated over the years, giving early detection of *Mycobacterium leprae* and epidemiological study of relapse renewed relevance in attempts to eliminate the disease.

Methods. A molecular epidemiological survey for drug-resistant *M. leprae* was conducted in the central and highland regions of Vietnam. A total of 423 samples taken from patients, including 83 patients with new cases, 321 patients receiving treatment, and 19 patients with relapse, were studied for detection of *M. leprae* with mutations relating to drug resistance by sequencing the drug resistance determining region of the *folP1*, *rpoB*, and *gyrA* genes, which are responsible for dapsone, rifampicin, and ofloxacin resistance, respectively.

Results. Nineteen mutations were found in the *folP1* gene samples, and no mutations relating to drug resistance were found in either the *rpoB* or *gyrA* genes. Samples from patients with relapse showed *folP1* mutation rates as high as 57%, and the mutation rates in samples from new and recent cases were <10%. Patients with relapse who had histories of treatment with dapsone monotherapy showed high mutation rates (78%), compared with patients with relapse who had previously only received multidrug therapy (33%).

Conclusions. Our study indicated high rates of dapsone resistance in patients with relapse, compared with patients with new and recent cases of leprosy. Moreover, it was observed that many of the patients with relapse who had dapsone-resistant mutations had histories of treatment with dapsone monotherapy.

Leprosy is a chronic infectious disease caused by infection with *Mycobacterium leprae*. The present strategy for leprosy control is based on the multidrug therapy (MDT), recommended by the World Health Organization (WHO) [1], which has successfully reduced the number of leprosy cases in the world. However, transition in the number of registered cases and new cases amounting to ~210,000 and ~250,000, respectively, has almost come to a standstill [2]. Drug-resistant strains were first found in 1964, 1976, and 1997 [3–5]. MDT was designed to prevent the emergence and spread of drug-resistant strains. However, a strain showing resistance to both dapsone and rifampicin was reported in 1993 [6], and at present, there are further reports indicating the emergence of *M. leprae* strains resistant to multiple drugs [5, 7]. At present, the rapid detection and control of such drug-resistant strains is essential in countries approaching leprosy elimination levels, such as Vietnam.

MDT has been quite successful in Vietnam, and elimination of leprosy (prevalence rate, < 1/10,000 population) was achieved on the national level in 1995 [8]. The prevalence rate per 10,000 population in 2006

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was .07 [8, 9]. However, the majority of patients with leprosy are found in the central and highland regions of Vietnam [10], consisting of 11 provinces, including 4 provinces in the highland region and 7 provinces in the delta region. In 2005, the number of patients with leprosy was 236, spread through 4 provinces of the highland region; the prevalence rate of newly detected cases was 3.5 cases/10,000 population, although the overall prevalence rate was .25 cases/100,000 population on the national level. The rate of newly detected cases in the 7 delta region provinces was 1.38 cases/10,000 population [8, 9]. These cases not only present the danger of being possible infectious sources for leprosy but also harbor the risk of developing into relapse cases. However, little is known regarding the effects of drug-resistant *M. leprae* in patients with leprosy, especially in cases of relapse.

Therefore, in the present study, molecular epidemiological studies on drug-resistant strains were conducted in 11 provinces primarily in the central and highland regions that represent the areas where leprosy is endemic in Vietnam.

MATERIALS AND METHODS

Sensitivity of Polymerase Chain Reaction

The number of bacilli isolated from nude mice footpads was counted using the method described by Shepard et al [11]. Serial 10-fold dilutions of the enumerated *M. leprae* bacilli were used for polymerase chain reaction (PCR) in our study.

Clinical Specimens

Samples (from slit-skin smears or punch biopsies) were taken from patients with leprosy after receipt of informed consent in primarily the central and highland regions of Vietnam (including 11 provinces: Danang, Quangnam, Quangngai, Binhdinh, Phuyen, Khanhhoa, Ninhthuan, Kontum, Gialai, Daknong, and Daklak), and the samples were classified as new (before starting MDT), recent (receiving MDT), and relapse cases. Relapse was defined as development of new skin lesions after completion of MDT and increase in bacterial index by >2 log units in any lesion.

