Molecular studies on virulence and drug resistance of leprosy: Molecular and sero-diagnosis of leprosy

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Summary:

Leprosy is a notifiable disease in Taiwan. During 2002 to 2011, we confirmed 81 new leprosy cases, with 5 to 12 cases reported annually. There were 56 (69.1%) multibacillary cases, 21 (25.9%) paucibacillary cases and 4 (4.9%) were not determined. Female individuals (50/81, 61.7%) are more venerable than male (P= 0.033). The majority (42, 51.9%) of cases was in the age group of 21-40 (P< 0.001), followed by 22 (27.2%) in the age group senior than 60 years old. Furthermore, 13 relapse indigenous cases were notified in 2002-2011. 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. The objectives of this project are to improve clinical diagnosis of leprosy, to develop diagnostic methods for leprosy and to participate in drug-resistance surveillance of leprosy. We conduct molecular diagnosis using *M. leprae* specific gene, drug-resistant gene sequencing of dapsone, rifampicin and ofloxacin, and serological diagnosis using MMP-II ELISA. Positive results were found in 4 cases using RLEP real-time PCR. Gene sequencing was performed to detect drug resistance of 4 cases. We found one case (D-10) was resistant to dapsone with mutation at codon 55 (CCC to CTC) of the *folP1* gene. The positive cut-off value was 0.233. The sensitivity and specificity of the MMP-II ELISA was 83.7% and 80%, respectively. We established a differential diagnosis algorithm for leprosy, and reveal drug-resistant patterns of *Mycobacterium leprae*. In addition, a sero-diagnostic test was also established for screening of leprosy. A strengthened laboratory detection system can improve clinical treatment and management of leprosy patients.

I. Purpose

The purposes of this collaborative project are to establish an algorithm for diagnosis of *Mycobacterium leprae*. We established molecular tests, a real-time and a nested PCR for detecting *M. leprae* and drug resistance to dapsone, rifampicin and ofloxacin. In addition, sero-diagnosis using MMP-II ELISA was evaluated in this study.

II. Methods

Study samples

In this study, 159 blade specimens and 110 blood samples were collected from suspected and/or enrolled leprosy cases from contract hospitals and/or from annual on-site health examination carried out by local health bureaus.

Molecular diagnosis of *Mycobacterium leprae*

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.

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[1] 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 12.5μl of TaqMan® 2X universal master mix (Applied Biosystems, USA), 2μl of 10 μM RLEP-TM-F primer (5'-GCA GTA TCG TGT TAG TGA A-3'), 2μl of 10 μM RLEP-TM-R primer (5'-CGC TAG AAG GTT GCC GTA TG-3'), 2μl of 10 μM RLEP-TM probe (5'-FAM-TCG ATG ATC CGG CCG TCG GCG –TANRA-3'), 1.5μl RNase- and DNase-free water, and 5μl sample DNA, in a final total volume of 25μl per single well reaction. It was recommended to use at least 10 ng DNA from either purified DNA or inactivated bacterial lysate directly.

Drug-resistant gene sequencing

For drug-resistant detection, we sequenced the *folp1* gene for dapsone, the *rpoB* gene for rifampin and the *gyrA* gene for ofloxacin following published protocols[2-3]. Briefly, the first set of PCR amplification primer was used, and then a nested-PCR was performed with 10-fold diluted first PCR product. A resulting fragment was sequenced further with oligonucleotide primers. 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). In addition, site-directed mutagenesis technique was adopted to determine the effect of novel mutations on the dapsone susceptibility. 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).

