A Doctoral Dissertation

Patterns of *rpoB, rpoC,* and *pncA* mutations in drug-resistant *Mycobacterium tuberculosis* isolated from patients in South Korea

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August, 2017

한국인 환자에서 분리한 약제내성 결핵균의 *rpoB, rpoC, pncA* 유전자 돌연변이 분석

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Patterns of *rpoB, rpoC,* and *pncA* mutations in drug-resistant *Mycobacterium tuberculosis* isolated from patients in South Korea

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ABSTRACT

Background Rifampin (RIF) is one of the primary first-line combination antibiotics indicated for *Mycobacterium tuberculosis*, which greatly reduces the length of chemotherapy. Pyrazinamide (PZA) is also an antimicrobial agent, especially effective against multi-drug-resistant (MDR) tuberculosis (TB), resistant to isoniazid (INH) and RIF. *M. tuberculosis* acquires resistance to RIF through mutations in the *rpoB* gene, while compensatory mutations in the rpoC gene restore the fitness of RIF-resistant *M. tuberculosis*. *M. tuberculosis* acquires its resistance to PZA by having mutations in the *pncA gene*. A total of 93 *M. tuberculosis* isolates attained from patients were analysed to examine the mutation patterns of *rpoB*, *rpoC*, and *pncA* in South Korea.

Methods Antibiotic susceptibility was determined by carrying out bacterial cultures of drug-resistant mycobacterial isolates. Mutations in the *rpoB*, *rpoC* and *pncA* genes were identified by sequencing analysis, while the attributes of mutations were determined by comparing a relevant wild-type DNA sequence with that of a mutant allele. (H37Rv, American Type Culture Collection 25618).

Results A drug susceptibility test was performed for the total of 93 *M. tuberculosis* isolates that had been successfully cultured. Of these 93 isolates that were subjected to drug susceptibility testing (DST), 75 were found to be resistant to multiple drugs. Of these 75 isolates, 20 were MDR-TB; 7 were MDR-Plus; 36 were extensively drug-resistant XDR-TB; and 12 were drug-resistant (DR)-TB. A total of 66 cultured *M. tuberculosis* isolates were found to be RIF-resistant; 40 cultured isolates were found to be PZA-resistant; 39 cultured isolates were found to be both RIF- and PZA-resistant; and 18 were identified as being pan-susceptible (pan-S). Substitutions or multiple-site

mutations in the *rpoB* region were identified in 56 isolates (56/80, 70.0%), of which 91.1% (51/56) were resistant to RIF and 9 distinctive-site mutations were identified. Fifteen (15) different types of *rpoC* mutations were identified in 24 isolates (24/93, 25.8%), all of which were resistant to both INH and RIF. The mutation rates in MDR- and XDR-TB were 37.0% (10/27) and 38.9% (14/36), respectively. Substitutions of a single nucleotide (22/24, 91.7%) or substitutions of multiple-site mutations (2/24, 8.3%) in the *rpoC* region were identified, and neither deletion nor insertion mutation was detected in any of the isolates. No mutations were identified in the *rpoC* region of any drug-susceptible strains. Various mutations were identified in the *pncA* gene in 46 isolates: Nucleotide substitutions, deletions, insertion, multiple-site mutations and 25 different mutation sites were found. Of these various mutations detected in 46 isolates, substitution of a single nucleotide was most common (27/46, 58.7%), followed by multiple-site mutations (4/46, 8.7%) and insertion (4/46, 8.7%). Frameshifts caused by an insertion or a deletion of a single or multiple nucleotides at various sites accounted for 15.2% (7/46) of all mutations.

Conclusion Mutations of the *rpoB, rpoC* and *pncA* genes are the essential mechanism of RIF and PZA resistance in drug-resistant *M. tuberculosis* isolates. Detection of *rpoB, rpoC* and *pncA* gene mutations can complement in vitro DST and DNA-based diagnosis of RIF and PZA resistance, and is a promising method for the rapid detection of drug resistance.

Key Words: *Mycobacterium tuberculosis,* multi-drug resistance, *rpoC* mutations, *rpoB* mutations, *pncA* mutations, MDR, and XDR

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1. INTRODUCTION

In 1882, Robert Koch discovered the causative agent of tuberculosis (TB), an airborne infectious disease caused by *Mycobacterium tuberculosis*. TB still continues to be a major cause of morbidity and mortality, primarily in deprived or moderately poor countries (World Health Organization, 2015) in 2016. Having primarily a pulmonary pathophysiology, *M. tuberculosis* may be manifested as extra-pulmonary TB as part of a primary or late, generalized systemic infection. Also, the clinical manifestations of TB may be widely extended from asymptomatic infection to a life-threatening malady (Barry, et al., 2009, Esmail, et al., 2014). From a clinical and public health perspective, TB may be pragmatically classified into two: (1) asymptomatic non-transmissible latent TB infection (LTBI) and (2) transmissible active-pulmonary TB, for which culture-based or molecular diagnostics can be used. Patients with active TB may present general symptoms, such as fever, fatigue, lack of appetite and weight loss, while those with pulmonary TB can experience persistent cough and hemoptysis of an advanced ailment. However, some patients with active, culture-positive disease may be asymptomatic and are best described as having subclinical TB (Barry, et al., 2009, Esmail, et al., 2014).

