($p<0.05$). As compared to RBT-resistant MDR isolates, RBT-susceptible ones harbored mutation at codon 526 tended to have higher resistant rates to EMB, ETH and three injectable second-line drugs; however, lower resistant rate to OFX was found (Table 2). In addition, isolates with a mutation at codon 531 had higher resistant rate to OFX ($p<0.05$) and ETH ($p<0.05$), and lower resistant rate to KAN ($p<0.05$). Furthermore, isolates with a mutation at codon 533 had lower resistant rate to PZA ($p<0.05$), STR ($p<0.05$), and ETH ($p<0.05$). In contract to MDR isolates harbored mutations at codons 513, 516, 526 and 531 had high resistant rates (16.3% to 46.2%) to ETB, none of MDR isolates with mutated codon 533 was resistant to ETH ($p<0.01$) (Table 2). Moreover, MDR isolates with codons 513 and 533 mutations were not found in isolates resistant to AM or CAP.

Discussion

RBT is as effective as RIF for TB treatment and is an alternative for treating MDR-TB patients, TB cases with serious side-effects, and HIV co-infected TB cases. However, RBT is not included in routine DST of first-line drugs for *M. tuberculosis* isolates, and subsequent testing of isolates is time-consuming. In addition, the cross-resistant rate is usually high between RIF and RBT. In this study, the cross-resistant rate between RIF and RBT is 87.0%. It was comparable to that of Australia et al. (88%, 18/22) [3] and Turkey et al. (85.4%, 35/41) [4] studies, however, higher than another research later from study of Turkey et al.(73.1%) [5]. We revealed 13 novel mutations and deletions in RIF-resistant *M. tuberculosis* clinical isolates. Moreover, we found RIF-resistant isolates with specific single mutation at codon 143, 511, 516, 522, 529 and ones with amino acid changes at codon 526 (H to C, L, T, N) of the *rpoB* gene were susceptible to RBT, and can be used as robust markers for RBT-susceptible isolates. Furthermore, we observed association of predominant mutations in the *rpo*B genes and other anti-tuberculosis drugs, and that might be used as predictors for probable resistance to various drugs during treatment and for selection of treatment options.

Previous studies revealed that variations of MICs of RBT in RIF-resistant strains carrying *rpo*B mutations depend on specific mutations in the *rpo*B gene. It has been reported that V176F (*M. tuberculosis* numbering), Q513K, Q513L, Q513P, S522W, H526R, H526Y, H526D, H526Q, H526P, S531L, S531W (*E. coli* numbering) coherently confer resistance to both RIF and RFB; whereas, D516Y, D516V, S522L, H526C confer resistance, and L511P confers low level resistance only to RIF [2, 6, 7, 8, 9, 10]. However, resistance to RIF or RBT remains controversial in isolates with

H526L and L533P [9, 10]. Our data were consistent with pervious findings, and we also confirmed 11 isolates with H526L were RBT-susceptible without ambiguous. It was postulated that simple amino acid substitution could interfere or enhance protein polar/hydrophobic enzyme and RIF interactions; while, induction of hydrogen bonding and changes in van der Waals interaction between protein and drug also had profound influence on drug resistance [11, 12, 13].

RIF had drug-drug interactions with several drugs, including antiretrovirals. Some mechanisms that RIF influences the susceptibilities of different structural drugs were postulated. RIF exposure induces multidrug-resistant gene (MDR1) expression which encodes an efflux pump contributing to fluconazole resistance in *Candida albicans*, compared to non-exposed control was up to 122-fold dose-dependent induction. However, RBT and rifamycin are not active [14]. RIF induces enzymes that transport and metabolize moxifloxacin [15]. RBT is mostly used in HIV co-infected patients because it has fewer drug interactions with antiretroviral agents than RIF. Furthermore, Srivastava et al. revealed that resistance to INH, EMB, RIF, ciprofloxacin (CIP), STR, tetracyclines (TETs), OFX and KAN were found to be related to mechanisms of efflux pump systems [16]. However, one antibiotic may induce a pump that also extrude other antibiotics or induce a particular single pathway which then leads to inductions of many different efflux pumps. Srivastava et al. proposed an evolution of simultaneous resistance to anti-tuberculosis drugs [16]. This may able to explain that some resistant strains do not harbor any mutation at resistance-related genes and partial cross-resistance phenomenon.