The total of 423 samples included those from 83 patients with new cases, 321 patients with recent cases (receiving treatment), and 19 patients with relapse (collection period: March 2004–August 2009). Among 16 patients with relapse who had positive results of *M. leprae*–specific PCR, 9 cases were determined to be relapse after dapsone monotherapy (7–20 years), 3 as relapse after complete MDT, 2 as second relapse (the first after dapsone monotherapy and the second after MDT), and 2 as relapse after ofloxacin treatment. Samples were obtained from the skin lesions of patients (smear on blade or biopsy soaked in 1 mL of 70% ethanol at room temperature in the field, before being sent to Quyhoa National Leprosy & Dermato-Venereology Hospital laboratory).

DNA Extraction, Nested PCR, and Sequencing

M. leprae templates from both dilutions of *M. leprae* bacilli and slit-skin smears were prepared by treatment with lysis buffer at 60°C overnight, as described elsewhere [12]. Nested PCR amplification of the RLEP regions of *M. leprae* was performed under conditions described elsewhere with minor modifications, using the primers listed in Table 1 [13]. In brief, PCR amplification using special reagents (20 mM Tris-HCl [pH, 7.5], 8 mM magnesium chloride, 7.5 mM DTT, 2.5 mg BSA, 150 μM deoxynucleotides, 1.5 mM magnesium sulphate, and 2.5 units KOD-plus-Ver.2 DNA polymerase [Toyobo]) was performed using sample DNA as templates. Both first and second PCR conditions were as follows; strand separation at 94°C for 4 min, denaturing at 94°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 20 s plus 1-s increment per cycle for 25 cycles. Products from the first PCR (0.5 uL) were used as templates in the second PCR. The nested PCR for DRDR was performed using the primer pairs listed in Table 1. Mutations were measured on the *folP1* gene for dapsone [14], the *rpoB* gene for rifampicin, and the *gyrA* gene for ofloxacin [15, 16]. Nested PCR conditions for drug resistance were different from that for RLEP-nested PCR. In brief, PCR amplification using standard reagents (10 mM Tris-HCl [pH, 8.3], 2 mM magnesium chloride, 250 μM dNTPs, and 2.5 units TaKaRa Ex Taq DNA polymerase [Takara shuzo]) was performed using sample genomic DNA as templates. The primer pairs used to amplify the specific drug-resistant genes are shown in Table 1. The reaction condition was 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 35 cycles.

The amplicons were visualized by agarose gel electrophoresis, and DNA was recovered from the gel using Mini-Elute gel extraction kits (Qiagen). The recovered DNA molecules were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems) and run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The sequence data were analyzed by DNA analysis program Genetyx-MAC, version 15 (GENETYX), and were compared with those in the GenBank database.

RESULTS

PCR Sensitivity

Serial dilutions of the bacilli of 1×10^8–1×10^0 were prepared to determine PCR sensitivities. Genomic DNAs were extracted from the diluents with use of methods described under Materials and Methods [11]. The previously reported RLEP-nested PCR (named RLEP-L) was capable of detecting 1×10^2 bacilli in samples (Figure 1a) [13]. The newly designed RLEP-nested PCR, using K1 and K2 primers for the first PCR and LP1 and LP2 primers for the second PCR (named RLEP-K), is capable of detecting comparable counts of bacilli (Figure 1b), and RLEP-K

