Molecular characteristics of second-line drug resistance in multidrug-resistant *Mycobacterium tuberculosis*

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Directed by Professor Kyungwon Lee

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ABSTRACT

Molecular characteristics of second-line drug resistance in multidrug-resistant *Mycobacterium tuberculosis*

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Tuberculosis (TB) is an infectious disease that usually attacks the respiratory system. Almost 10 million people worldwide contract TB each year. In Korea, TB incidence is 97 cases per 100,000 populations, which is highest among the member countries of the Organisation for Economic Cooperation and Development. Although there have been many efforts and resources contributed to control TB worldwide, an increase in drug-resistant TB cases is one of the major obstacles. Multidrug-resistant TB (MDR-TB) is defined as TB that is resistant to both isoniazid and rifampicin, which are the most important first-line drugs for TB. It takes approximately two years

to cure MDR-TB with second-line drugs. Moreover, some MDR-TB cases are resistant to second-line drugs, which are called extensively drug-resistant TB (XDR-TB). It is extremely difficult to select drugs to treat XDR-TB; thus, treatment outcomes are poor. For successful control of drug-resistant TB, assessment of drug resistance rates and rapid and accurate drug susceptibility testing (DST) are necessary. Since conventional DST has many drawbacks, molecular DST has been introduced for detecting MDR-TB. However, there is little evidence of feasibility for molecular DST to detect XDR-TB. Therefore, the present study aims to analyze drug resistance in Korea and to evaluate rapid molecular detection of second-line drug resistance.

DST results in 2009 were collected and divided into six groups: smear-negative new cases of health centers (HCs), smear-positive new cases of HCs, previously treated cases of HCs, health examination, referred cases from hospitals, and patients of the Korean National TB Association clinics. Also, we collected MDR-TB isolates whose mutations in seven loci associated with second-line drug resistance were identified and compared with conventional DST results.

The resistance rate to isoniazid was highest, and the MDR rate of smear-positive new cases was 3.5%. XDR-TB accounted for 1% of the total cases and 12% of the MDR-TB cases. Resistance rates in MDR-TB varied greatly based on the drug and group. First-line drugs other than isoniazid and rifampicin showed very high resistance rates, with injectable agents demonstrating the lowest resistance rates among the drugs tested. The rates of resistance to fluoroquinolones were 2-4-fold higher in the private sector than in the public sector.

Mutations in *rpsL*, 16S rRNA gene, and *gyrA* and *gyrB* were common in TB isolates with resistance to streptomycin, aminoglycosides, and fluoroquinolones, respectively. On the other hand, mutations in *eis* and *gidB* were not only present in resistant isolates, but also in susceptible isolates.

This study demonstrates an increasing trend in MDR-TB rate among new cases of Korea. Also, we found that the proportion of XDR in MDR-TB cases was lower than the previously reported rate of 15%. A higher resistance rate of fluoroquinolones in the private sector underlines cautious use of these drugs and need for improvement in the treatment success rate for MDR-TB cases. Detecting major mutations which confer resistance to second-line drugs would be a reliable and specific method of identifying these strains. In summary, these results will be useful in helping to establish a treatment strategy for MDR-TB, and the data suggest that molecular DST can be used as a surrogate for conventional DST.

Key words: multidrug-resistant tuberculosis, extensively drug-resistant tuberculosis, second-line drug resistance, drug susceptibility testing, mutations, rapid diagnosis

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I. INTRODUCTION

The World Health Organization (WHO) estimated that 9.4 million people worldwide developed tuberculosis (TB) and 1.7 million people died of TB in 2009.[1] In Korea, more than 47,000 TB cases were notified in 2009 and the incidence rate was 97/100,000 population, which was lower than global incidence but much higher than those of other industrialized countries, like the USA or Japan. Many countries are running national TB control programs (NTP) and contribute many resources to these programs. However, the incidence and prevalence of TB seldom decrease, and TB remains as the leading cause of death.[1] The emergence of HIV infection and drug-

resistant TB are jeopardizing TB control worldwide.[1] Because drug-resistant TB develops due to the improper treatment of drug-susceptible TB, it is a man-made disaster.[2] Multidrug-resistant TB (MDR-TB) is defined as TB that is resistant to at least isoniazid (INH) and rifampicin (RIF), which are the most important first-line drugs. The WHO estimate that the MDR-TB rate among new TB cases was 3.3% in 2009.[1] According to the last drug resistance survey (DRS) conducted in Korea, 2.7% of new smear-positive cases in 2004 were MDR-TB.[3]

Patients with MDR-TB should be treated with second-line drugs, such as fluoroquinolones (FQs), and injectable agents. Moreover, some TB bacilli are resistant to second-line drugs, which are defined as extensively drug-resistant TB (XDR-TB). XDR-TB was originally defined as MDR-TB with bacillary resistance to three or more of the six classes of second-line drugs.[4] In 2006, the definition of XDR-TB was revised by the WHO as MDR-TB with resistance to any fluoroquinolone and at least one second-line injectable drug (amikacin, capreomycin, or kanamycin), emphasizing the important roles of FQs and injectables for MDR-TB treatment.[5]

The duration of treatment for MDR/XDR-TB is quite long.[5] The efficacies of second-line drugs are lower than those of first-line drugs, and adverse reactions to these drugs are common. Therefore, the treatment outcome of MDR/XDR-TB is worse than that of susceptible TB.[5,6] Accurate drug susceptibility testing (DST) is crucial to detect MDR-TB and for the selection of an appropriate regimen for MDR-TB patients.[2,7]

DST proficiency tests conducted by the supranational reference laboratories network

(SRLN) revealed that concordance rates for INH and RIF between SRLs are very high.[8] However, reliabilities of antituberculosis drugs other than INH and RIF are relatively poor due to a weak correlation with clinical outcome and a lack of procedure standardization.[7,9,10] Moreover, conventional DST has several issues. First, weeks or even months are required for the determination of test results, resulting in diagnostic delays due to a long generation time.[10,11] Biosafety is another important issue for DST laboratories since manipulation of TB isolates for DST is a hazardous procedure.[12] In addition, it is difficult to maintain appropriate proficiency for DST because of its technical complexity. Therefore, TB laboratories performing DST must maintain a high level of biosafety and qualified staff.

Genetic resistance to an antituberculosis drug develops due to spontaneous chromosomal mutations which occur at a frequency of 10^{-6} to 10^{-8} mycobacterial replications.[2] Molecular mechanisms of drug resistance have been elucidated for antituberculosis drugs.[13,14] RIF resistance is mainly associated with mutations in an 81-bp RIF resistance-determining region of the *rpoB* gene.[2] Mutations in *katG*, promoter of *inhA*, and *ahpC* have been identified in INH-resistant isolates.[2] Rapid molecular DSTs for RIF and INH were developed to detect MDR-TB strains and have shown very good performance in many studies.[10,15] Eventually, the WHO endorsed the use of molecular DST for rapid diagnosis of MDR-TB strains.

Mutations conferring resistance to fluoroquinolones (FQ) are known to accumulate in a short discrete region of the *gyrA* and *gyrB* genes called the quinolone resistance-determining region (QRDR).[2] Mutations in *rrs* also confer broad resistance to

injectable agents.[2] However, the drug resistance mechanisms of many TB isolates are not understood. In recent years, new mechanisms of second-line drug resistance have been identified.[11,16-18] However, only a few studies have focused on these mechanisms, and the results are inconsistent.

In this study, we analyzed the results of drug susceptibility testing to assess the status of drug resistance in Korea and to help physicians choose an MDR-TB treatment regimen. We also evaluated a variety of genes associated with second-line drug resistance by comparing genotype results with conventional DSTs and minimal inhibitory concentrations (MIC).

II. MATERIALS AND METHODS

1. Analysis of the drug resistance profiles

The Korean Institute of Tuberculosis (KIT) is the central laboratory of the National TB Control Program (NTP) in Korea. KIT carries out DST with TB isolates from public health centers (HCs), private hospitals and clinics of the Korean National Tuberculosis Association (KNTA). In 2009, a total of 22,409 DSTs were performed by the KIT. Among them, duplicates, nontuberculous mycobacteria and growth failures were excluded, and the remaining 16,751 test results were analyzed. Cases were divided into the following six groups according to the institution type and treatment history: smear–negative new cases of HCs (SS- New), smear-positive new cases of HCs (SS+ New), previously treated cases of HCs (Retreat), health examination (Health exam), referred cases from hospitals (Referred) and patients of KNTA clinics (KNTA).

2. *Mycobacterium tuberculosis* clinical isolates

A total of 205 multidrug-resistant *Mycobacterium tuberculosis* (MTB) isolates were collected from June 2009 through October 2009. MDR-TB isolates were grouped as XDR-TB (XDR) (n = 36), MDR with FQ resistance (MDR-F) (n = 43), MDR with injectable agents resistance (MDR-I) (n = 16) or others (n = 110), and the members of the others group were excluded from analysis. Fully susceptible isolates (n = 10) were also collected for use as a control group.

3. Drug susceptibility testing (DST)

A. Conventional DST

Conventional DST was performed using the absolute concentration method with Lowënstein-Jensen (LJ) media. Fresh MTB colonies were scraped from LJ slants and transferred into a McCartney bottle with 10% Tween 80 buffer and 20-30 glass beads. The suspension was homogenized by vortexing, and 2-4 mL of supernatant was transferred to a sterile tube. The turbidity of the suspension was adjusted to a McFarland No. 1 standard solution and then diluted 1:10 with phosphate buffer. The DST kit was inoculated with 25 μL of the suspension in each well containing LJ media with the following critical concentrations of 14 antituberculosis drugs: 0.2 μg/mL INH; 40 μg/mL RIF, kanamycin (KM), amikacin (AMK), capreomycin (CAP) and prothionamide (PTH); 4 and 10 μg/mL streptomycin (SM); 2 μg/mL ethambutol (EMB), ofloxacin (OFL), levofloxacin (LEV) and moxifloxacin (MOX); 1 μg/mL *para*-aminosalycilic acid (PAS); 30 μg/mL cycloserine (CS); and 20 μg/mL rifabutin (RBT). Growth greater than 1% of that of the control was regarded as drug resistance. Resistance to pyrazinamide (PZA) was determined using the pyrazinamidase (PZase) assay. PZase activity was detected using the Wayne method as described previously.[19] Briefly, Dubos agar tubes containing 100 μg/mL PZA were heavily inoculated and incubated for seven days. Then 1 mL ferrous ammonium phosphate solution was added to the tube and refrigerated for 4 hours. If no pink band was observed, the isolate had no PZase activity, implying PZA resistance.