The frequency of major mutations was shown in Table 1. The most common mutation was at codon 531 (64%), codon 526 (17.1%) and codon 526 (7.1%).

However, only 1% of isolates with codon 531 mutation were multiple mutation cases while isolates with codon 511 or 146 were 70% and 45.5% respectively. In addition, 71.4% and 84.6% of multiple mutation cases with one common mutation at codon 511 and 516 changed their susceptibilities to RFB and became resistant.

In conclusion, RIF had high cross-resistant rate with RBT. However, RBT remains potent to isolates with certain genetic alterations. Besides, genetic mutation of certain condon of the *rpoB* gene might be used to predict drug-drug interaction to other anti-TB drugs.

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Table 1. MDR *Mycobacterium tuberculosis* isolates with multiple mutations in the *rpoB* gene

Mutated position	No. of isolates with any mutation (%)	No. of isolates with multiple mutations (%)
146	11 (1.4)	5 (45.5)
511	10 (1.3)	7 (70.0)
513	25 (3.1)	3 (12.0)
516	57 (7.1)	13 (22.8)
526	137 (17.1)	13 (9.5)
531	512 (64.0)	5 (1.0)
533	39 (4.9)	12 (30.8)
Total	800	58 (7.5)

Table 2. Associations of various mutation codons at the *rpo*B gene and the first and the second-line drugs of 568 multidrug-resistant *Mycobacterium tuberculosis* isolates

Mutation[f]		No. of MDR isolates	EMB	PZA	STR	PAS	OFX	ETH	KAN	CM	AMK
			No. of resistant isolates (%)								
513[a]	wt	547	289 (52.8)	186 (34.0)	241(44.1)	61 (11.2)	131 (23.9)	159 (29.1)	54 (9.9)	28 (5.1)	31 (5.7)
	mut	21	11 (52.4)	7 (33.3)	5 (23.8)	4 (19.0)	2 (9.5)	6 (28.6)	2 (9.5)	0 (0.0)	0 (0.0)
516[b]	wt	537	283 (52.7)	177 (33.0)	229 (42.6)	56 (10.4)	123 (22.9)	154 (28.7)	49 (9.1)	26 (4.8)	28 (5.2)
	mut	31	17 (54.8)	**16 (51.6)***	17 (54.8)	**9 (29.0)**[g]**	10 (32.3)	11 (35.5)	**7 (22.6)**[g]**	2 (6.5)	3 (9.1)
526[c]	wt	482	263 (54.6)	170 (35.3)	200 (41.5)	57 (11.8)	120 (24.9)	151 (31.3)	45 (9.3)	25 (5.2)	25 (5.2)
	mut	86	**37 (43.0)***	23 (26.7)	**46 (53.5)***	8 (9.3)	**13 (15.1)***	**14 (16.3)****	11 (12.8)	3 (3.5)	6 (7.0)
526[d]	wt	555	291 (52.4)	189 (34.1)	237 (42.7)	64 (11.5)	133 (24.0)	159 (28.6)	53 (9.5)	26 (4.7)	28 (5.0)
	mut	13	9 (69.2)	4 (30.8)	**9 (69.2)**[g]**	1 (7.7)	**0 (0.0)**[g]**	6 (46.2)	3 (23.1)	2 (15.4)	**3 (23.1)**[g]**
531[e]	wt	183	93 (50.8)	58 (31.7)	84 (45.9)	25 (13.7)	33 (18.0)	41 (22.4)	25 (13.7)	8 (4.4)	12 (6.6)
	mut	385	207 (53.8)	135 (35.1)	162 (42.1)	40 (10.4)	**100 (26.0)***	**124 (32.2)***	**31 (8.1)***	20 (5.2)	19 (4.9)