Table 1. Sequences of Primers Used in this Study

Name	Usage	Gene	Sequence, 5′ →3′	Reference	Size, bp
K1	First PCR (F)	RLEP	CGTGGGTGTGAGGATAGTTGT-	Present study	268
K2	First PCR (R)	RLEP	GATCATCGATGCACTGTTCACT-	Present study	
LP1	First or second PCR (F)	RLEP	TGCATGTCATGGCCTTGAGG-	13	129
LP2	First or second PCR (R)	RLEP	CACCGATACCAGCGGCAGAA	13	
LP3	Second PCR (F)	RLEP	TGAGGTGTCGGCGTGGTC	13	99
LP4	Second PCR(R)	RLEP	CAGAAATGGTGCAAGGGA	13	
F1	Second PCR (F)	*folP1*	GCAGGTTATTGGGGTTTTGA	Present study	312
F2	First PCR(R)	*folP1*	CCACCAGACACATCGTTGAC	Present study	
F3	Second PCR (F)	*folP1*	CTTGATCCTGACGATGCTGT	Present study	245
F4	Second PCR(R)	*folP1*	ACATCGTTGACGATCCGTG	Present study	
F5	Sequencing primer (F)	*folP1*	ATCCTGACGATGCTGTCCA	Present study	–
F4	Sequencing primer (R)	*folP1*	ACATCGTTGACGATCCGTG	Present study	–
R1	First PCR (F)	*rpoB*	CAGACGCTGATCAATATCCGT	Present study	358
R2	First PCR (R)	*rpoB*	CAGCGGTCAAGTATTCGATC	Present study	
R3	Second PCR (F)	*rpoB*	CAATATCCGTCCGGTGGTC	Present study	337
R4	Second PCR (R)	*rpoB*	GTATTCGATCTCGTCGCTGA	Present study	
R5	Sequencing primer (F)	*rpoB*	ACGCTGATCAATATCCGTCC	Present study	–
R6	Sequencing primer (R)	*rpoB*	CGACAA TGAACCGATCAGAC	Present study	–
G1	First PCR (F)	*gyrA*	ACGCGATGAGTGTGATTGTGG	Present study	336
G2	First PCR (R)	*gyrA*	TCCCAAATAGCAACCTCACC	Present study	
G3	Second PCR(F)	*gyrA*	GATGGTCTCAAACCGGTACA	Present study	291
G4	Second PCR (R)	*gyrA*	CCCAAATAGCAACCTCACCA	Present study	
G3	Sequencing primer (F)	*gyrA*	GATGGTCTCAAACCGGTACA	Present study	–
G4	Sequencing primer (R)	*gyrA*	CCCAAATAGCAACCTCACCA	Present study	–

products are visualized more clearly with less smear bands. Therefore, the new RLEP-K system was used for detection in further experimentation with use of clinical samples.

Using DNAs extracted from the serial dilutions of *M. leprae*, we determined the sensitivity of the nested PCR for DRDRs. The limit of amplification by PCR was 1×10^3–1×10^4 bacilli (Figure 1 c–e).

RLEP-nested PCR for Clinical Samples

The PCR methods were applied on 423 clinical samples collected from areas of endemicity in Vietnam. First, we tested RLEP-K for detection of *M. leprae* after extraction of DNA from smear samples. Positive bands were obtained by gel electrophoresis using RLEP-K on 290 samples. The positivity rate was 69%. The patients supplying the 290 samples were divided into 3 categories: new, relapse, and recent cases. Positive rates of RLEP-K by category were 75%, 84%, and 66%, respectively (Table 2).

Mutations in Clinical Samples

Samples positive by RLEP-nested PCR were applied for mutation experiments on the DRDRs of *folP1*, *rpoB*, and the *gyrA* gene. Nineteen mutations were found in 187 *folP1* samples, but no mutations related to drug resistance were noted in 163 *rpoB* and 147 *gyrA* gene samples. The mutations detected on *folP1* were as follows: 6 cases of ACC to ATC in codon 53(threonine to isoleucine), 9 cases of CCC to CGC in codon 55 (proline to arginine), and 4 cases of CCC to CTC (proline to leucine). Two new cases, 8 relapse cases, and 9 recent cases had mutations on *folP1*. Mutation rates in the 3 categories were 6.1%, 57%, and 6.4%, respectively (Table 3).