Sero-diagnosis of loprosy

MMP-II ELISA

The ELISA for the detection of anti-MMP-II immunoglobulin G (IgG) antibodies was performed as leprosy center suggested[4-7]. Ninety-six well plates (Immunosorb, Nunc) were coated overnight, with MMP-II at a concentration of 4 μg/mL in coating buffer (pH 9.5) (Protein Detector ELISA kit, KPL). After blocking with blocking solution and incubated at 37 ℃ for 1 h., the plates were washed with phosphatebuffered saline containing 0.1% Tween 20 (PBST), and human sera diluted 100-fold were added and incubated at 37 ℃ for 2 h. After washing with PBST, biotinylated anti-human IgG (KPL) was added at a 1:1000 and incubated for 1 h. The plates were incubated with reagents ABC peroxidase staining kit (Thermo) for 30 min. After further washing with PBST, a substrate solution consisting of OPD/citric acid and

0.02% H2O2 in 0.1M citrate buffer was added until a yellow color developed and the OD was measured at 450nm using a spectrophotometer. Plate-to-plate variations in OD readings were controlled using a standard pool positive serum and if the positive control reading varied more than 0.05 O.D., then the test was repeated.

Statistical analyses

For statistical analysis, Receiver Operator Characteristics (ROC) curves were drawn to describe the relation between sensitivity and specificity at various cut-off levels using the MedCalc software.

III. Results

Epidemiology of Leprosy in Taiwan

During 2002 to 2011, we confirmed 81 new leprosy cases, with 5 to 12 cases reported annually[8]. There were 56 (69.1%) multibacillary (MB) cases, 21 (25.9%) paucibacillary (PB) cases and 4 (4.9%) were not determined. Female individuals (50/81, 61.7%) are more venerable than male (P= 0.033). Geographically, 42 (51.9%), 26 (32.1%), 11 (13.6%) and 2 (2.5%) cases were reported from northern, southern, central and eastern Taiwan, respectively. Of the 81 cases, 37 (45.7%) were indigenous cases and 44 (55.3%) were imported cases (Table 1). Majority (43/44, 97.7%) of imported cases were originated from South-East Asia, particularly from Indonesia (32/44, 72.7%) (P< 0.001), which reported 20,023 new cases in 2011[9]. In addition, of the total 425,660 foreign worker entered Taiwan as of December 2011, 175,409 (41.2%) were from Indonesia. Among 50 female cases, 37 (74%) (P< 0.001) were imported cases including 29 (58%) from Indonesia, 4 from Vietnam, 3 from Philippines and 1 from China. While among 31 male cases, 7 (22.6%) were imported cases including 3 from Indonesia, 3 from Thailand and 1 from Myanmar. The median age of male between indigenous and imported were 60.5 (range 31-83 years) and 33 (range 24-60 years) (P= 0.001) and that of female were 67 (range 45-81 years) and 27 (range 20-52 years) (P< 0.001), respectively. We did not observed any leprosy case younger than 19 years old. The majority (42, 51.9%) of cases was in the age group of 21-40 (P< 0.001), followed by 22 (27.2%) in the age group senior than 60 years old. Furthermore, 13 relapse indigenous cases were notified in 2002-2011. Of the 13 cases, 2 were reactivated after 5 years, one after 14 years, 6 after 35 years and 4 were unknown.

Molecular diagnosis and drug-resistance detection of *M. leprae*

We analyzed 159 blade specimens from skin smears each from individual cases collected from 8 (A to H) sites in Taiwan in 2012 (Table 2). Positive results were found in 4 cases using RLEP real-time PCR. Gene sequencing was performed to detect drug resistance of 4 cases. We found one case (D-10) was resistant to dapsone with mutation at codon 55 (CCC to CTC) of the *folP1* gene (Table 3). Increased Ct value of the RLEP was observed from 23.86 in 2008 to 37.00 in 2012. Neither the *rpoB* gene conferring rifampicine resistance nor the *gyrA* gene conferring ofloxacin resistance was found in all 4 cases (Table 3).

Sero-diagnosis of leprosy

Of the 110 cases agreed to provide blood samples for MMP-II ELISA testing, 75 (68.2%) were male and the median age is 78.5 years old. There were 76 (69.1%) MB cases, 30 (27.3%) PB cases and 4 were unknown. The ROC curve indicated that the positive cut-off value is 0.233 (Figure 1A). The average O.D. values are 0.439, 0.318 for MB and PB, respectively (Figure 1B); while the average O.D. value for 10 health control is 0.149. The sensitivity and specificity of the MMP-II ELISA was 83.7% and 80%, respectively.

