## Abstract

The purposes of this collaborative project are to establish an algorithm for diagnosis of *Mycobacterium leprae* and to detect its drug-resistance. Molecular techniques such as nested PCR was adopted from the Leprosy Research Center, National Institute of Infectious Disease (NIID), Japan, and a modified real-time PCR assay was developed by the National Reference Laboratory of Mycobacteriology at Taiwan Centers for Diseases Control (TCDC). The detection limit of real-time PCR was 100fg/$\mu$l and 1 pg/$\mu$l. In this pilot study, 13 biopsy samples and 48 blade specimens from skin smears each from individual confirmed leprosy cases were analyzed. We did not obtain any positive result from skin smears. Nevertheless, of the 13 biopsy samples, 5 and 8 positive results were found in nested PCR and real-time PCR, respectively. One case (case 4) was resistant to dapsone with mutation at codon 53 (T to R) of the *folP1* gene. Thus, drug regimen for case 4 was adjusted for effective treatment. In conclusion, molecular assays can provide better diagnosis, treatment and management of leprosy.

## Introduction

The causative agent of leprosy is *Mycobacterium leprae* which is named after Dr Gerhard Henrik Armauer Hansen. *M. leprae* is acid-fast, rod-shaped bacillus, with 5-year incubation period. It affects all ages and both sexes and affects skin, peripheral nerves, mucosa of upper respiratory tract and eyes.

Leprosy is a curable chronic infectious disease. Close and frequent contacts through skin to skin and airborne are known transmission routes. World Health Organization (WHO) has provided free multidrug therapy (MDT) treatment since 1995. MDT treatment consists of 3 drugs including dapsone, rifampicin and clofazimine. No resistance to anti-leprosy medicines when MDT was used. Leprosy has been eliminated from 119 of 122 countries in 2010.

Leprosy is a notifiable infectious disease in Taiwan. According to the Statistics of Communicable Diseases and Surveillance Report issued by Taiwan Centers for Disease Control, confirmed and registered leprosy cases were 8 and 1,103 in 2008; 7 and 1,214 in 2009; and 5 and 1,183 in 2010, respectively (Figure 1).

Since *M. leprae* can not be cultivated *in vitro*, smear microscopy is currently the only test used in clinical bacteriological laboratories for bacteriological diagnosis in Taiwan. However, the majority of people with leprosy have a negative smear. The objectives of this project are to learn and join the global leprosy program, to improve leprosy clinical diagnosis, to develop leprosy diagnostic methods and to participate in leprosy drug-resistance surveillance. This

is a laboratory-based study that will use samples collected from leprosy contract hospitals. We will conduct molecular diagnosis using *M. leprae* specific gene, drug-resistant gene sequencing of dapsone, rifampicin and ofloxacin, and serological diagnosis using PLG-1 and MMP-II antigens. This project will establish a differential diagnosis algorithm for leprosy, and reveal drug resistant patterns of *Mycobacterium leprae* using molecular methods. The sero-prevalence rate of leprosy patients will be determined. The laboratory detection, clinical treatment and management of leprosy patients in Taiwan will be improved.

## Materials and Methods

### Study samples

We retrospectively analyzed 13 biopsy samples from National Taiwan University and 48 blade specimens from skin smear from Lo-Sheng Sanatorium, Taiwan.

### Molecular assays

### DNA extraction

Paraffin-embedded or blade specimens was placed in microcentrifuge tubes containing 180 μl of buffer ATL, 20 μl proteinase K, mixed by vortexing, and incubated at 56°C until the tissue is completely lysed. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 μl Buffer AL to the sample. Mix again by vortexing for 15 sec., and incubate at 70°C for 10 min. Add 200 μl ethanol (96–100%) to the sample, and mix by vortexing for 15 sec. After mixing, carefully apply the mixture to the QIAamp Mini spin column. Close the cap, and centrifuge at 8,000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate. Then add 500 μl Buffer AW1 and repeat the centrifuge program. And 500 μl Buffer AW2 added at full speed (14,000 rpm) for 3 min. Finally 200 μl Buffer AE added at room temperature for 1 min, and then centrifuge at 8000 rpm for 1 min. The filtrate solution was collected for molecular assays.