Some missense mutations, of which the association with drug resistance is unknown, were detected in the *rpoB* gene from clinical samples. The mutations were detected in 7 patients at codons 517, 532, and 556. One patient with relapse showed a mutation from CAG (glutamine) to CAT (histidine) at codon 517. One new patient showed 2 mutations at codon 517 from CAG (glutamine) to CAT (histidine) and at codon 532 from GCG (alanine) to TCG (serine). Sequence electropherograms indicated double peaks of a second nucleotide at codon 556 in 3 patients categorized as having recent cases. One peak was G (identical to that of wild-type), and the other peak was T, which changed the amino acid from glycine (GGC) to valine (GTC; data not shown).

The Relation between Treatment and Drug-Resistant Mutations in Patients with Relapse

Patients with relapse were categorized into 4 groups, by treatment history (Table 4). Group 1 comprised those treated with dapsone monotherapy. Group 2 was treated with MDT for 24 months. Group 3 included patients who had received

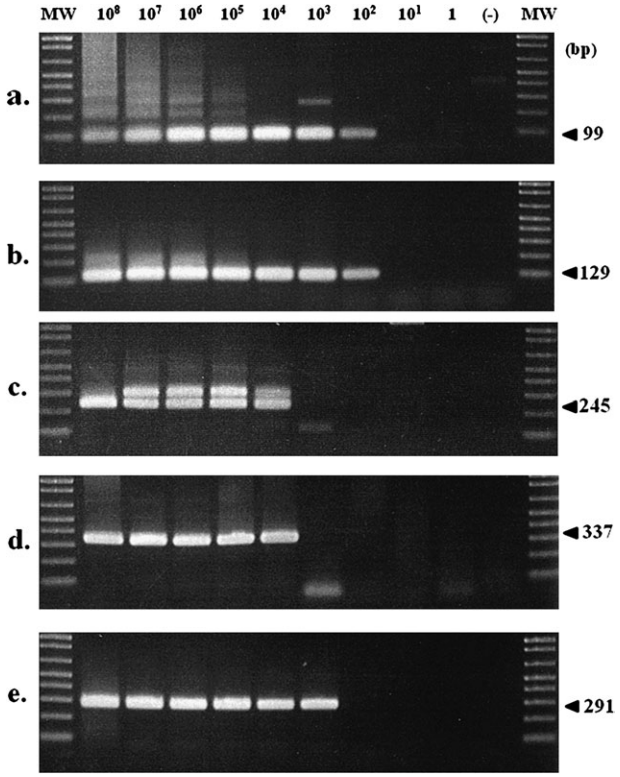

Figure 1. Sensitivity of nested polymerase chain reaction (PCR). The nested PCR products were visualized on 2 % agarose gel. A, RLEP-nested PCR (RLEP-L) using primers, LP1-LP4 (final products size, 99 bp). B, RLEP-nested PCR (RLEP-K) using primers, K1, K2, LP1, and LP2 (final products size, 129bp). C, *folP1*-nested PCR using F1-F4. D, *rpoB*-nested PCR using R1-R4. E, *gyrA*-nested PCR using G1-G4.

a diagnosis of second relapse—once after treatment with dapsone monotherapy and, subsequently, after MDT for 24 months. Group 4 was treated with ofloxacine monotherapy. Eight of the 14 patients with *folP1*-amplified relapse cases (57%) had mutations on the *folP1* gene. Seven (78%) of 9 patients with relapse who were categorized in groups 1 and 3 also had *folP1* mutations. However, 2 patients in group 4 had no mutations on any of the 3 genes.

Monitoring of Mutations in Patients

One hundred seven slit-skin smear samples from 43 patients were taken with consents at different times from each patient for monitoring mutations under treatment. Table 5 shows the difference in mutation results between 5 such patients. The other 38 patients showed no mutation during monitoring. Patients A, B, and C, who had new cases, showed a similar pattern, with no mutation at first testing and mutation in codon 53 on the *folP1* gene during MDT. However, double peaks of T and C in the second base were observed on *folP1* in the 3 patients. Patients D and E, who had relapse cases and finished dapsone monotherapy 20 years earlier, had a mutation on *folP1* in 2005 and no mutation after MDT.