B. Determination of minimal inhibitory concentrations

The MICs of OFL, LEV, MOX, KM, AMK, CAP and linezolid (LIN) were determined using the broth microdilution method in Middlebrook 7H9 broth supplemented with 5% oleic acid-albumin-dextrose-catalase (OADC), according to CLSI guideline.[20] The oxidation-reduction dye, 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (Kyokuto Seiyaku, Tokyo, Japan) was added to the broth media at a concentration of 100 μg/mL. Ranges of drug concentrations were as follows: KM (0.06 to 64 μg/mL), AMK (0.06 to 64 μg/mL), CAP (0.06 to 64 μg/mL), OFL (0.06 to 64 μg/mL), LEV (0.06 to 64 μg/mL), MOX (0.03 to 32 μg/mL) and LIN (0.03 to 32 μg/mL).

Media plates were incubated at 37ºC in ambient air and interpreted after two weeks of incubation. When color of the media well turned red, it was regarded as growth of *M. tuberculosi*s.

4. Mutations in genes associated with second-line drug resistance

DNA was extracted from isolates using the heating method. The colonies were collected from LJ slants and suspended in a tube with 1 mL distilled water (DW). The tube was boiled for 10 min and centrifuged at 10,000 x *g* for 5 min. The supernatant was transferred to a sterile tube and preserved at -70ºC before use.

The following seven loci were amplified using polymerase chain reaction (PCR): *rpsL*, *gidB* (SM), *rrs* (SM, KM, AMK and CAP), *eis* (KM), *tlyA* (CAP), *gyrA*, *gyrB* (OFL, LEV and MOX). PCR was performed using AccuPower® HF PCR PreMix

(Bioneer, Daejeon, Korea). Each PCR reaction contained 45 μL DW, 1 μL the forward and reverse primers, and 3 μL template DNA in a Premix PCR tube. Primers used for amplification and sequencing are shown in Table 1.[17,21-24]

PCR conditions for *gidB*, *tlyA*, *rpsL*, *gyrA* and *gyrB* were as follows: initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 30 sec and elongation at 72°C for 1 minute, with a final elongation at 72°C for 7 min.

For *rrs*, the PCR conditions were as follows: initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 90 sec, with a final elongation at 72°C for 7 min.

For *eis*, the PCR conditions were as follows: initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec, with a final elongation at 72°C for 7 min.

The PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit, and sequencing was performed on the 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequence data produced by the ABI 3130xl Genetic Analyzer were analyzed to detect the presence of mutations through comparison with sequences of H37Rv using the BLAST web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

If no mutations were found in the QRDR of *gyrA* in FQ-resistant isolates, the whole *gyrA* gene was sequenced using primers gyrA2 F, gyrA2 R and gyrA2 seq. The PCR conditions for the whole *gyrA* gene were the same as those described for *rrs* PCR.

Table 1. Primers used for PCR amplification and sequencing

Primer	Target gene	Nucleotide sequence (5'-3')	Product size (bp)	Reference
rpsL F	*rpsL*	CCAACCATCCAGCAGCTGGT	572	21
rpsL R		GTCGAGAGCCCGCTTGAGGG		21
tlyA F	*tlyA*	GCATCGCACGTCGTCTTT	947	22
tlyA R		GGTCTCGGTGGCTTCGTC		22
gyrA F	*gyrA*	GATGACAGACACGACGTTGC	398	21
gyrA R		GGGCTTCGGTGTACCTCAT		21
gyrA2 F		GGGCAACTTCGGCTCGC	1,883	This study
gyrA2 R		GCAGATAGGTGCCTTCACG		This study
gyrA2 seq		CGGGTCGGTTTACGCATCG		This study
GYRB-1	*gyrB*	CCACCGACATCGGTGGATT	428	23
GYRB-2		CTGCCACTTGAGTTTGTACA		23
gidB F	*gidB*	GTCCCTCCACTCGCCATC	662	17
gidB R		GCGGAGTGCGTAATGTCTC		17
16S Univ F	*rrs*	AGAGTTTGATCCTGGCTCAG	1,524	24
16S Univ R		AAGGAGGTGATCCAGCCGCA		24
eis F	*eis*	ATCGGTGAAACTGGCCGCGG	257	This study
eis R		CGGGGTATGCGTCGACGTGG		This study

III. RESULTS

1. Antituberculosis drug resistance patterns

A. The rates of resistance to first- and second-line drugs

We analyzed 16,751 non-duplicated DST results. In general, the drug resistance rate was highest in the KNTA group and lowest in the SS- New group. While the first-line drug resistance patterns of Health exam group were very similar with those of Referred group, Heath exam showed lower resistance rates to second-line drugs than those of Referred. Overall resistance rate to INH was 15.5%, which was highest among the tested drugs (Table 2). INH resistance rates of the Health exam, SS- New, SS+ New, Retreat, Referred and KNTA groups were 14.6%, 10.0%, 11.2%, 17.8%, 15.6% and 55.8%, respectively, while the RIF resistance rates were 7.3%, 3.9%, 4.2%, 11.7%, 8.9% and 52.3%. In total, 8.9% (1,499) of all cases were resistant to RIF. The overall SM resistance rate was 5.2%, and there was little difference in SM resistance rates between patient groups, except for that of the KNTA group. The EMB resistance rate ranged from 2.5% to 34.4%. PZA showed the lowest resistance rate (4.9%) among the first-line drugs.

Injectable agents showed very good activities against MTB, and the rates of resistance to KM, AMK and CAP were 1.4%, 1.2% and 1.1%, respectively (Table 3). However, FQ-resistance rates were relatively high (3.8% for OFL, 3.5% for LEV and 2.5% for MOX). The overall rates of resistance against RBT, PTH, CS and PAS were 6.4%, 3.8%, 1.3% and 3.4%, respectively.

A total of 1,347 (8.0%) MDR-TB cases were identified in 2009 (Table 4). The proportions of MDR-TB in SS- New and SS+ New were low as 3.1% and 3.5%. Almost 10% of Retreat group was MDR-TB. MDR-TB prevalence was highest in the KNTA (48.8%).

Table 2. Resistance to first-line antituberculosis drugs among *M. tuberculosis* isolates in 2009

Group	No. of isolates	No. (%) of resistant isolates				
		INH	RIF	SM	EMB	PZA
Health exam	274	40 (14.6)	20 (7.3)	15 (5.5)	16 (5.8)	15 (5.5)
SS- New	2,586	258 (10.0)	100 (3.9)	82 (3.2)	65 (2.5)	49 (1.9)
SS+ New	1,850	207 (11.2)	77 (4.2)	74 (4.0)	58 (3.1)	36 (1.9)
Retreat	650	116 (17.8)	76 (11.7)	34 (5.2)	48 (7.4)	26 (4.0)
Referred	10,911	1,706 (15.6)	975 (8.9)	575 (5.3)	746 (6.8)	534 (4.9)
KNTA	480	268 (55.8)	251 (52.3)	95 (19.8)	165 (34.4)	168 (35.0)
Total	16,751	2,595 (15.5)	1,499 (8.9)	874 (5.2)	1,098 (6.6)	828 (4.9)

INH, isoniazid; RIF, rifampicin; SM, streptomycin; EMB, ethambutol; PZA, pyrazinamide; Health exam, health examination; SS- New, smear-negative new cases from health centers; SS+ New, smear-positive new cases from health centers; Retreat, previously treated cases from health centers; Referred, referred cases from private hospitals; KNTA, cases from clinics of the Korea National Tuberculosis Association.

Table 3. Resistance to second-line antituberculosis drugs among *M. tuberculosis* isolates in 2009

Group	No. of isolates	No. (%) of resistant isolates									
		KM	AMK	CAP	OFL	LEV	MOX	RBT	PTH	CS	PAS
Health exam	274	2 (0.7)	2 (0.7)	1 (0.4)	2 (0.7)	2 (0.7)	1 (0.4)	16 (5.8)	5 (1.8)	1 (0.4)	12 (4.4)
SS- New	2,586	10 (0.4)	9 (0.3)	8 (0.3)	25 (1.0)	19 (0.7)	14 (0.5)	73 (2.8)	57 (2.2)	5 (0.2)	46 (1.8)
SS+ New	1,850	10 (0.5)	7 (0.4)	9 (0.5)	21 (1.1)	18 (1.0)	13 (0.7)	51 (2.8)	44 (2.4)	2 (0.1)	27 (1.5)
Retreat	650	10 (1.5)	6 (0.9)	5 (0.8)	12 (1.8)	11 (1.7)	7 (1.1)	62 (9.5)	20 (3.1)	3 (0.5)	21 (3.2)
Referred	10,911	165 (1.5)	138 (1.3)	131 (1.2)	422 (3.9)	395 (3.6)	290 (2.7)	692 (6.3)	411 (3.8)	145 (1.3)	384 (3.5)
KNTA	480	46 (9.6)	37 (7.7)	38 (7.9)	148 (30.8)	136 (28.3)	102 (21.3)	183 (38.1)	105 (21.9)	61 (12.7)	85 (17.7)
Total	16,751	242 (1.4)	199 (1.2)	192 (1.1)	630 (3.8)	581 (3.5)	427 (2.5)	1,077 (6.4)	642 (3.8)	217 (1.3)	575 (3.4)

KM, kanamycin; AMK, amikacin; CAP, capreomycin; OFL, ofloxacin; LEV, levofloxacin; MOX, moxifloxacin; RBT, rifabutin; PTH, prothionamide; CS, cycloserine; PAS, *para*-aminosalycilic acid. Other abbreviations are as in Table 1.