533	wt	549	290 (52.8)	191 (34.8)	242(44.1)	65 (11.8)	129 (23.5)	165 (30.1)	55 (10.0)	28 (5.1)	31 (5.6)
	mut	19	10 (52.6)	**2 (10.5)***	**4(21.1)***	0 (0.0)	4 (21.1)	**0 (0.0)****	1 (5.3)	0 (0.0)	0 (0.0)
Total		568	300 (52.8)	193 (34.0)	246 (43.3)	65 (11.4)	133 (23.4)	165 (29.0)	56 (9.9)	28 (4.9)	31 (5.5)

* P value <0.05

** P value <0.01

[a] Include codon 513 CAA to AAA, CTA, GAA, and CCA

[b] Include codon 516 GAC to TAC, GGC, GTC, and TTC

[c] Include codon 526 CAC to CGC, TAC, GAC, CAA, and CCC, which were associated with RFB resistance

[d] Include codon 526 CAC to TGC, CTC, AAC, and ACC, which were not associated with RFB resistance

[e] Include codon 531 TCG to TTG and TGG

[f] wt, wild-type; mut, mutant

[g] Significance may not be valid, because the sample size is too small.

Molecular and serological identification and drug resistance detection of leprosy

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Summary

The prevalence rate of leprosy has been less than one case per 10,000 populations in Taiwan since 2004. Since *Mycobacterium leprae* can not be cultivated *in vitro*, smear microscopy is currently the only test used in clinical laboratories for bacteriological diagnosis in Taiwan. The objectives of this study are to develop diagnostic methods and to establish drug-resistance surveillance of leprosy. We conducted molecular diagnosis using *M. leprae*-specific repetitive element (RLEP) and drug-resistant gene sequencing of dapsone (*folP1*), rifampicin (*rpoB*) and ofloxacin (*gyrA*). A real-time PCR was established for rapid diagnosis of *M. leprae* with detection limit of 100 fg. Results of real-time PCR were consistent with histological classification of lepromatous leprosy. Of the 170 new, cured or on-treatment cases tested, 7 were *M. leprae* positive cases. Drug-resistant gene sequencing was performed to determine drug-resistant *M. leprae* of 7 confirmed cases. We found 2 cases (N and S) were resistant to dapsone with single mutation at either codon 53 (T to R) or codon 55 (P to L) of the *folP1* gene, respectively. Nevertheless, we did not identify any mutation in the *rpoB* and *gyrA* genes. The new marker, MMP-I, seems to be a better antigen than PGL-1 and MMP-II for serodiagnosis of leprosy. The sensitivity and specificity of the well-established MMP-II ELISA was 79.1% and 100% for leprosy diagnosis, respectively. For strengthening leprosy control, rapid and definite detection of leprosy and determination of drug-resistant *M. leprae* can ensure favorable treatment outcome.

Purposes

The purposes of this collaborative study are to understand epidemiology of leprosy and to establish methods for detecting *Mycobacterium leprae.*

Materials and Methods

Study samples

We retrospectively analyzed 13 biopsy samples from National Taiwan University and 48 blade specimens from skin smear from the Lo-Sheng Sanatorium, Taiwan. Besides, we also prospectively analyzed paraffin specimens of four suspect leprosy cases in 2013. For serodiagnosis, serum samples of 110 registered leprosy cases were obtained from the 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 12.5μl 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 –TAMRA-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.

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 100μl. 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).

Serodiagnosis

MMP-II ELISA

The ELISA for the detection of anti-MMP-II immunoglobulin G (IgG) antibodies was performed as leprosy center suggested[1-4]. 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 phosphate buffered 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.