Table 2. Polymerase Chain Reaction Positivity in New, Relapse, and Recent Cases

Case category	No.	RLEP	*folP1*	*rpoB*	*gyrA*
New	83	62 (75%)	33	39	43
Relapse	19	16 (84%)	14	15	13
Recent	321	212 (66%)	140	109	91
Total	423	290 (69%)	187 (64%)	163 (56%)	147 (51%)

DISCUSSION

The most popular PCR method for *M. leprae* detection with high sensitivity and specificity is probably the RLEP-nested PCR method, because the RLEP regions are specific for *M. leprae,* with >28 copies dispersed through the *M. leprae* genome [17]. New primers were designed for the RLEP-nested PCR in our study. This system using the new primers was termed RLEP-K. RLEP-K products appear to be a somewhat sharper and stronger band on agarose gel electrophoresis, compared with that that of previous RLEP-nested PCR (ie, RLEP-L). The RLEP-K detected *M. leprae* in 69% of the Vietnam samples. The remaining 31% of the samples were deduced as being cases either cleared of *M. leprae* by chemotherapy or those having <100 bacilli, which was below the detection limit of RLEP-K. We also designed new primers for amplification and sequencing of DRDR in the drug-resistance related genes *folP1*, *rpoB*, and *gyrA,* which were applied in examining the Vietnam samples. The mutation rates of *folP1* in new and recent cases were 6.1% and 6.4%, respectively. In contrast, the mutation rate in relapse cases was quite high, at 57%. The result indicated a strong correlation between mutation rate and relapse. Two possible reasons were conceived regarding the high positive rate of dapsone resistance in patients with relapse: (1) reinfection by the primary drug-resistant strain (7 of 8 samples indicating relapse were collected in the province in central Vietnam, which had the highest prevalence of leprosy and high rate of relapse (data not shown) and (2) reactivation of dapsone-resistant strains capable of

Table 3. Number of Mutations on *folP1*

Case category	No. of PCR-positive cases	No. of mutations (mutation ratio)	No. of mutation in mutation types
New	33	2 (6.1%)	2 (55th: CCC-CGC)
Relapse	14	8 (57%)	2 (53rd: ACC-ATC)
			3 (55th: CCC-CGC)
			3 (55th: CCC-CTC)
Recent	140	9 (6.4%)	4 (53rd: ACC-ATC)
			4 (55th: CCC-CGC)
			1 (55th: CCC-CTC)

Table 4. Mutations Noted in RLEP-Positive Relapse Cases, by Treatment Group

Group	Past treatment	No.	Mutation on *folP1*	Mutation on *rpoB*	Mutation on *gyrA*
1	DDS	7	5	0	0
2	MDT (24 months)	3	1	1[a]	0
3	DDS plus MDT (24 months)	2	2	0	0
4	OFX	2	0	0	0
All	…	14	8	1[a]	0

Abbreviations: DDS (diaminodiphenylsulfone), dapsone monotherapy; MDT,multidrug treatment; OFX, Ofloxacine monotherapy.

[a] Unknown DR mutation

persisting after chemotherapy, discussed below. Although it is still unclear whether the relapses are caused by reinfection by *M. leprae* or by reactivation of persistent *M. leprae*, close correlation between drug resistance and relapse have been recognized in several studies [18, 19].

The proportion of samples showing mutation on the *folP1* gene related to dapsone resistance was 10.2% (19 of 187) in samples from the central and highland regions of Vietnam (Table 3). Comparison with previous reports from South Korea (19.2%) indicates lower rates of relapse in these regions of Vietnam [20].