The first-line anti-TB agents that constitute a standard treatment regimen are isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB). Bacterial resistance to multiple-drugs are reality and the notion of multidrug-resistant TB (MDR-TB) to INH and RIF has been accepted worldwide (World Health Organization. 2015). Extensively drug-resistant TB (XDR-TB) strains, which cause even more severe clinical manifestations, are resistant to not just INH and RIF but also fluoroquinolones and aminoglycosides. The worldwide emergence of MDR-TB and XDR-TB threatens global efforts to contain tuberculosis (Gandhi, et al., 2010, World Health Organization. 2015). The combination of INH and RIF is an effective primary first-line anti-TB

regimen (Abate et al., 2014, Jeon et al., 2015, Park, et al., 2016). MDR-TB strains, resistant to INH and RIF, have placed an increasing burden on South Korea (Jeon, et al., 2015, Park, et al., 2016, Tauhid, et al., 2014). *M. tuberculosis* can acquire resistance to RIF through mutations in *rpoB*, encoding the β subunit of RNA polymerase (Cavusoglu, et al., 2002, Yue, et al., 2003, Yun, et al., 2005). Mutations in the *rpoC* gene, encoding the β′ subunit of RNA polymerase, were also associated with increased *in vitro* fitness. Such mutations were overrepresented among patients inflicted with MDR-TB isolates in the high MDR-TB burdened countries (Comas, et al., 2011, de Vos, et al., 2013). Mutations in the *rpoC* gene were overrepresented among MDR-TB strains and one study showed that *M. tuberculosis* isolates harbouring *rpoB* mutations also carried nonsynonymous mutations in the *rpoC* gene (de Vos, et al., 2013). PZA is an effective anti-tubercular agent as well as an important treatment option in cases with MDR-TB strains resistant to INH and RIF. PZA, administered concurrently with a first-line drug regimen of INH and RIF, shortens the duration of anti-tubercular treatment (Mphahlele et al., 2008). *M. tuberculosis* can acquire resistance to PZA through mutations in the *pncA* gene, which encode pyrazinamidase (PZase). PZA is a prodrug that must be enzymatically converted to the active form pyrazinoic acid by PZase reaction. PZase activities, revealed through study findings of drug resistance to PZA, are apparently the pathophysiologic mechanism responsible for PZA resistance. PZA-resistant strains having mutations in the *pncA* gene contribute to the loss of its activity (Scorpio, et al., 1996). Furthermore, mutations in *pncA* have demonstrated a solid correlation between the loss of PZase activities and PZA resistance in *M. tuberculosis* (Hirano, et al., 1998, Kim, et al., 2012, Mphahlele, et al., 2008,). In this study, we investigated the patterns of *rpoB*, *rpoC* and *pncA* mutations in drug-resistant and susceptible *M. tuberculosis* among patients in South Korea.

2. MATERIALS AND METHODS

2.1. *Mycobacterial* isolates and susceptibility testing

Ninety three (93) *M. tuberculosis* isolates with clinically observed drug resistance or with susceptibility to anti-tuberculosis drugs were collected at National Masan Hospital and Pusan National University Colleague of Medicine in South Korea. Each isolate was cultured on Löwenstein-Jensen (LJ) medium at 37°C for 3-4 weeks and tested for resistance at critical concentrations of capreomycin (CPM) (40 μg/mL), EMB (2.0 μg/mL), INH (0.2 μg/mL), kanamycin (KM) (40 μg/mL), ofloxacin (OFX) (2 μg/mL), streptomycin (SM) (4 μg/mL), PZA (100 μg/mL, Wayne's pyrazinamidase assay) (Wayne et al., 1974), and RIF (40 μg/mL).

M. tuberculosis H37Rv (American Type Culture Collection (ATCC) 27294) was used as a positive control for all experiments. Regarding drug resistance profiles, MDR was defined as having resistance to both RIF and INH; XDR, MDR plus resistance to any of the second-line injectable drugs and fluoroquinolones; DR, any drug resistance other than MDR or XDR; and Pan-S, susceptible to all drugs. Sixty-six (66) isolates were RIF-resistant *M. tuberculosis* (Table 1). This study was approved by the institutional review board (IRB) of the International Tuberculosis Research Centre, and informed consent was obtained from all subjects.