Table 4. Prevalence of multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis in 2009

Group	No. of isolates	No. (%)		XDR/MDR ratio (%)
		MDR-TB	XDR-TB	
Health exam	274	18 (6.6)	1 (0.4)	5.6
SS- New	2,586	79 (3.1)	5 (0.2)	6.3
SS+ New	1,850	64 (3.5)	3 (0.2)	4.7
Retreat	650	64 (9.8)	4 (0.6)	6.3
Referred	10,911	888 (8.1)	105 (1.0)	11.8
KNTA	480	234 (48.8)	44 (9.2)	18.8
Total	16,751	1,347 (8.0)	162 (1.0)	12.0

MDR-TB, multidrug-resistant tuberculosis; XDR, extensively drug-resistant tuberculosis. Other abbreviations are as in Table 1.

B. Antituberculosis drug resistance patterns in the MDR and XDR-TB isolates

A total of 1,375 MDR-TB cases were identified, and drug resistance profiles of these isolates were analyzed. First-line drugs other than INH and RIF showed relatively high resistance in MDR cases (Fig. 1). More than half of MDR cases were resistant to PZA, and only 29.1% were susceptible to RBT. The EMB resistance rate was 66.4%. Resistances to SM, EMB and RBT did not vary between patient groups. Injectable agents were the most active drugs to MDR-TB, and variations in resistance among patient groups were relatively low. The rates of resistance to KM, AMK and CAP were 16.5%, 13.7% and 12.2%, respectively. On the other hand, FQ resistance was higher than that of any other second-line drug. OFL resistance rates ranged from 11.1% to 53.8%, and the Referred and KNTA groups showed 2- to 4-fold higher resistance rate than did the other groups. LEV and MOX exhibited similar resistance patterns to that of OFL. The overall rates of resistance to PTH, CS and PAS were 28.4%, 15.3% and 26.4%, respectively.

There were 162 (1.0%) XDR-TB cases, 105 (64.8%) of which were from hospitals, 44 (27.2%) were from KNTA clinics, and 12 (7.4%) were from public HCs. The proportion of XDR-TB cases among MDR-TB cases was 12.0% (Table 4). Interestingly, the resistance rate for SM (45%) was lowest in XDR-TB, followed by CS (47%) (Fig. 2). More than 90% of XDR-TB isolates were resistant to EMB, PZA, KM, OFL, and LEV.

Eight pandrug-resistant TB (PDR-TB) cases, which were resistant to all tested drugs, were also identified. Of these, one was from a KNTA clinic, three were from a

TB hospital, and four were from tertiary teaching hospitals. The ages of the PDR-TB patients ranged from 24 to 74 years.

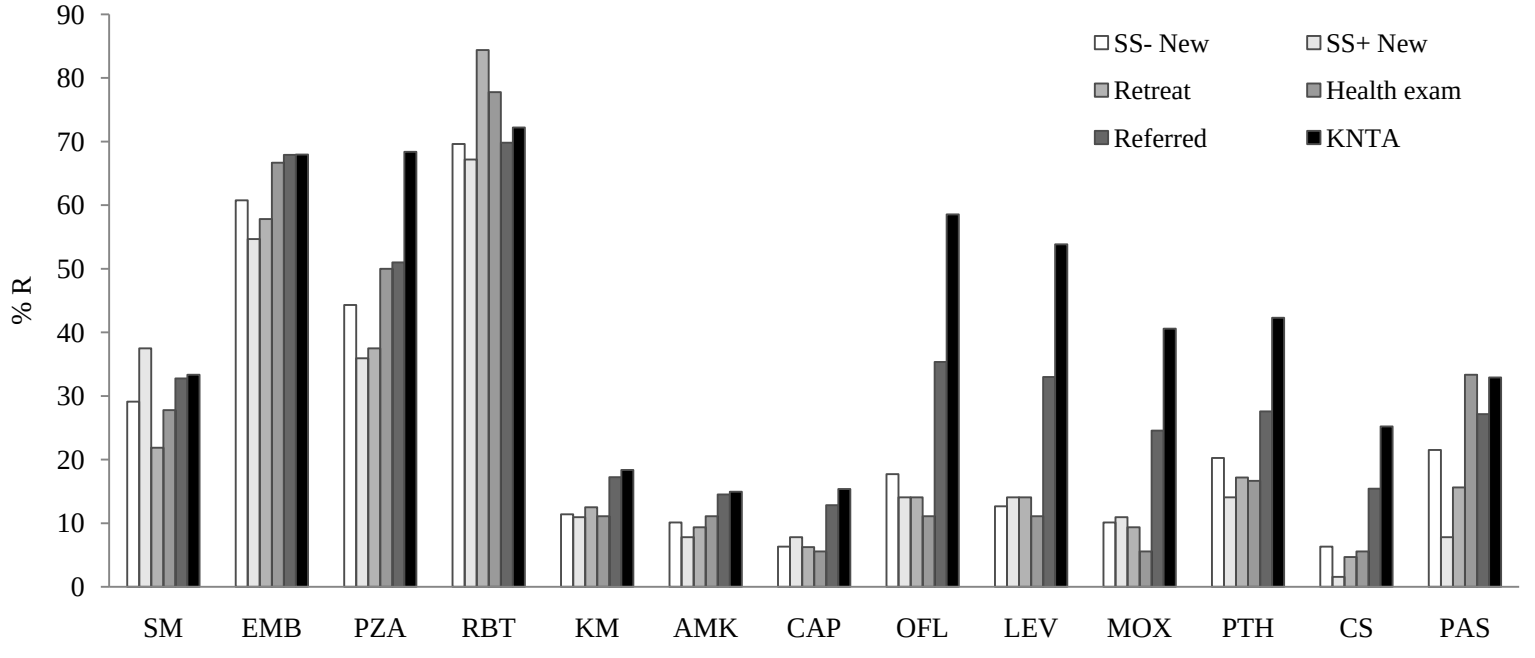


Figure 1. Resistance patterns of multidrug-resistant tuberculosis cases according to patient group. The rates of resistance against first-line drugs were also high in multidrug-resistant tuberculosis. Injectable agents showed the lowest level of drug resistance. Fluoroquinolone resistance rates were 2-4-fold higher in the private sector than they were in the public sector. Abbreviations are as Tables 1 and 2.

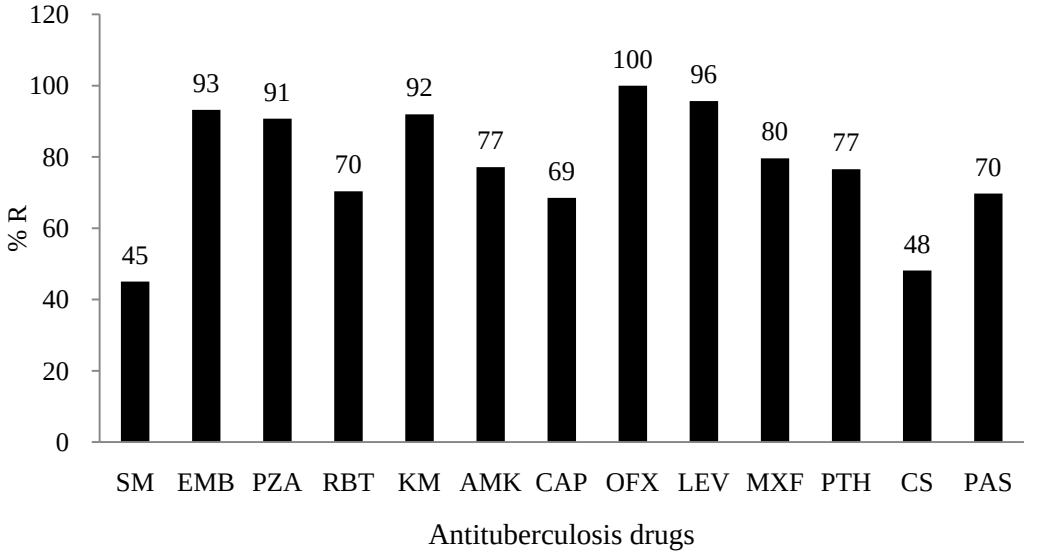


Figure 2. Drug resistance patterns of extensively drug-resistant tuberculosis cases. Streptomycin resistance was lowest among extensively drug-resistant tuberculosis isolates, followed by cycloserine resistance. The majority of extensively drug-resistant tuberculosis isolates were resistant to ethambutol, pyrazinamide, kanamycin, ofloxacin, and levofloxacin. Abbreviations are as in Tables 2 and 3.

C. Cross-resistance between antituberculosis drugs

(A) Injectable agents

A total of 270 cases were resistant to at least one injectable agent. Of these, 161 (59.6%) were resistant to all three drugs. All of the AMK-resistant cases (n = 199) were also resistant to KM, whereas 38 (19.1%) cases were still susceptible to CAP (Fig. 3). Among cases with resistance to KM (n = 242), 78 (32.2%) were still susceptible to CAP and 43 (17.8%) to AMK.

(B) Fluoroquinolones

A total of 630 cases were resistant to at least one FQ. Of these, all cases were resistant to OFL, and 426 (67.6%) were resistant to all three drugs. All cases with resistance to LEV or MOX were co-resistant to OFL, whereas 32.2% and 7.8% of OFL resistant cases were still susceptible to MOX and LEV, respectively. There were no LEV or MOX mono-resistant cases.

(C) Rifampicin and rifabutin

A total of 1,074 (6.4%) cases were resistant to RIF or RBT, and 15,674 (93.6%) cases were susceptible to both drugs. Among cases resistant to RIF (n = 1,499), 425 (28.4%) were susceptible to RBT (Table 5). Three cases were resistant to RBT but susceptible to RIF. The concordance rate between RIF and RBT was 97.4%, and the kappa value was 0.82 (95% CI, 0.80-0.84).

(D) Isoniazid and prothionamide

Of 16,751 cases, 632 (3.8%) were resistant to INH or PTH, and 14,146 (84.4%) cases were susceptible to both drugs (Table 6). The concordance rate between the two drugs was 88.2%, and the kappa value was 0.35 (95% CI, 0.33-0.37). Almost all PTH-resistant cases were also resistant to INH.