No mutation was found in the DRDR regions of *rpoB* in all samples. Mutation frequencies of the *rpoB* gene are also very low in other reports. Regarding other areas in Southeast Asia, no cases of rifampicin resistance have been detected in the Philippines, 1 (1.9%) of 54 cases in Myanmar, and 4 (3.3%) of 121 cases in Indonesia. However, in Japan, where the prevalence of leprosy is very low, the reported rate of rifampicin resistance is very high, at 29.5% (26 of 88 cases) [21]. The long-term use of drugs outside the standard MDT regimen in Japanese leprosy cases might have been instrumental in promoting this rifampicin resistance.

As such, no mutations have been found in the DRDR of the *M. leprae rpoB* gene derived from patients with leprosy, including relapse cases in Vietnam. A possible explanation for this could be the success of leprosy control in Vietnam and efficacy of properly administered MDT in which rifampicin—with its bactericidal properties—was effective in suppressing the occurrence of drug-resistant bacilli. In contrast, dapsone (not bactericidal in itself, although capable of suppressing growth), which had previously been used as monotherapy, may have enabled bacteria surviving in the patient receiving treatment to develop mutations, giving them resistance against the drug. Although occurrence of drug-resistant *M. leprae* was kept very low after application of MDT, 7 of 9 samples with drug-resistant mutations had previously been treated by dapsone monotherapy (Table 4). Jing et al [22] reported that patients with multibacillary leprosy who were retreated with MDT after dapsone monotherapy may have lower risk of early relapse while continuing to carry the risk of late relapse. Our observations suggest the possibility that efficacy of MDT may be hampered in some patients by the presence of surviving dapsone-resistant *M. leprae* in their bodies, which could develop into late relapse. Similar observations have been reported, suspecting involvement of the effects of dapsone monotherapy in patients with relapse [23].

There was no mutation in the major sites for drug resistance on the *rpoB* gene. However, we observed mutations at 3 positions, codons 517, 532, and 556, which have not been associated with rifampicin resistance. These mutations in the *rpoB* gene are a finding calling for further clarification.

Table 5. Monitoring of 5 Patients with Multibacillary Leprosy for *folP1* Mutation

Patient	Case category	Date of sample obtainment	Sample site (method of obtainment)	*folP1* mutation
A	New	2006 April 3	Abdomen (biopsy)	None[a]
		2007 January 30	Earlobe (smear)	53rd (ACC→ATC/ACC)
		2007 January 30	Abdomen (smear)	53rd (ACC→ATC/ACC)
B	New	2005 May 31	Earlobe (smear)	None
		2006 March 24	Skin (smear)	None
		2007 November 2	Skin (smear)	53rd (ACC→ATC/ACC)
C	New	2006 July 20	Skin (smear)	None
		2007 January 30	Skin (smear)	53rd (ACC→ATC/ACC)
		2007 January 30	Skin (smear)	53rd (ACC→ATC/ACC)
D	Relapse	2005 November	Earlobe (smear)	55th (CCC→CGC)
		2007 January	Skin (smear)	None
E	Relapse	2007 Jannuary 17	Arm (smear)	None
		2007 January 30	Earlobe (smear)	55th (CCC→CGC)
		2007 January 30	Arm (smear)	None

[a] ACC ATC/ACC indicates double peaks in second base at codon 53.

To reveal the possible relation between treatment and gene mutation, some patients with leprosy were monitored for gene mutations in light of drug treatments. The results showed incidence of dapsone-resistant *M. leprae* in patients receiving MDT, suggesting that some of the patients with relapse who were previously treated with dapsone monotherapy might have persistent infections with dapsone-resistant *M. leprae*. Furthermore, samples derived from different sites of lesions in the same patient sometimes showed different results (Table 5). The results suggest that we need to know the relation between the situation of patients with leprosy and drug resistance.

Overall, our study indicated a high ratio of dapsone resistance in patients with relapse, compared with the other patients with leprosy. In contrast, an unexpected outcome of our study was that we were unable to find mutations on the *rpoB* gene in patients with relapse. Moreover, it was shown that many of the patients with relapse who had dapsone-resistant mutations had histories of treatment with dapsone monotherapy. To clarify the relationship between relapse, drug resistance, and dapsone monotherapy, it might be necessary to investigate persistence of drug-resistant *M. leprae* through large-scale surveillance.