IV. Discussion

In this study, we established a diagnosis algorithm for leprosy, and reveal drug resistant patterns of *Mycobacterium leprae* using molecular methods. A real-time PCR was established with detection limit of 100 fg. We identified 4 leprosy cases in 2012. In addition, drug-resistant gene sequencing was performed to determine drug resistance of 4 *M. leprae* infected cases in 2012. After confirmation of one case for dapsone resistance, this patient can be treated with second-line medication to improve outcome. Nevertheless, we did not identify any mutation to rifampicin and ofloxacin in 4 confirmed cases. Taiwan has implemented national leprosy control program to eliminate leprosy sine the 1930s. Annual on-site health examination of cases, diagnosis and treatment with MDT remain key elements in our control strategies. In addition, foreign workers was requested by the Enforcement Rules of the Communicable Disease Control Act implemented in 1985 to provide health certificate at entry, and to have their health-check including leprosy at the sixth, the eighteenth and the thirtieth month during their stay in Taiwan. The prevalence rate has been less than 1 leprosy case per 10,000 populations in Taiwan since 2004. MDT treatment provides a simple yet highly effective cure for all types of leprosy. Results of

real-time PCR were consistent with histological classification of lepromatous leprosy. In addition, we proved that sero-diagnosis using MMP-II ELISA is useful for screening leprosy with a satisfactory sensitivity of 83.7%. For providing appropriate treatment, determination of drug-resistance for each confirmed case can ensure good treatment outcome become a leprosy diagnosis policy in Taiwan.

V. References

1. Truman RW, Andrews PK, Robbins NY, Adams LB, Krahenbuhl JL, Gillis TP. Enumeration of *Mycobacterium leprae* using real-time PCR. *PLoS Negl Trop Dis.* 2008;**2**:e328.
2. Kai M, Nguyen Phuc NH, Nguyen HA, Pham TH, Nguyen KH, Miyamoto Y, et al. Analysis of drug-resistant strains of *Mycobacterium leprae* in an endemic area of Vietnam. *Clin Infect Dis.* 2011;**52**:e127-32.
3. Matsuoka M., Drug resistance in leprosy, invited review, Jpn. J. Infect. Dis. 63:1-7, 2010
4. Maeda Y., Mukai T., Kai M, Fukutomi Y., etal., Evaluation of major protein-II as a tool for serodiagnosis of leprosy. FEMS Microbial. Lette. 272: 202-205, 2007.
5. Kai M. Phuc N. H. N., Thi T. H. H., etal., Serological diagnosis of leprosy in patients in Vietnam by enzyme-linked immunosorbent assay with *Mycobacterium leprae*-derived major membrane protein II. Clinical and Vaccine Immunology, 15: 1755-59, 2008.
6. Maeda Y., Mukai T., Kai M, Fukutomi Y., etal., Evaluation of major protein-II as a tool for serodiagnosis of leprosy. FEMS Microbial. Lette. 272: 202-205, 2007.
7. Kai M. Phuc N. H. N., Thi T. H. H., etal., Serological diagnosis of leprosy in patients in Vietnam by enzyme-linked immunosorbent assay with *Mycobacterium leprae*-derived major membrane protein II. Clinical and Vaccine Immunology, 15: 1755-59, 2008
8. Taiwan Centers for Disease Control. *Notifiable Infectious Diseases Statistics System.* Available at http://nidss.cdc.gov.tw/
9. WHO: *Weekly epidemiological record, Global leprosy situation.* 2012;**87**:317-28.

Tables and Figures

Table 1. Demographic characteristics of 81 leprosy cases in Taiwan, 2002-2011

	Imported cases (N=44)	Indigenous cases (N=37)
Sex		
Male No. (%)	7 (15.9)	24 (64.9)
Female No. (%)	37 (84.1)	13 (35.1)
Male		
Median age (range), y	33 (24-60)	60.5 (31-83)
≦20	0	0
21-40	5	3
41-60	2	9
≧61	0	12
Female		
Median age (range), y	27 (20-52)	67 (45-81)
≦20	1	0
21-40	34	0
41-60	2	3
≧61	0	10
Nationality		
Taiwan	0	36
Indonesia	32	1
Vietnam	4	0
Others[a]	8	0
Type		
Multibacillary	31	25
Paucibacillary	10	11
Not determined	3	1

[a]Includes Thailand (3), Philippines (3), Myanmar (1), and China (1).