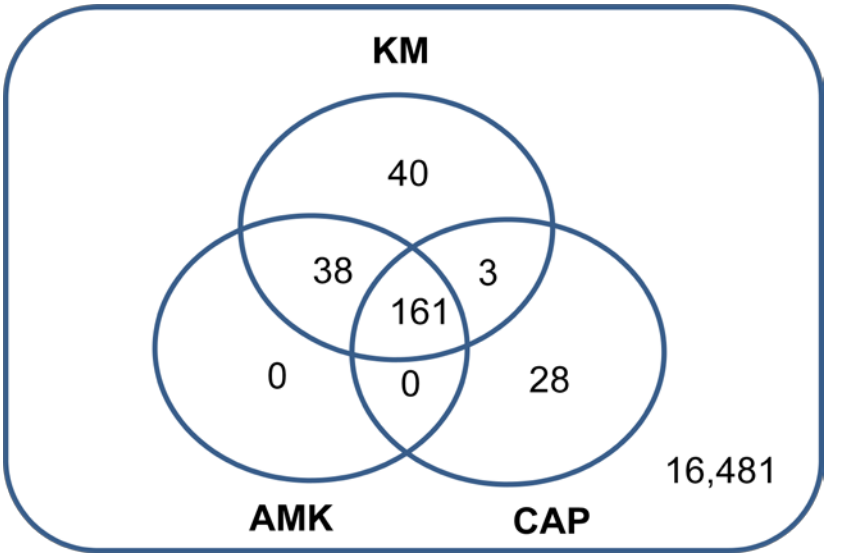


Figure 3. Cross-resistances among injectable agents in *M. tuberculosis* isolates from 2009. Each circle corresponds to the number of isolates resistant to the drug. Forty isolates were mono-resistant to KM, and 28 were mono-resistant to CAP. All AMK-resistant isolates were co-resistant to KM. KM, kanamycin; AMK, amikacin; CAP, capreomycin.

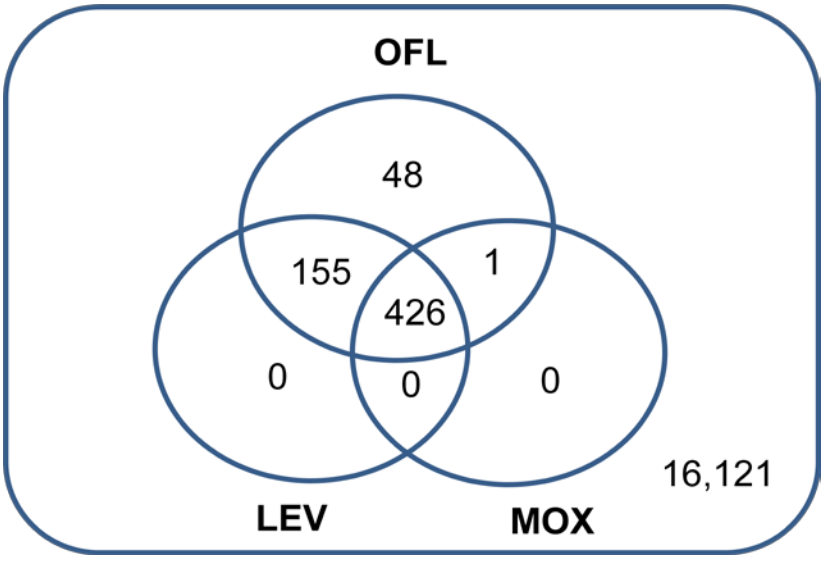


Figure 4. Cross-resistances among fluoroquinolones in TB isolates from 2009. Each circle corresponds to the number of isolates resistant to the drug. Although 48 isolates were mono-resistant to OFL, none of the LEV- or MOX-resistant isolates were susceptible to OFL. OFL, ofloxacin; LEV, levofloxacin; MOX, moxifloxacin

Table 5. Cross-resistance between rifampicin and rifabutin

Rifampicin	Rifabutin		Total
	Resistant	Susceptible	
Resistant	1,074 (6.4)	425 (2.5)	1,499 (8.9)
Susceptible	3 (0.02)	15,249 (91.0)	15,252 (91.1)
Total	1,077 (6.4)	15,674 (93.6)	16,751 (100)

Concordance rate = 97.4%, kappa value= 0.82 (95% CI, 0.80-0.84).

Table 6. Cross-resistance between isoniazid and prothionamide

Isoniazid	Prothionamide		Total
	Resistant	Susceptible	
Resistant	632 (3.8)	1,963 (11.7)	2,595 (15.5)
Susceptible	10 (0.1)	14,146 (84.4)	14,156 (84.5)
Total	642 (3.8)	16,109 (96.2)	16,751 (100)

Concordance rate = 88.2%, kappa value= 0.35 (95% CI, 0.33-0.37).

2. Mutation analysis

We analyzed 95 MDR-TB isolates and sequenced seven loci to detect any mutations. Mutation types were compared with conventional DST results and MIC results.

A. Streptomycin

Two different critical concentrations (4 and 10 μg/mL) were used for SM susceptibility testing. When an isolate was grown on LJ media with 10 μg/mL of SM, it was deemed to be high-level resistant to SM (Table 7). A total of 39 (41.1%) isolates were resistant to a higher concentration of SM, and seven isolates (7.4%) were only resistant to a lower concentration of SM. Three loci were analyzed in these isolates. The most prevalent mutation was Lys43Arg in *rpsL,* which was found in 19 isolates (20%), followed by Lys88Arg in *rpsL*. These two mutations were only found in highly SM-resistant isolates. Most of the TB isolates with a mutation in *rpsL* showed very high MIC. Mutations in the 530 loop and 912 region of *rrs* were detected in 12 (12.6%) isolates. However, these mutations were not only found in resistant isolates, but also in susceptible isolates. Three isolates with the A514C substitution and one isolate with the A907T substitution were resistant to 10 μg/mL of SM. Three of five isolates with C517T were resistant to 10 μg/mL of SM, whereas the remaining two isolates were susceptible to SM.

We identified various *gidB* mutations that were known to be associated with low level SM resistance. A total of 18 (18.9%) isolates harbored the *gidB* mutation, four of which possessed concomitant mutations in *rrs* or *rpsL*. Fifteen of these mutations

were missense mutations and three were nonsense mutations. Among the 14 isolates with a single mutation in *gidB*, the resistance levels varied greatly. Nine were susceptible to SM and four were resistant to 4 µg/mL SM. Only two isolates with Val124Ala and Tyr22 (stop) mutations were highly resistant to SM. The MICs of isolates with *gidB* mutations ranged from 0.25 to 16 µg/mL. Of the 39 isolates with high-level resistance to SM, four (10.3%) had no mutations in *rpsL*, *rrs* or *gidB.*

Table 7. Correlations between streptomycin resistance and mutations in *rpsL*, *rrs* and *gidB* in multidrug-resistant tuberculosis isolates

Mutations in gene			SM susceptibility			No. of isolates
rpsL	*rrs**	*gidB*	R (10 μg/mL)	R (4 μg/mL)	S	
Lys43Arg	ND	ND	18	-	-	18
Lys43Arg	ND	Pro75Thr	1	-	-	1
Lys88Arg	ND	ND	7	-	-	7
ND	T166C	Pro78Arg	-	-	1	1
ND	A514C	ND	2	-	-	2
ND	A514C	Val77Ala	1	-	-	1
ND	C517T	ND	3	-	2	5
ND	C905T	ND	-	1	-	1
ND	A907T	ND	1	-	-	1
ND	A908G	Ala161Thr	-	-	1	1
ND	ND	Ser70Asn	-	-	1	1
ND	ND	Pro75Ser	-	1	-	1
ND	ND	Pro75Thr	-	-	1	1
ND	ND	Val77Ala	-	-	1	1
ND	ND	Leu79Ser	-	1	1	2
ND	ND	Val88Ala	-	-	1	1
ND	ND	Thr98Pro	-	-	1	1
ND	ND	Val124Ala	1	-	-	1
ND	ND	Ala133Pro	-	1	-	1
ND	ND	Lys163Asn	-	-	1	1
ND	ND	Tyr22 stop	1	-	-	1
ND	ND	Glu113 stop	-	1	-	1
ND	ND	Gln125 stop	-	-	1	1
ND	ND	ND	4	2	37	43
Total			39	7	49	95

*Mutations in the 530 loop and 912 region of *rrs.*

ND, not detected; SM, streptomycin.

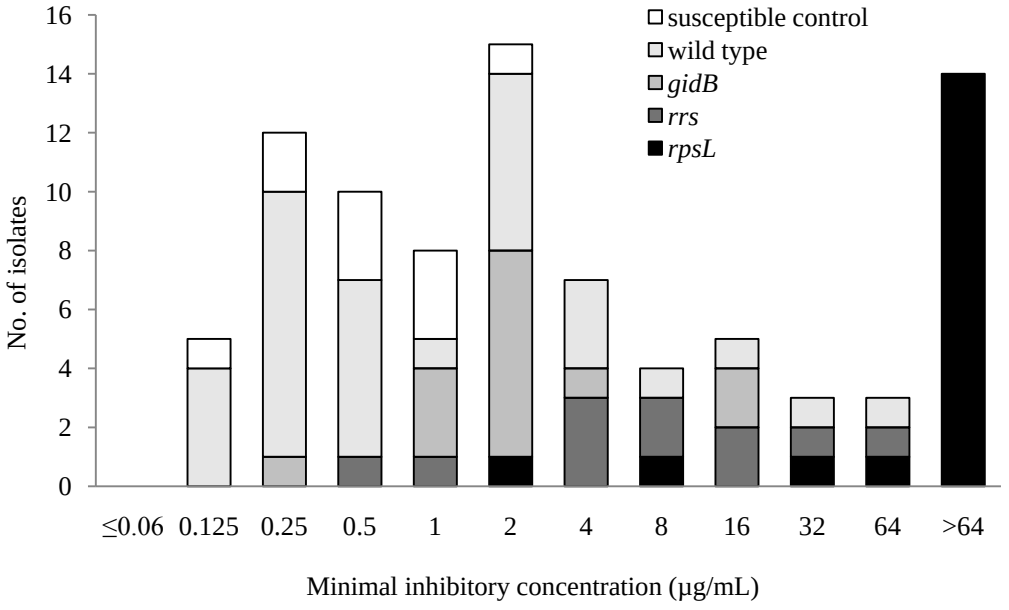


Figure 5. The minimal inhibitory concentration (MIC) distribution for streptomycin in 86 *M. tuberculosis* isolates. The MICs of isolates with *gidB* mutations ranged from 0.25 to 16 µg/mL, whereas most of the isolates with *rpsL* mutations showed high-level MIC (≥ 64 µg/mL).