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Potential conflicts of interest. All authors: no conflicts.

References

1. WHO Study Group. Chemotherapy of leprosy for control Programmes. Geneva: Tech Resp Ser, 1982: 675.
2. World Health Organization (WHO). Global leprosy situation. Wkly Epidemiol Rec **2009**; 84:333–40.
3. Rees RJ. Mycobacterial disease in man and animals. Studies on leprosy bacilli in man and animals. Proc R Soc Med **1964:**; 57:482–3.
4. Jacobson RR, Hastings RC. Rifampin-resistant leprosy. Lancet **1976**; 2:1304–5.
5. Cambau E, Perani E, Guillemin I, Jamet P, Ji B. Multidrugresistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. Lancet **1997**; 349:103–4.
6. González AB, Maestre JL, Hernández O, et al. Survey for secondary dapsone and rifampicin resistance in Cuba. Lepr Rev **1993**; 64:128–35.
7. Matsuoka M, Kashiwabara Y, Namisato MA. *Mycobacterium leprae* isolate resistant to dapsone, rifampin, ofloxacin and sparfloxacin. Int J Lepr Other Mycobact Dis **2000**; 68:452–5.
8. World Health Organization (WHO). Global leprosy situation. Wkly Epidemiol Rec **2008**; 83:217–24.
9. Bang PD, Suzuki K, Ishii N, Kang TH. Leprosy situation in Vietnam-reduced burden of stigma. Jpn J Lep **2008**; 77:29–36.
10. Quyhoa NDH. Annual report (2004–2009). http://www.quyhoandh.org.vn/quyhoandh/vn/portal/index.jsp. Accessed 29 December 2010.
11. Shepard CC. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. J Exp Med **1960**; 112:445–54.
12. de Wit MY, Faber WR, Krieg SR, et al. Application of a polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissues. J Clin Microbiol **1991**; 29:906–10.
13. Donoghue HD, Holton J, Spigelman M. PCR primers that can detect low levels of *Mycobacterium leprae* DNA. J Med Microbiol **2001**; 50:177–82.
14. Kai M, Matsuoka M, Nakata N, et al. Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. FEMS Microbiol Lett **1999**; 177:231–5.
15. Honore N, Cole ST. Molecular basis of rifampin resistance in *Mycobacterium leprae*. Antimicrob Agents Chemother **1993**; 37:414–8.
16. Maeda S, Matsuoka M, Nakata N. Multidrug resistant *Mycobacterium leprae* from patients with leprosy. Antimicrob Agents Chemother **2000**; 45:3635–9.
17. Cole ST, Eiglmeier K, Parkhill J, et al. Massive gene decay in the leprosy bacillus. Nature **2001**; 409:1007–11.
18. Kaimal S, Thappa DM. Relapse in leprosy. Indian J Lepr **2009**; 75:126–35.
19. Lopez-Roa RI, Fafutis-Morris M, Matsuoka M. A drug-resistant leprosy case detected by DNA sequence analysis from a relapsed Mexican leprosy patient. Rev Latinoam Microbiol **2006**; 48:256–9.
20. You EY, Kang TJ, Kim SK, et al. Mutations in genes related to drug resistance in *Mycobacterium leprae* isolates from leprosy patients in Korea. J Infect **2005**; 50:6–11.
21. Matsuoka M, Budiawan T, Aye KS, et al. The frequency of drug resistance mutations in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from Myanmar, Indonesia and the Philippines. Lepr Rev **2007**; 78:343–52.
22. Jing Z, Zhang R, Zhou D, Chen J. Twenty five years follow up of MB leprosy patients retreated with a modified MDT regimen after a full course of dapsone mono-therapy. Lepr Rev **2009**; 80:170–6.
23. Chakma JK, Girdhar A, Natrajan M, et al. Two microbiological relapses in a patient with lepromatous leprosy. Lepr Rev **2008**; 79:331–4.