2.2. DNA preparation for plymerase chain reaction (PCR)

The bead beater-phenol extraction method was utilized to extract DNA (Kim, et al., 1999). Two or three fragmented specimens were suspended in 200 μL of distilled water in Screw Cap Microcentrifuge tubes filled with 200 μL (packed volume) of glass beads

(diameter, 0.1 mm; Biospec Products; Bartlesville, Okla) and 200 μL of phenol-chloroform-isopropyl alcohol (50:49:1). The tube was oscillated on a Mini-Bead Beater (Biospec Products) for 1 minute to disrupt the tissues and bacteria, and then centrifuged (12,000 ×*g*, 5 min). After the aqueous phase was transferred to another clean tube, 10 μL of 3 M sodium acetate and 250 μL of ice-cold ethanol were added, and the mixture was kept at -20°C for 10 minutes. The obtained DNA pellets were then washed with 70% ethanol. Then, the solution was dissolved in 60 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and used it as a template for PCR.

2.3. Polymerase chain reaction (PCR) and sequencing of the *rpoB*

The *rpoB* DNAs (342 bp), encompassing the Rif^r region, which is associated with RIF resistance in *M. tuberculosis*, were amplified by PCR with the GeneAmp PCR System 9600 (PerkinElmer, Foster City, CA, USA) using MF,) (Kim, et al., 1999). Briefly, the PCR parameters were 5 minutes at 95°C, followed by 40 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 60 seconds at 72°C, with a final extension at 10 minutes at 72°C. The PCR products were purified using the QIAEX II Gel Extraction Kit (Qiagen Inc., Mainz, Germany) according to the manufacturer's instructions and sequenced using the BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). Nucleotide sequences were analyzed using the BioEdit software (version 5.0.9.1, Ibis Biosciences, Carlsbad, CA, USA), Chromas version 2.33 (Technelysium, Brisbane, QLD, Australia) (http://www.technelysium.com.au/chromas.html), and Basic Local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, MD, USA) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mutations in *rpoB* encoding regions were

defined as any nucleotide difference compared with those in the RIF-susceptible strain H37Rv (ATCC 25618).

2.4. PCR and sequencing of the *rpoC*

The *rpoC* region (1730 bp) was amplified by PCR with the GeneAmp PCR System 9600 (PerkinElmer, Foster City, CA, USA) using the primers 5'-CGAAAACCTCTACCGCGAAC-3' and 5'-CACGGAAGGAGGACTTGACC-3 (de Vos, et al., 2013). Briefly, the PCR parameters were 5 minutes at 95°C, followed by 40 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 60 seconds at 72°C, ending with a final extension of 10 minutes at 72°C. The PCR product was purified using the QIAEX II Gel Extraction Kit (Qiagen Inc., Mainz, Germany) according to the manufacturer's instructions and sequenced using the BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) using primers 5'-CGAAAACCTCTACCGCGAAC-3' and 5'-CACGGAAGGAGGACTTGACC-3 (Comas, et al., 2011). Nucleotide sequences were analysed using the BioEdit software (version 5.0.9.1; Ibis Biosciences, Carlsbad, CA), Chromas version 2.33 (Technelysium, Brisbane, QLD, Australia) (http://www.technelysium.com.au/chromas.html), and the Basic Local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, MD, USA) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mutations in the *rpoC*-encoding regions were defined as any nucleotide difference leading to translational changes of RpoC compared with those in the RIF-susceptible strain, H37Rv (ATCC 25618).

2.5. PCR and sequencing of the *pncA*

The *pncA* (670 bp) region was amplified by PCR with the GeneAmp PCR System 9600 (PerkinElmer, Foster City, CA, USA) using primers 5'-GGCGTCATGGACCCTATATC-3' and 5'-CAACAGTTCATCCCGGTTC-3 (Kim, et al., 1999, Kim, et al., 2012, Yun, et al., 2005). Briefly, the PCR parameters were 5 minutes at 95°C, followed by 40 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 60 seconds at 72°C, with a termination using a final extension step at 72°C for 10 minutes. The PCR products were purified using the QIAEX II Gel Extraction Kit (Qiagen Inc., Mainz, Germany) according to the manufacture6r's instructions and sequenced using the BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). Nucleotide sequences were analyzed using the BioEdit software (version 5.0.9.1; Ibis Biosciences, Carlsbad, CA, USA), Chromas version 2.33 (Technelysium, Brisbane, QLD, Australia) (http://www.technelysium.com.au/chromas.html), and the Basic Local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, MD, USA) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mutations in *pncA* encoding regions were defined as any nucleotide difference compared with those in the PZA-susceptible strain H37Rv (ATCC 25618).