B. Injectable agents

The most frequent mutation type was the A1401G in *rrs*, which was found in 39 (75.0%) resistant isolates and was not present in susceptible isolates (Table 8). Two isolates harbored the A1338C substitution in *rrs*, which were susceptible to all injectable agents. Four *tlyA* mutations (Leu70Pro, Leu180Arg, Ins55T and Ins665A) were found in five isolates, two of which were resistant to all injectable agents and three of which were mono-resistant to CAP. Mutations in the promoter region of *eis* were found in 12 isolates, eight of which were susceptible to KM, AMK and CAP, three of which were only resistant to KM and the remaining one isolate was resistant to all drugs. Deletion of thymidine at *eis* 45th nucleotide was observed in two isolates, which were susceptible to all drugs. Of the 52 isolates with resistance to injectable drugs, four (7.7%) possessed no mutations in *rrs*, *tlyA* or *eis*. The A1401G mutation in *rrs* showed high-level resistance to both KM and AMK. Without exception, MICs of isolates with the A1401G mutation in *rrs* were ≥ 64 μg/mL for KM and AMK. However, the CAP MICs of isolates with this mutation ranged from 0.5 to 16 μg/mL (Fig. 5). Nine of ten isolates with the *eis* mutation were inhibited at 4 μg/mL of KM. Most of the wild-type MDR isolates were inhibited at 8 μg/mL of KM, and one isolate showed a high MIC level for KM. The MIC of KM against susceptible isolates was 1 or 2 μg/mL. The MIC_{90} of AMK for wild-type isolates was lower than that of KM. The MIC_{90} of AMK for susceptible isolates was also lower than that of KM. The MICs of CAP were not clearly distinguishable among mutation patterns. The CAP MICs of two isolates containing a single *tlyA* mutation were 2 and 8 μg/mL, which

were within the MIC range of wild-type isolates (0.25 to 8 μg/mL). Susceptible controls were found to have a narrow MIC range of CAP (1 to 2 μg/mL), which overlapped with those of the other groups.

Table 8. Mutations in *rrs*, *tlyA* and *eis* in multidrug-resistant tuberculosis isolates

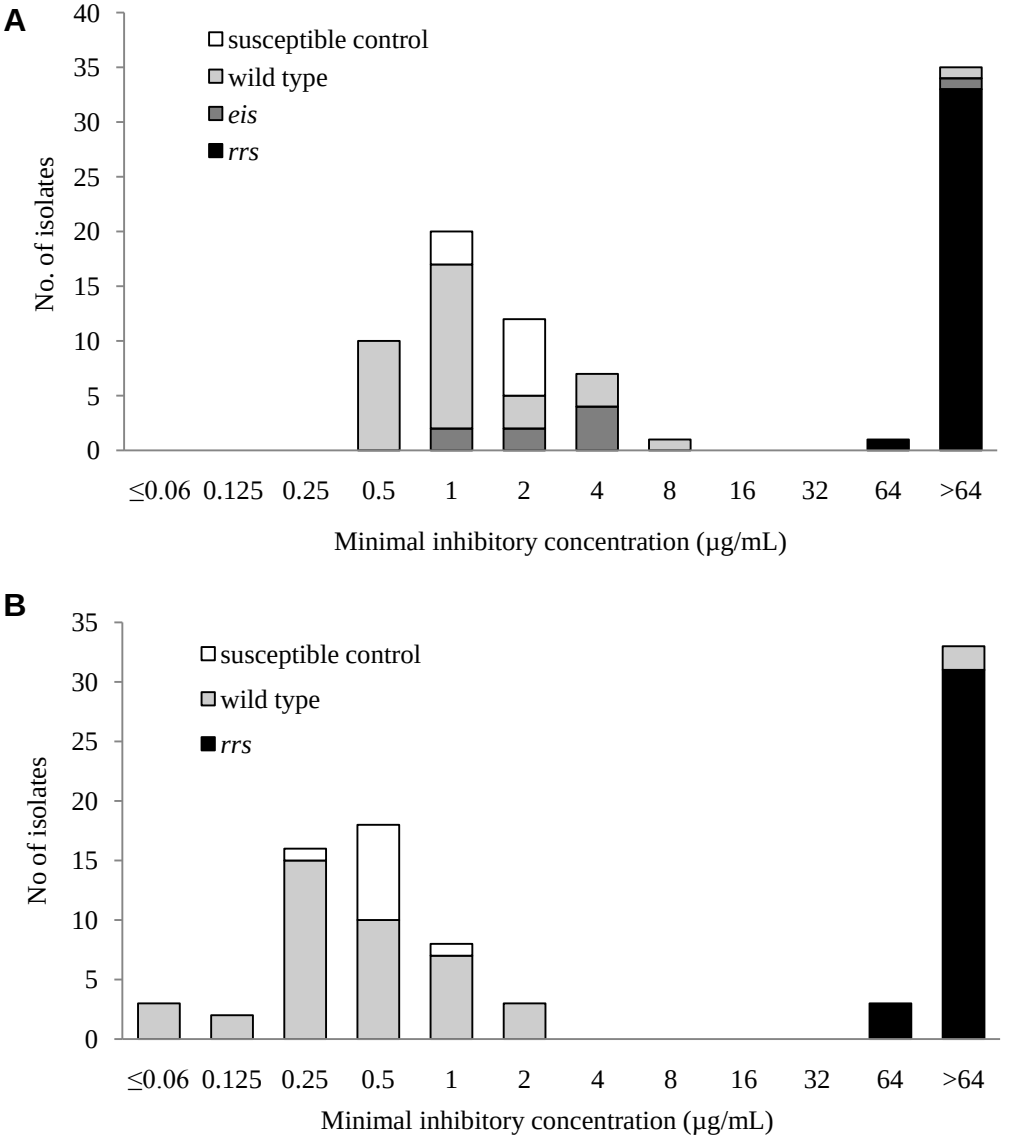
Mutations in gene			Susceptibility to injectable agents‡					No. of isolates
*rrs**	*tlyA*	*eis*	RRR	RRS	RSS	SSR	SSS	
A1401G	ND	ND	36	3	-	-	-	39
A1338C	ND	ND	-	-	-	-	1	1
A1338C	ND	G-10A & WT	-	-	-	-	1	1
ND	Leu70Pro	ND	-	-	-	1	-	1
ND	Leu180Arg	ND	2	-	-	-	-	2
ND	Ins55T†	ND	-	-	-	1	-	1
ND	Ins665A†	ND	-	-	-	1	-	1
ND	ND	G-10C	-	-	-	-	1	1
ND	ND	C-12T	-	-	-	-	3	3
ND	ND	C-14T	1	-	1	-	-	2
ND	ND	G-37T	-	-	2	-	3	5
ND	ND	47delT†	-	-	-	-	2	2
ND	ND	ND	2	1	1	-	32	36
Total			41	4	4	3	43	95

*Mutations in the 1400 region of *rrs*.

† Frameshift.

‡ In order of kanamycin, amikacin and capreomycin.

WT, wild-type; ND, not detected.


A
susceptible control
wild type
eis
rrs
No. of isolates
40
35
30
25
20
15
10
5
0
≤0.06 0.125 0.25 0.5 1 2 4 8 16 32 64 >64
Minimal inhibitory concentration (µg/mL)

B
susceptible control
wild type
rrs
No of isolates
35
30
25
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≤0.06 0.125 0.25 0.5 1 2 4 8 16 32 64 >64
Minimal inhibitory concentration (µg/mL)

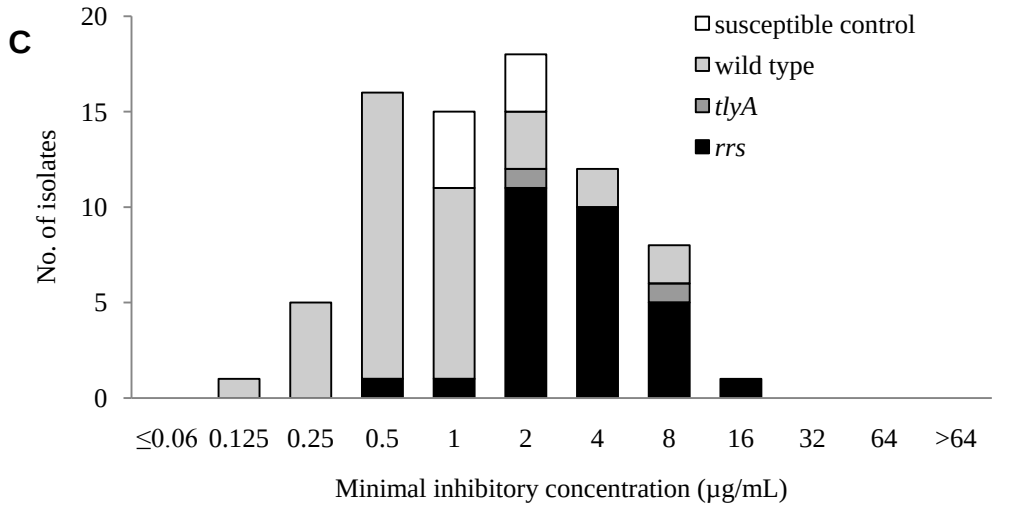

Figure 6. The minimal inhibitory concentration (MIC) distributions for injectable agents in 86 *M. tuberculosis* isolates. (A) Kanamycin MIC distribution. Isolates with the A1401G substitution in *rrs* show a very high MIC of kanamycin, whereas most of the isolates with a mutation in the promoter of *eis* exhibited low MIC values ranging from 1 to 4 μg/mL, which are similar to that of the susceptible control or wild-type isolates. (B) Amikacin MIC distribution. The A1401G substitution in *rrs* confers high-level resistance to amikacin. Two wild-type isolates showed high level resistance to amikacin (MIC > 64 μg/mL). (C) Capreomycin MIC distribution. The A1401G substitution in *rrs* does not cause high level resistance to capreomycin.