### Diagnosis of *M. leprae*

### Real-time PCR

For identification of *M. lepare,* we designed primers set based on *M. leprae*-specific repetitive element (RLEP). We developed a modified real-time TagMan PCR method using probes designed at the Leprosy Research Center of National Institute of

Infectious Disease (NIID), Japan for rapid screening of *M. leprae*. Primer-probe sets of RLEP were synthesized by Applied Biosystems for the ABI 7500 real-time PCR system. Probes were labeled with TaqMan® MGB FAM™ markers. For each sample, the real-time PCR was performed by using 12.5 μl of TaqMan® 2X universal master mix (Applied Biosystems, USA), 2 μl of 10 μM RLEP-TM-F primer (5'-GCA GTA TCG TGT TAG TGA A-3'), 2 μl of 10 μM RLEP-TM-R primer (5'-CGC TAG AAG GTT GCC GTA TG-3'), 2 μl of 10 μM RLEP-TM probe (5'-FAM-TCG ATG ATC CGG CCG TCG GCG –TAMRA-3'), 1.5 μl RNase- and DNase-free water, and 5 μl sample DNA, in a final total volume of 25 μl per single well reaction. It was recommended to use at least 10 ng DNA from either purified DNA or inactivated bacterial lysate directly.

**Nested PCR**

For nested PCR of *M. lepare,* we adopted *M. leprae*-specific repetitive element (RLEP) target as an amplified gene. For first round of PCR, amplification primer were LP1(5′-TGC ATG TCATGG CCT TGA GG-3′) and LP2 (5′-CAC CGA TAC CAG CGG CAG AA-3′). And the PCR products were diluted 1:10 in sterile water, and final volume was 100 μl. Second round of PCR were amplified by primer LP3(5′-TGA GGT GTC GGC GTG GTC-3′) and primer LP4 (5′-CAG AAA TGG TGC AAG GGA-3′). The PCR reactions were performed as follows: 95°C for 4 min, followed by 25 cycles at 95°C for 40 sec; annealing at 55°C for 1 min; and elongation at 72°C for 20 sec (increase 1 sec/cycle), and a final cycle of 72°C for 1 min to complete elongation of the intermediate PCR products. The PCR products were visualized by gel electrophoresis. The detection limit of nested PCR is 1

organism/mL.

**Detection of drug-resistance**

For drug-resistant analysis, we sequenced the *folp1* gene for dapsone, *rpoB* gene and *gyrA* gene for ofloxacin.

**DNA sequencing of the *folp1* gene.** Two primer sets were used to analyze the variation at the *folp1* gene. First PCR amplification primer were NF2 (5′-GCA GGT TAT TGG GGT TTT GA-3) and FH2 (5′-CCA CCA GAC ACA TCG TTG AC-3), and then Nest-PCR by primer FK1(5′-CTT GAT CCT GAC GAT GCT GT-3), FK2(5′-ACA TCG TTG ACG ATC CGT G-3). A 245-bp fragment was sequenced with the oligonucleotide primers FH1 (5′-ATC CTG ACG ATG CTG TCC A -3′). The PCR reactions were performed as follows: 95°C for 10 min, followed by 30 cycles at 95°C for 1 min; annealing at 60°C for 1 min; and elongation at 68°C for 1 min, and a final cycle of 68°C for 6 min to complete elongation of the intermediate PCR products.

**DNA sequencing of the *rpo*B gene.** Two primer sets were used to analyze the variation at the *rpo*B gene. First PCR amplification primer were Rif-1 (5′-CAG ACG CTG ATC AAT ATC CGT-3′) and RpoBR05 (5′-CAG CGG TCA AGT ATT CGA TC-3′), and Nest-PCR by primer RH1(5′-CAA TAT CCG TCC GGT GGT C-3′), RH2(5′-GTA TTC GAT CTC GTC GCT GA-3′). A 337-bp fragment was sequenced with the oligonucleotide primers RK1 (5′-ACG CTG ATC AAT ATC CGT CC -3′). The PCR reactions were performed as follows: 95°C for 10 min, followed by 30 cycles at 95°C for 1 min; annealing at 60°C for 1 min; and elongation at 68°C for 1

min, and a final cycle of 68°C for 6 min to complete elongation of the intermediate PCR products.

**DNA sequencing of the *gyrA* gene.** Two primer sets were used to analyze the variation at the *gyrA* gene. First PCR amplification primer were GH1(5′-ACG CGA TGA GTG TGA TTG TGG-3′) and GH2 (5′-TCC CAA ATA GCA ACC TCA CC-3′), and Nest-PCR by primer GK1(5′-GAT GGT CTC AAA CCG GTA CA-3′), GK2(5′-CCC AAA TAG CAA CCT CAC CA-3′). A 291-bp fragment was sequenced with the oligonucleotide primers GK1. The PCR reactions were performed as follows: 95°C for 10 min, followed by 30 cycles at 95°C for 1 min; annealing at 60°C for 1 min; and elongation at 68°C for 1 min, and a final cycle of 68°C for 6 min to complete elongation of the intermediate PCR products.

Thereafter, the PCR products were analyzed with an ABI 3730 automated sequencer (Applied Biosystems, USA), and the sequence data were assembled and edited using the Sequencing Analysis 5.2.0 software (Applied Biosystems, USA).