Results

Leprosy in Taiwan, 2002-2011

Leprosy has been significantly reduced through the implementation of intensified control strategies. Particularly, World Health Organization (WHO) has provided free multidrug therapy (MDT) treatment, consisting of diaminodiphenylsulfone (dapsone), rifampicin and clofazimine, since 1995.[5] Consequently, leprosy has been successfully eliminated from 119 of 122 countries in 2010.[6] The elimination of leprosy is defined as having leprosy prevalence rate of less than 1 case per 10,000 populations. The enhanced global strategy for further reducing the disease burden due to leprosy 2011-2015 has been carried out in endemic countries.[7] The prevalence of leprosy in the WHO Western Pacific Region, where Taiwan is located, is 0.05 and that of South-East Asia Region is 0.64 in the first quarter of 2012.[8]

Leprosy is caused by *Mycobacterium leprae* that is an acid-fast bacillus with an incubation period ranged from several months to several decades after infection. Leprosy causes severe, disfiguring skin sores and nerve damage in the arms and legs. Skin become stiffness and dryness, and lesions show erythematous plaques (annular), hyperpigmented plaques and hypopigmented patches. Sensory manifestations include paresthesia, pain and feeling loss. In Taiwan, leprosy is a notifiable disease defined mainly by smear microscopy, histological examination and clinical judgment. A comprehensive leprosy control program to eliminate leprosy has been implemented in Taiwan sine the 1930s. The first sanatorium was established in 1930 to quarantine leprosy patients. Nevertheless, leprosy cases have been treated as out-patient in 5 Taiwan Centers for Disease Control (CDC) designated integrated medical service settings since 2009. Dapsone and MDT have been respectively prescribed since 1952 and 1983.[9]

Epidemiology of leprosy in Taiwan

According to the Statistics of Communicable Diseases and Surveillance Report issued by Taiwan CDC, the prevalence of leprosy decreased from 1.33/10,000 in 2002 to 0.50/10,000 in 2011 (Figure 1A). The registered cases were 2,987 and 1,131 in 2002 and 2004, respectively. After national census conducted in 2004, the prevalence rate was decreased to 0.50 and was considered as a country with leprosy elimination. During 2002 to 2011, we confirmed 81 new leprosy cases, with 5 to 12 cases reported annually.[10] 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). 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 (Figure 1B). 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.[8] 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.

The National Reference Laboratory of Mycobacteriology of the Taiwan CDC participates in national laboratory-based surveillance for *M. leprae* which started in 2012 and involves 5 sentinel hospitals in Taiwan.

Molecular diagnosis of leprosy

Since the majority of leprosy patients have a negative smear, the laboratory established molecular assays for leprosy using a PCR-based analysis. The results of PCR were well correlated with biopsy findings. For *M. lepare* identification, primers set based on the *M. leprae*-specific repetitive element was developed with a modified real-time TaqMan PCR method.[11] Of the 13 cases tested, 2 cases were resistant to dapsone with mutations in the *folP* gene, and all cases were susceptible to rifampicin (the *rpoB* gene) or fluoroquinolone (the *gyrA* gene) using the nested PCR and sequencing.[8] Minocylcine was added in addition to MDT thereafter for one case that showed a striking improvement in the following several months. The other case is a relapse case with diabetes mellitus that initially responded poorly to MDT and moxifloxacin was included in the prescription thereafter. In 2013, of the four suspected leprosy cases, cases 1 and 3 had negative results using the RLEP real-time PCR, and cases 2 and 4 had positive results with real-time PCR (Table 1). Due to low DNA content of *Mycobacterium leprae* in the specimen, we were not able to perform drug-resistant gene sequencing for case 2. While the *folp1*, *rpoB* and *gyrA* drug-resistant gene sequencing was performed to detect drug resistance of case 4, no mutation was found.

Serodiagnosis of leprosy

Antigens, PGL-1, MMP-I and MMP-II were used to determine the sensitivity for