C. Fluoroquinolones

The presence of mutations in the QRDR of *gyrA* and *gyrB* was determined for 95 MDR-TB isolates. Seventy-one (89.9%) FQ-resistant isolates had a mutation in the QRDR of *gyrA* or *gyrB*, whereas no mutations were found in FQ-susceptible isolates (Table 9). Asp94Gly in *gyrA* was the most common mutation type (31/95, 30.5%), followed by Ala90Val (16.8%) and Ser91Pro (9.5%). Three isolates were found to possess double mutations (2 Ala90Val/Asp94Gly, 1 Ala90Val/Ser91Pro) in the QRDR of *gyrA*. One isolate had a G/T double peak at *gyrA* 280 and A/G at 281. Nine *gyrB* mutation types were found in 14 (14.7%) FQ-resistant isolates. Of these, 12 isolates also had a *gyrA* mutation, and two isolates harbored only a *gyrB* mutation. For 25 isolates including eight resistant isolates with no mutation in either loci, the whole *gyrA* gene was sequenced and two had additional mutations (Ile234Val and Ans282Lys) other than that in the QRDR of *gyrA*. However, these mutations were found in isolates also containing mutations in the QRDR. The MIC_{90} of OFL and LEV for isolates was 16 μg/mL, whereas the MIC_{90} of MOX was 2 μg/mL (Table 10). Eleven isolates containing mutations in both loci had an MIC of 2 to 8 μg/mL for OFL, 1 to 4 μg/mL for LEV, and 0.5 to 2 μg/mL for MOX. All wild-type isolates were inhibited at 2 μg/mL of OFL and LEV but at 1 μg/mL of MOX. Susceptible controls were inhibited at low concentrations of all three drugs, and the MIC_{90}s for OFL, LEV and MOX were 0.25, 0.25 and 0.125 μg/mL, respectively.

Table 9. Mutations in the *gyrA* and *gyrB* genes in multidrug-resistant tuberculosis isolates

Mutations in gene		Susceptibilities to fluoroquinolones†				No. of isolates
gyrA	*gyrB*	RRR	RRS	RSS	SSS	
Gly88Ala	Asn500Lys	1	-	-	-	1
Ala90Val	ND	6	4	-	-	10
Ala90Val	Met485Ile	-	1	-	-	1
Ala90Val	Asp472Asn	1	-	-	-	1
Ala90Val	Gly523Arg	-	1	-	-	1
Ser91Pro	ND	6	1	1	-	8
Asp94Ala	ND	5	1	-	-	6
Asp94Ala	Leu518Phe	1	-	-	-	1
Asp94Gly	ND	22	-	-	-	22
Asp94Gly	Asn500Lys	1	-	-	-	1
Asp94Gly	Ala515Val	1	-	-	-	1
Asp94Gly	Gly523Arg	4	-	-	-	4
Asp94Gly	Gly551Arg	1	-	-	-	1
Asp94His	ND	1	1	-	-	2
Asp94Asn	ND	5	-	-	-	5
Double peaks at 280, 281*	ND	1	-	-	-	1
Ala90Val, Asp94Gly	ND	2	-	-	-	2
Ala90Val, Ser91Pro	ND	-	1	-	-	1
ND	Asp472Lys	1	-	-	-	1
ND	Asn500Asp	-	-	1	-	1
ND	ND	4	2	2	16	24
Total		63	12	4	16	95

*G/T at *rrs* nt 280 and A/G at *rrs* nt 281.

† In the order of ofloxacin, levofloxacin and moxifloxacin.

ND, not detected.

Table 10. Minimal inhibitory concentrations of ofloxacin, levofloxacin, and moxifloxacin according to the mutation patterns of *gyrA* and *gyrB*

Mutation type	No. of isolates	Ofloxacin			Levofloxacin			Moxifloxacin		
		MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
gyrA only	44	1 – 32	4	16	0.5 – 16	2	8	0.25 – 4	1	2
gyrA + *gyrB*	11	2 – 8	4	8	1 – 16	2	4	0.5 – 8	1	2
gyrB only	2	1 – 8	1	8	0.5 – 8	0.5	8	0.5 – 2	0.5	2
Wild-type	19	≤0.06 – 4	0.25	2	≤0.06 – 2	0.25	0.5	≤0.03 – 1	0.06	0.25
Susceptible control	10	≤0.06 – 0.5	0.25	0.25	≤0.06 – 0.25	0.125	0.25	≤0.03 – 0.25	0.06	0.125
Total	86	≤0.06 – 32	2	8	≤0.06 – 16	1	8	≤0.03 – 4	0.5	2

MIC, minimal inhibitory concentration.

3. Linezolid susceptibilities of MDR and XDR-TB isolates

The MICs of linezolid were determined for four groups of 86 TB isolates using broth microdilution (XDR, MDR-F, MDR-I, and susceptible control). Most isolates showed very low MIC values for linezolid. Distributions of MICs were very similar among the groups (Table 11). The MIC_{90}s of XDR, MDR-F, MDR-I and susceptible controls were 0.5, 1, 0.5 and 1 μg/mL, respectively. However, one isolate was found to have an MIC of 16 μg/mL. This patient had been treated at one of the KNTA clinics and had no history of linezolid. The 23S rRNA gene and ribosomal protein L3, L4 genes were sequenced and found to contain no mutations.

Table 11. Linezolid susceptibility test results

Group	No. of isolates tested	Minimal inhibitory concentration (μg/mL)		
		Range	50%	90%
XDR	26	≤0.03 – 1	0.25	0.5
MDR-F	35	0.125 – 16	0.5	1
MDR-I	15	0.125 – 1	0.25	0.5
Susceptible controls	10	0.25 – 1	0.5	1
Total	86	≤0.03 – 16	0.5	1

XDR, extensively drug-resistant tuberculosis; MDR-F, multidrug-resistant tuberculosis with fluoroquinolone resistance; MDR-I, multidrug-resistant tuberculosis with injectable agent resistance.

IV. DISCUSSION

INH is a first-line drug for tuberculosis and has been used for more than 60 years. The INH resistance rate is highest among the test drugs. In this study, the overall INH resistance was 15.5%, and more than 10% of all new cases were resistant to INH. INH is prescribed for prevention in latent TB infection (LTBI) patients. This result emphasizes the importance of the careful prescription of INH to new TB cases and LTBI patients. RIF is the most important drug for short course regimen for TB.[2] The overall rate of RIF resistance in the present study was 8.9%, which was following INH-resistance rate. Because of relatively high resistance rate to INH and RIF, DST for INH and RIF should be mandatory for every TB patient.

Although SM had long been used as a first-line drug, the WHO recently grouped this drug into the Group 2 injectable agents. After the introduction of the standard short course regimen in the 1980s in Korea, SM has not been included in the initial regimen for TB. Because of the infrequent use of SM, the resistance rate was relatively low, and variation in SM resistance among groups was less than those of the other first-line drugs. On the other hand, in China and Viet Nam, where SM has been used for initial treatment, a high resistance rate to SM was measured among new cases.[25,26] The average resistance rate to PZA (4.9%) was lower than that of any other first-line drug.

In Korea, the MDR-TB rate had been increasing among new cases. According to previous DRS, the MDR-TB rate of new smear-positive cases increased from 1.6% in 1994 to 2.7% in 2004.[3] The present study revealed that 3.5% of SS+ New cases were

MDR-TB, which was a statistically significant increase compared to the result of previous surveys.

MDR-TB patients typically require second-line antituberculosis drugs for duration of approximately 2 years.[5,27] While conventional DST requires 2-4 weeks to obtain results, there is little evidence of molecular methods that detect the resistance of antituberculosis drugs other than RIF and INH. Therefore, it might be practical and effective to use a standardized regimen based on the drug resistance profiles of MDR-TB strains. Our results revealed that MDR-TB isolates from Korea had relatively high resistances to SM, EMB, PZA and RBT. These drugs may not be suitable for empirical treatment of MDR-TB. Injectable agents were shown to have relatively good activity against MDR-TB isolates, with resistance rates less than 20%. However, more than 30% of MDR isolates were resistant to OFL and LEV. Although MOX was more active against MDR-TB than were OFL and LEL, the resistance rate reached 25%. Moreover, the Referred and KNTA groups showed much higher resistance rates than did the new and retreated cases of HC, which might suggest that FQ resistance is amplified in the private sector.

Because of an inconvenient administration route, injectable agents are usually prescribed during the intensive phase of MDR-TB treatment. This might explain the low resistance to SM in Korea. FQs are pivotal drugs for the treatment of MDR-TB and are also widely used for urinary tract infections, pneumonia, and other infectious diseases.[28,29] This allows TB bacilli more exposure to FQs compared with its exposure to other second-line drugs. Because HCs refer MDR-TB patients to private hospitals,

TB hospitals or KNTA clinics, high resistance rates in the Referred and KNTA groups may imply frequent acquisition of FQ resistance during treatment. This emphasizes the importance of MDR-TB patient control and restricted use of FQs.

Since XDR-TB was first reported in South Africa, it has been found in 58 countries.[1,4,30-32] The US CDC conducted a survey on XDR-TB, in which 2000-2004 DST data from 48 countries were analyzed.[4] According to the survey report, 1.7% of isolates from Korea in 2004 were XDR, and the proportion of XDR among MDR cases was 15.4%, much higher than the global average (9.9%).[4] Although the definition of XDR at the time was different than the current definition, the XDR rate and proportion of MDR-TB cases were higher than those in 2009. In fact, we found several duplicates in the 2009 DST data, suggesting that the previous data from 2004 may contain a significant number of duplicates. Moreover, our results revealed that XDR proportions varied according to patient group. Among the XDR-TB cases, eight were found to be resistant to all drugs. Those patients were suspected to be chronic cases and may have undergone several episodes of treatment. Although there are no consensus criteria for PDR-TB, it is an extreme threat to public health. This finding underlines the need to improve the treatment success rate of MDR-TB and XDR-TB.

Many studies have reported cross-resistance among TB drugs. Because KM and AMK share structural similarity, a relatively high-level of cross-resistance between the two drugs is possible. In this study, all of the AMK-resistant strains were cross-resistant to KM, whereas KM mono-resistant strains were identified. Because mutations in the promoter region of *eis* are related to only KM resistance, *eis*

mutations may contribute to KM mono-resistance. CAP, the cyclic polypeptide antibiotic, inhibits ribosome like other injectables. The majority of isolates with resistance to CAP showed cross-resistance to KM or AMK but some did not. This may be explained by mutations in the *tlyA* gene. It is interesting that there were no MOX mono-resistant or LEV mono-resistant isolates among the OFL-resistant isolates. Therefore, susceptibility testing for OFL and KM/CAP may be sufficient for detecting XDR-TB.