## Results

### Establishment of PCR assays

For identification of *M. lepare,* we designed primers set based on *M. leprae*-specific repetitive element (RLEP). We modified and developed a real-time TagMan PCR method for rapid screening of *M. leprae*. The detection limit of this assay is 100 fg/$\mu$L (Figure 2). In comparison, real-time PCR can be used to detect DNA in Ct valves less than approximately 35, while that for nested PCR is 28 (Table 1). Of the 13 biopsy samples, 5 (38.5%) and 8 (61.5%) positive results were found in nested PCR and real-time PCR, respectively. Preliminary results showed that real-time PCR is more sensitive than nested PCR assay (Table 1).

### Molecular diagnosis of *M. leprae*

Thirteen biopsy samples and 48 blade specimens from skin smears each from individual were analyzed. Based on histological classification of those 13 cases with biopsy samples, 6 cases (cases 1-3, 5-7 and 11) were lepromatous, 3 cases (cases 8, 12, 13) were tuberculoid, 2 cases (cases 9 and 10) were borderline lepromatous (BL) – mid-borderline lepromatous (BB), and case 4 was BB. We did not obtain any positive result from skin smears. Nevertheless, of the 13 biopsy samples, 5 (cases 1-4 and case 7) and 8 (cases 1-7 and case 11) positive results were found in PCR and real-time PCR, respectively (Table 1).

### Detection of drug resistance

Gene sequencing was performed to detect drug resistance of 5 cases (cases 1-4 and 7). We found one case (case 4) was resistant to dapsone with mutation at codon 53 (T to R) of the *folP1* gene. Neither the *rpoB* gene conferring rifampicine resistance

nor the *gyrA* gene conferring ofloxacin resistance was identified (Table 2). Discrepant results obtained between 2 laboratories needed to be further evaluated.

## Discussion

In Taiwan, leprosy is a notifiable disease defined mainly by smear microscopy, histological examination and clinical judgment. The goal of the first-year collaborative project is to establish a differential diagnosis algorithm for leprosy and to reveal drug resistant patterns of *M. leprae* using molecular methods.

Two molecular assays, nested PCR and real-time PCR have been established in TCDC. Real-time assay has advantages in lesser labor-intensive and faster turnaround time. In addition, preliminary results demonstrated that real-time PCR is more sensitive than nested PCR method. Therefore, we will adopt real-time PCR assay in our routine service.

Since the amount of DNA extracted from biopsy or blade samples were low, cycles of the PCR reaction might need to be adjusted according to results of smear microscopy. In this study, results of real-time PCR were consistent with histological classification of lepromatous leprosy (Table 1).

For detecting of drug resistance, discrepant results were observed in 2 laboratories (Table 2). Improvement of conditions and protocol for increasing the successful rate of PCR reactions is still needed. Case 4 identified as resistant to dapsone was a treatment failure case. For providing appropriate treatment, determination of drug-resistance for each confirmed case can ensure good treatment outcome.

We are currently working on sero-diagnosis in parallel at laboratories at NIID

and TCDC. Through this collaborative project, laboratory detection, clinical treatment and management of leprosy patients in Taiwan will be improved.

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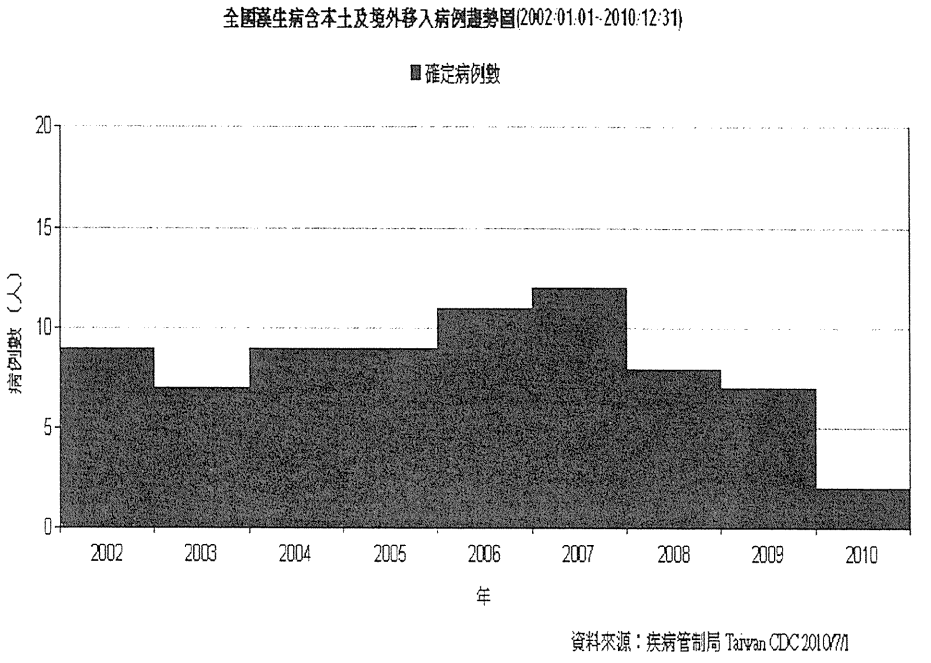
## Publication list for this work