Table 2. Results of RLEP real-time PCR ,2012

Site	**Cases no.**	**RLEP real-time PCR positive, Case no.**
A	7	0
B	10	0
C	14	0
D	11	1
E	15	1
F	9	2
G	3	0
H	90	0
Total	159	4

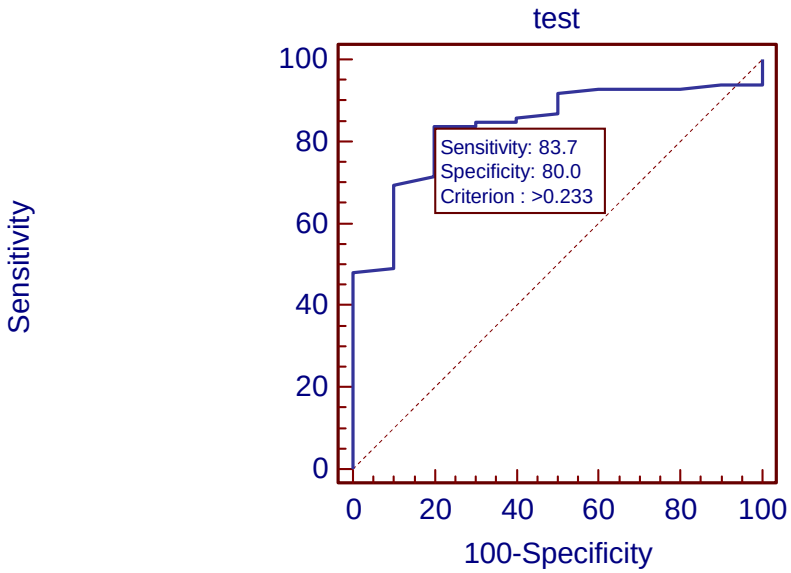
Table 3. Sequencing results of the drug-resistant genes of the RLEP positive cases, 2012

ID	Ct of RLEP	drug-resistant genes		
		*fol*P1	*rpo*B	*gyr*A
D-10 (2008)	23.86	R*	S	S
D-10 (2010)	29.00	R	NA	S
D-10 (2012)	37.00	NA	NA	NA
E-13	27.45	S	S	S
F-4	26.09	S	S	S
F-8	38.20	NA	NA	NA

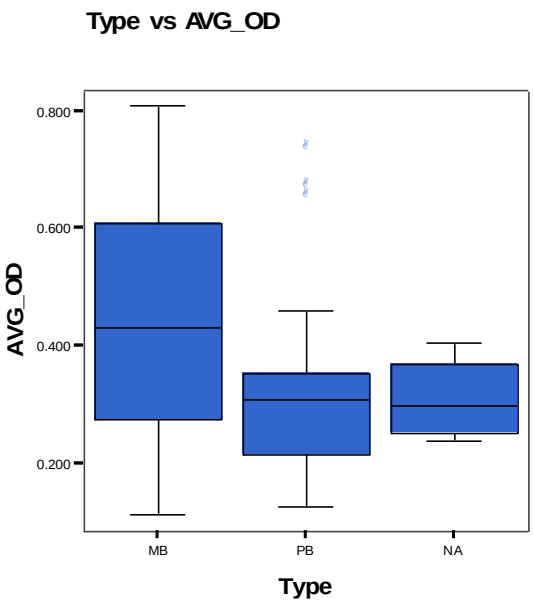
* indicate codon 55 of the *fol*P1 gene harbored CCC (Pro) to CTC (Leu)

Figure 1. (A)Receiver Operator Characteristics (ROC) curves describes sensitivity and specificity and the cut-off level; (B) OD values of MB and PB leprosy cases in Taiwan

(A)



(B)



VI. Publication list for this work

The poster has been accepted at the 23rd European Congress of Clinical Microbiology and Infectious Diseases.