In this study, we collected a total of 205 MDR isolates and analyzed the sequences of genes associated with drug resistance in 95 MDR-TB isolates with resistance to FQs or injectables. SM is an aminoglycoside with which mutations in the *rrs*, *rpsL* and *gidB* genes have been associated. Specifically, *rpsL* mutations confer high-level resistance to SM. In this study, we identified two missense mutations in *rpsL*, which were only found in isolates resistant to 10 μg/mL of SM. Mutations in the 530 loop region and the 912 region of *rrs* are known to cause SM resistance. [17,21,33,34] Most isolates with mutations in those regions of *rrs* were resistant to 10 μg/mL and 4 μg/mL of SM. Although we identified various *gidB* mutations, correlations between mutation and SM resistance were not clear. More than 60% of isolates containing a single *gidB* mutation were susceptible to SM. Even though two isolates had double mutations (T166C in *rrs* + Pro78Arg in *gidB* and A908G in *rrs* + Ala161Thr in *gidB*), all were found to be susceptible to SM.

Previous studies revealed that the A1401G substitution in *rrs* confers high-level resistance to aminoglycosides and is the most common mutation among

aminoglycoside-resistant TB isolates. [22,34-37] Of the 52 isolates resistant to at least one injectable, 39 (75.0%) possessed the A1401G substitution. This substitution was the most frequent mutation type and was not found in the susceptible isolates group. The MICs of KM and AMK for isolates with the A1401G substitution were very high (MIC_{90} >64 μg/mL). Like previous studies, the A1401G substitution in *rrs* was determined to be associated with CAP resistance, while the MICs of CAP were lower than those of KM or AMK. Jugheli et al. revealed that the A1401G substitution confers variable resistance to CAP, and the C1402T substitution in *rrs* is associated with high-level resistance to CAP.[35]

The e*is* gene encodes Eis protein, an aminoglycoside acetyltransferase, which acetylates and inactivates KM and AMK.[11,18] It is possible that a mutation in the -10 and -35 promoter regions of the *eis* gene could cause low-level resistance to KM.[11,18] In this study, we identified five mutations in the promoter region of *eis*. However, isolates with these mutations were mostly susceptible to KM and MIC values as the isolates were similar to wild-type strains and susceptible control strains. Zaunbrecher et al. reported that isolates with C-14T, G-37T, or G-10A *eis* mutations are likely to be resistant to KM. Our results showed only C-14T and G-37T mutations were found in isolates resistant to KM.[18] Deletion of thymidine at position 47 in *eis* was identified in two isolates but was not correlated with KM resistance.

CAP binds the 30S and 50S ribosome subunits. TlyA is a methyltransferase, modifying helix 44 of 16S rRNA and helix 69 of 23S rRNA, which are the binding site of CAP.[22,33-35] Therefore, *rrs* and *tlyA* mutations may cause resistance to CAP.

Because of the infrequent use of CAP, the resistance rate to this drug is usually lower than those to other injectable agents. Data on *tlyA* mutations in CAP-resistant TB isolates from Korea were limited. A previous study conducted in Korea reported several *tlyA* mutations that were found only in CM susceptible isolates.[34] In present study, four mutations in the *tlyA* gene were detected in five CAP-resistant isolates, three of which were mono-resistant to CAP. Because CAP is seldom used in Korea due to its high cost, it seems that these *tlyA* mutations developed spontaneously.

Correlation between mutations in the QRDR of *gyrA* and *gyrB* and FQ resistance has been well described. In this study, we analyzed the sequences of the QRDRs of *gyrA* and *gyrB* for 95 MDR-TB isolates. Among the FQ resistant isolates, 71/79 (89.9%) had one or more mutations in *gyrA* and *gyrB*, respectively. The most common mutation occurred at codon 94 of *gyrA*. It is noteworthy that no mutation was found in susceptible isolates, suggesting that the molecular DST for FQ would be highly specific. Kam et al. reported that the Asp94Gly mutation in *gyrA* is associated with a higher level of resistance to OFL and MOX.[38] However, our results revealed that isolates with the Asp94Gly mutation showed the same or a 2-fold higher MIC_{50} of OFL compared to those of other isolates (data not shown). We also compared the MICs of FQs for the *gyrA* mutation, *gyrA/B* double mutation and *gyrB* groups and failed to identify any differences between groups. We sequenced the whole *gyrA* gene to identify any polymorphisms other than that in the QRDR, identifying two such mutations. However, these mutations were combined with mutations in the QRDR of *gyrA* or *gyrB*, and thus it was not possible to assess the contributions of these

mutations to FQ resistance.

Due to the relatively high sensitivity (89.9%) and specificity (100.0%), sequencing of the QRDRs in *gyrA* and *gyrB* would be a good alternative to the DST method. However, the resistance mechanisms of injectables are more complicated than that of FQs and involve many genes. Therefore, choosing a well-evaluated mutation is important for analysis. For example, the A1401G substitution in *rrs* had sensitivities of 73.5%, 80.0% and 75.0% for detecting resistance to KM, AMK and CAP, respectively, and the specificities for KM and AMK were 100.0% and 94.1% for CAP. Mutations in the promoter of *eis* could be added for detecting KM resistance. However, these mutations confer low-level resistance to KM, and MICs of isolates with these mutations were not clearly distinct from those of wild-type isolates or susceptible controls. Although *tlyA* mutations revealed a diagnostic value for the CAP susceptibility test in this study, mutations occur throughout the locus, and several mutations have been reported. Therefore, further studies are necessary to understand the significance of *eis* and *tlyA* mutations. Some isolates resistant to FQs or injectables did not have a mutation at any of the seven tested loci. Other resistance mechanisms, like an efflux pump, are therefore possible.[39,40]

Recently, a line probe assay for second-line drug resistance (GenoType MTBDR*sl*) was developed by Hain Lifescience. MTBDR*sl* can detect resistance to SM, FQs, injectables and EMB.[33,41,42] Brossier et al. evaluated the performance of MTBDR*sl* and reported relatively good sensitivity and specificity for FQs, SM and injectables,[33] similar to our results.

It is very difficult to find drugs to treat XDR-TB cases since the WHO recommends the use of at least four drugs in these patients. Therefore, physicians sometimes have to prescribe linezolid, clofazimine, clarithromycin and other WHO group 5 drugs. Linezolid is the only oxazolidinone, is very active against Gram-positive bacteria and been found to have good *in vitro* and *in vivo* activities against MTB. In this study, we evaluated the susceptibility of linezolid by determining the MICs for MDR and XDR-TB isolates. Like previous reports, almost all isolates were inhibited at 1.0 μg/mL of linezolid. Only one isolate showed high-level LIN resistance, a KNTA patient with no previous history of LIN treatment. This is the first report of linezolid-resistant MTB in Korea. However, this isolate was found to have no mutation in the 23S rRNA gene or the ribosomal protein L3, 4 genes. Activation of efflux pumps or a reduced input rate of the drug might have caused linezolid resistance in this isolate.

V. CONCLUSION

The current study has focused on two topics: a trend of antituberculosis drug resistance in Korea and analysis of the molecular mechanism on second-line drug resistance. In addition, the authors assessed the susceptibility of LIN to MDR- and XDR-TB isolates from Korea. Analysis of drug susceptibility testing revealed a further increase in the MDR-TB rate among new cases and variable XDR-TB proportions in MDR-TB cases according to patient group. We identified major mutations that confer high-level resistance to SM, FQs and injectables, and a rapid DST to detect these mutations would be useful. However, further studies will be required to identify new mechanisms of drug resistance in MTB. DST for LIN revealed that LIN has good activity against MDR- and XDR-TB, which means LIN would be useful for treating drug-resistant TB.

REFERENCES

1. WHO. Global tuberculosis control: WHO report 2010 (WHO/HTM/TB/2010.7). Geneva, Switzerland: WHO press; 2010.
2. Zhang Y, Yew WW. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 2009;13:1320-30.
3. Bai GH, Park YK, Choi YW, Bai JI, Kim HJ, Chang CL, et al. Trend of anti-tuberculosis drug resistance in Korea, 1994-2004. Int J Tuberc Lung Dis 2007;11:571-6.
4. Shah NS, Wright A, Bai GH, Barrera L, Boulahbal F, Martin-Casabona N, et al. Worldwide emergence of extensively drug-resistant tuberculosis. Emerg Infect Dis 2007;13:380-7.
5. Kwon YS, Kim YH, Suh GY, Chung MP, Kim H, Kwon OJ, et al. Treatment outcomes for HIV-uninfected patients with multidrug-resistant and extensively drug-resistant tuberculosis. Clin Infect Dis 2008;47:496-502.
6. Johnston JC, Shahidi NC, Sadatsafavi M, Fitzgerald JM. Treatment outcomes of multidrug-resistant tuberculosis: a systematic review and meta-analysis. PLoS One 2009;4:e6914.
7. Kim SJ, Espinal MA, Abe C, Bai GH, Boulahbal F, Fattorin L, et al. Is second-line anti-tuberculosis drug susceptibility testing reliable? Int J Tuberc Lung Dis 2004;8:1157-8.
8. Van Deun A, Wright A, Zignol M, Weyer K, Rieder HL. Drug susceptibility testing proficiency in the network of supranational tuberculosis reference laboratories. Int J Tuberc Lung Dis 2011;15:116-24.
9. Kim SJ. Drug-susceptibility testing in tuberculosis: methods and reliability of results. Eur Respir J 2005;25:564-9.
10. Van Deun A, Martin A, Palomino JC. Diagnosis of drug-resistant tuberculosis: reliability and rapidity of detection. Int J Tuberc Lung Dis 2010;14:131-40.
11. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et

al. Molecular Detection of Mutations Associated with First and Second-Line Drug Resistance Compared with Conventional Drug Susceptibility Testing in *M. tuberculosis*. Antimicrob Agents Chemother 2011.