NIL

# Tables and Figures

## Figure 1 Leprosy cases in Taiwan, 2002-2010




資料來源：疾病管制局 Taiwan CDC 2010/7/1

**Figure 2 Standardized curve of real-time PCR for *Mycobacterium lepre* identification**

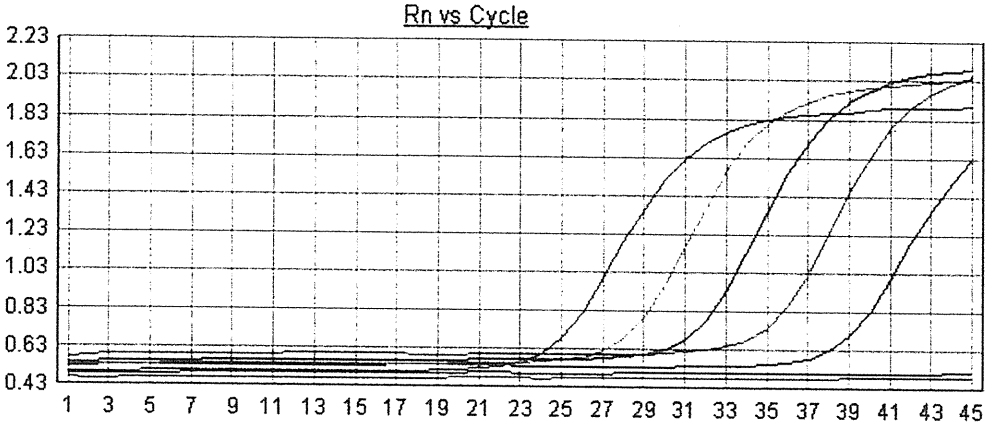


**Table 1 Results of PCR assays and classification of cases**

| ID | Age/Sex | TaqMan-RT-PCR | | Nested PCR | Clinical classification | Histological classification |
|---|---|---|---|---|---|---|
| | | Ct value | Result | Result | | |
| 001 | 62/F | 24.41 | positive | positive | erythema nodosum leprosum | lepromatous |
| 002 | 30/F | 25.19 | positive | positive | NA | lepromatous |
| 003 | 34/F | 21.04 | positive | positive | defer | lepromatous |
| 004 | 84/M | 28.03 | positive | positive | borderline leprosy | BB |
| 005 | 34/F | 33.04 | positive | negative | lepromatous leprosy | lepromatous |
| 006 | 42/M | 32.81 | positive | negative | lepromatous leprosy | lepromatous |
| 007 | 52/F | 25.99 | positive | positive | multibacilli leprosy | lepromatous |
| 008 | 55/M | 42.49 | negative | negative | leprosy | tuberculoid |
| 009 | 62/M | 43.3 | negative | negative | erythema nodosum leprosum | BL-BB |
| 010 | 62/M | undet. | negative | negative | erythema nodosum leprosum | BL-BB |
| 011 | 34/F | 34.1 | positive | negative | leprosy | lepromatous |

| | | | | | | |
|---|---|---|---|---|---|---|
| 012 | 55/M | 41 | negative | negative | NA | tuberculoid |
| 013 | 55/M | undet. | negative | negative | borderline leprosy | tuberculoid |

**Table 2 Sequencing results of the drug-resistant genes: *folP1, rpoB and gyrA***

**(A) Results from TCDC laboratory**

| ID | *folP1* | *rpoB* | *gyrA* |
|---|---|---|---|
| 001 | WT* | WT | WT |
| 002 | WT | WT | WT |
| 003 | WT | WT | WT |
| 004 | T53R | WT | WT |
| 007 | WT | WT | NA |

* WT, wild-type

**(B) Results from NIID laboratory**

| ID | *folP1* | *rpoB* | *gyrA* |
|---|---|---|---|
| 001 | WT* | WT | WT |
| 002 | WT | WT | WT |
| 003 | WT | WT | WT |
| 004 | T53R | WT | WT |
| 006 | NA | NA | NR |
| 007 | WT | NA | NA |
| 011 | WT | NA | NA |
| 012 | T53I (?) | WT | NA |
| 013 | WT | WT | NR |

# Sapovirus epidemiological study & Quick diagnostic system for diarrheal viruses

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