12. Kim SJ, Lee SH, Kim IS, Kim HJ, Kim SK, Rieder HL. Risk of occupational tuberculosis in National Tuberculosis Programme laboratories in Korea. Int J Tuberc Lung Dis 2007;11:138-42.
13. Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin Microbiol Rev 1995;8:496-514.
14. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis 1998;79:3-29.
15. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med 2010;363:1005-15.
16. Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, et al. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Mol Microbiol 2007;63:1096-106.
17. Spies FS, da Silva PE, Ribeiro MO, Rossetti ML, Zaha A. Identification of mutations related to streptomycin resistance in clinical isolates of *Mycobacterium tuberculosis* and possible involvement of efflux mechanism. Antimicrob Agents Chemother 2008;52:2947-9.
18. Zaunbrecher MA, Sikes RD, Jr., Metchock B, Shinnick TM, Posey JE. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 2009;106:20004-9.
19. Davies AP, Billington OJ, McHugh TD, Mitchison DA, Gillespie SH. Comparison of phenotypic and genotypic methods for pyrazinamide susceptibility testing with *Mycobacterium tuberculosis*. J Clin Microbiol 2000;38:3686-8.

20. CLSI. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard. CLSI document M24-A. Wayne, Pennsylvania: CLSI; 2003.
21. Sekiguchi J, Miyoshi-Akiyama T, Augustynowicz-Kopec E, Zwolska Z, Kirikae F, Toyota E, et al. Detection of multidrug resistance in *Mycobacterium tuberculosis*. J Clin Microbiol 2007;45:179-92.
22. Feuerriegel S, Cox HS, Zarkua N, Karimovich HA, Braker K, Rusch-Gerdes S, et al. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug-resistant tuberculosis patients undergoing treatment. Antimicrob Agents Chemother 2009;53:3353-6.
23. Wang JY, Lee LN, Lai HC, Wang SK, Jan IS, Yu CJ, et al. Fluoroquinolone resistance in *Mycobacterium tuberculosis* isolates: associated genetic mutations and relationship to antimicrobial exposure. J Antimicrob Chemother 2007;59:860-5.
24. Loffler FE, Sun Q, Li J, Tiedje JM. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating Desulfuromonas and Dehalococcoides species. Appl Environ Microbiol 2000;66:1369-74.
25. He GX, Zhao YL, Jiang GL, Liu YH, Xia H, Wang SF, et al. Prevalence of tuberculosis drug resistance in 10 provinces of China. BMC Infect Dis 2008;8:166.
26. Huong NT, Lan NT, Cobelens FG, Duong BD, Co NV, Bosman MC, et al. Antituberculosis drug resistance in the south of Vietnam: prevalence and trends. J Infect Dis 2006;194:1226-32.
27. Kim DH, Kim HJ, Park SK, Kong SJ, Kim YS, Kim TH, et al. Treatment outcomes and long-term survival in patients with extensively drug-resistant tuberculosis. Am J Respir Crit Care Med 2008;178:1075-82.
28. Gaba PD, Haley C, Griffin MR, Mitchel E, Warkentin J, Holt E, et al. Increasing outpatient fluoroquinolone exposure before tuberculosis diagnosis and impact on culture-negative disease. Arch Intern Med 2007;167:2317-22.

29. Ginsburg AS, Grosset JH, Bishai WR. Fluoroquinolones, tuberculosis, and resistance. Lancet Infect Dis 2003;3:432-42.
30. Eker B, Ortmann J, Migliori GB, Sotgiu G, Muetterlein R, Centis R, et al. Multidrug- and extensively drug-resistant tuberculosis, Germany. Emerg Infect Dis 2008;14:1700-6.
31. Shah NS, Pratt R, Armstrong L, Robison V, Castro KG, Cegielski JP. Extensively drug-resistant tuberculosis in the United States, 1993-2007. JAMA 2008;300:2153-60.
32. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet 2006;368:1575-80.
33. Brossier F, Veziris N, Aubry A, Jarlier V, Sougakoff W. Detection by GenoType MTBDRsl test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol 2010;48:1683-9.
34. Via LE, Cho SN, Hwang S, Bang H, Park SK, Kang HS, et al. Polymorphisms associated with resistance and cross-resistance to aminoglycosides and capreomycin in *Mycobacterium tuberculosis* isolates from South Korean Patients with drug-resistant tuberculosis. J Clin Microbiol 2010;48:402-11.
35. Jugheli L, Bzekalava N, de Rijk P, Fissette K, Portaels F, Rigouts L. High level of cross-resistance between kanamycin, amikacin, and capreomycin among *Mycobacterium tuberculosis* isolates from Georgia and a close relation with mutations in the rrs gene. Antimicrob Agents Chemother 2009;53:5064-8.
36. Maus CE, Plikaytis BB, Shinnick TM. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2005;49:3192-7.
37. Leung KL, Yip CW, Yeung YL, Wong KL, Chan WY, Chan MY, et al.

Usefulness of resistant gene markers for predicting treatment outcome on second-line anti-tuberculosis drugs. J Appl Microbiol 2010;109:2087-94.

38. Kam KM, Yip CW, Cheung TL, Tang HS, Leung OC, Chan MY. Stepwise decrease in moxifloxacin susceptibility amongst clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*: correlation with ofloxacin susceptibility. Microb Drug Resist 2006;12:7-11.

39. Escribano I, Rodriguez JC, Llorca B, Garcia-Pachon E, Ruiz M, Royo G. Importance of the efflux pump systems in the resistance of *Mycobacterium tuberculosis* to fluoroquinolones and linezolid. Chemotherapy 2007;53:397-401.

40. De Rossi E, Ainsa JA, Riccardi G. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS Microbiol Rev 2006;30:36-52.

41. Hillemann D, Rusch-Gerdes S, Richter E. Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol 2009;47:1767-72.

42. Kiet VS, Lan NT, An DD, Dung NH, Hoa DV, van Vinh Chau N, et al. Evaluation of the MTBDRsl test for detection of second-line-drug resistance in *Mycobacterium tuberculosis*. J Clin Microbiol 2010;48:2934-9.

ABSTRACT (IN KOREAN)

다제내성결핵에서 이차약제 내성의 분자생물학적 특성 규명

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김창기

결핵은 호흡기로 전파되는 전염성 질환으로 전세계적으로 매년 천만명에 가까운 환자가 발생하고 있다. 한국의 결핵발생률은 인구 10만 명당 97명으로 Organisation for Economic Cooperation and Development 회원국 중에서 가장 높다. 전세계적으로 결핵관리를 위해 많은 노력을 기울이고 있으나 최근 내성결핵의 증가는 결핵퇴치의 큰 걸림돌 중 하나이다. 다제내성결핵은 가장 중요한 항결핵제인 isoniazid와 rifampicin에 모두 내성인 결핵을 지칭하는데 이를 치료하기 위해서는 2차 약제를 장기간 투여해야 한다. 광역내성결핵은 다제내성결핵이면서 주요한 2차 약제에도 내성인 경우를 말하는데 치료약제의 선정이 어려워 치료 성공률이 낮다. 이들 내성

결핵을 성공적으로 관리하기 위해서는 현재 내성현황을 정확하게 파악하여 치료방침을 수립할 필요가 있다. 또한 주요 내성결핵을 신속하게 진단하고 감수성인 약제를 선정할 수 있어야 한다. 현재 다제내성결핵 진단을 위한 분자진단시약은 많이 사용되고 있으나 광역내성결핵을 진단하는 분자검사법의 유용성에 대한 자료가 많지 않다. 따라서 이번 연구에서는 국내 결핵의 항결핵제 현황을 파악하고 2차 약제 내성의 분자생물학적 검출의 유용성을 평가하고자 하였다.

2009년 결핵연구원에서 시행한 항결핵제 감수성시험결과를 수집하여 환자를 도말음성 보건소 신환자, 도말양성 보건소 신환자, 보건소 재치료환자, 건강검진, 수탁의뢰 그리고 결핵협회 복십자의원으로 구분하였다. 환자군에 따른 내성률을 분석하였고 다제내성결핵과 광역내성결핵의 비율을 확인하였고 2차 약제간 교차내성을 분석하였다. 또한 2차 약제 내성과 연관성이 알려진 7개의 유전자의 염기서열을 분석하여 유전자형과 감수성결과를 비교하였다.

항결핵제 중에서 isoniazid 내성률이 가장 높았고 도말양성 신환자 중에서 다제내성결핵의 비율은 3.5%이었다. 광역내성결핵은 전체 결핵의 1%였고 다제내성결핵 중에서는 12%이었다. 다제내성결핵의 항결핵제 내성은 약제와 환자군에 따라 크게 차이가 있었는데 기타 일차약제 내성률이 매우 높았고 주사약제 내성이 가장 낮았으며 fluoroquinolone 내성은 보건소 환자보다 민간환자에서 2-4배 높았다. 2차 약제내성과 연관된 7개의 유전자

의 변이를 분석한 결과 streptomycin은 *rpsL*, aminoglycoside는 16S rRNA 그리고 fluoroquinolone은 *gyrA*와 *gyrB* 유전자의 변이가 내성균주에서 흔하였으며 고도 내성과 연관이 있었다. 반면 최근에 규명된 *eis*와 *gidB* 유전자 변이는 내성균주와 감수성 균주에서 모두 발견되었고 최소억제농도도 야생형 균주 또는 감수성 대조군과 큰 차이가 없었다.

결론적으로 본 연구를 통해 국내 다제내성결핵의 비율이 2004년 내성률 조사 보다 증가했음을 확인하였다. 우리나라 다제내성결핵 중 광역내성의 비율이 15%였던 기존 보고 보다 낮았다. 민간에서 치료받는 다제내성결핵 환자의 높은 fluoroquinolone 내성률은 항결핵제의 적절한 사용과 치료 효율의 향상이 필요함을 역설하고 있다. 이차약제의 내성과 연관된 유전자를 분석한 결과 주요 유전자 변이를 규명하였고 진단에 적용할 경우 매우 높은 특이도를 기대할 수 있으며 고도내성 균주를 신속하게 진단할 수 있음을 확인하였다. 이상의 결과는 향후 내성결핵치료를 위한 전략수립에 유용하게 이용될 수 있으며 신속한 내성결핵진단법 개발의 기초자료로 활용될 수 있다고 판단된다.

핵심되는 말: 다제내성결핵, 광역내성결핵, 이차결핵약제, 감수성시험, 유전자 변이, 신속진단