3. RESULTS

Ninety three (93) clinical isolates, obtained from South Korean patients, were included in this study. In the drug-susceptibility testing (DST), 75 isolates were found to be multidrug-resistant. Twenty (20) were categorized as MDR-TBs; 7, MDR-Plus; 36, XDR-TB; and 12, DR-TB. Sixty-six (66) cultured *M. tuberculosis* isolates were found to be RIF-resistant, 40 cultured *M. tuberculosis* isolates were found to be PZA-resistant, 39 cultured *M. tuberculosis* isolates were found to be both RIF and PZA-resistant, and 18 were categorized as pan S. (Table 1).

3.1. The patterns of *rpoB* mutations

The *rpoB* PCR products were obtained from 80 cultured isolates, among the total of 93 isolates (80/93, 86.0%), and sequenced (Table 2). Substitutions or multiple-site mutations in the *rpoB* region were identified in 56 isolates (56/80, 70.0%), and found that 91.1% (51/56) were resistant to RIF (Table 2). The mutation rates in MDR- and MDR Plus-TB were 86.4% (19/22) and XDR-TB was 93.5% (29/31). Nine (9) different mutation sites were identified (Figure 1). Substitution of a single nucleotide was most common (52/56, 92.9%), and the most frequent mutation site was at codon 531 (nucleotide (nt) 1594), which resulted in amino acid substitution from Ser to Glu, Leu or Tyr in 34 isolates (34/52, 65.4%) (Table 2). Neither deletion nor insertion mutation was detected in any of the isolates, while no mutation was identified in 5 isolates, despite the fact that they were proven to be MDR- and MDR Plus-TB (3/22, 13.6%) or XDR-TB (2/31, 6.5%). Six (6) isolates (no. 22, 28, 30, 35, 37, and 77) had mutations in the *rpoB,* but were sensitive to RIF (Table 2). Some of the mutations and multi-site mutations revealed in this investigation had not been previously reported. These new mutations

were indicated in Table 2. The wild-type DNA sequences of *rpoB* and the mutation sites in this study were shown in Figure 1.

3.2. The patterns of *rpoC* mutations

The *rpoC* PCR products were amplified from 93 isolates, and sequenced. Fifteen (15) different types of mutations were identified in 24 isolates (24/93, 25.8%), all of which were resistant to both INH and RIF, multidrug-resistant tuberculosis and mutation rates in MDR- and XDR-TB were 37.0% (10/27) and 38.9% (14/36), respectively (Table 3). Substitutions of a single nucleotide (22/24, 91.7%) or substitutions of multiple-site mutations (2/24, 8.3%) in the *rpoC* region were identified. However, neither deletion nor insertion mutation was detected in any of the isolates. No mutation was identified in the *rpoC* region of any drug-susceptible strain.

A mutation at codon 452 (nt 1356), detected in 7 isolates, was the most common mutation (7/24, 29.2%) and a mutation at codon 531 (nt 1594), which is the nucleotide most frequently involved in *rpoB* mutation, were also detected in these isolates (Table 2 and 3) (Cavusoglu, et al., 2002, Yue, et al., 2003, Yun, et al., 2005). Twelve (12) different mutation sites (at codon 281 (nt 843), 416 (nt 1249), 434 (nt 1302), 446 (nt 1338), 561 (nt 1683), 575 (nt 1726), 581 (nt 1745), 728 (nt 2186), 747 (nt 2242), 801 (nt 2403), 812 (nt 2437), and 813 (nt 2441)) were first reported in this study (Comas, et al., 2011, de Vos, et al., 2013); these new mutations are indicated in Figure 2 and Table 3.

3.3. The patterns of *pncA* mutations

Of the 93 isolates, the *pncA* PCR products were obtained in 89 cultured isolates (89/93, 95.7%), and sequenced. Various mutations, identified by the pncA gene of 46 isolates, include nucleotide substitution, deletion, insertion, and multiple-site mutations

(Table 4). Twenty-five (25) different mutation sites were identified, and substitutions of single nucleotides were the most common (27/46, 58.7%), followed by multiple-site mutations (4/46, 8.7%) and insertions (4/46, 8.7%). Frameshifts caused by insertion or deletion of a single or multiple nucleotides in various sites accounted for 15.2% (7/46) of all mutations. The most frequently mutated sites were at nt 403, which showed a substitution from adenosine to cytosine, resulting in an amino acid substitution from Thr to Pro in 8 isolates that are resistant to PZA (8/46, 17.4%). One isolate was MDR-TB and 7 isolates were XDR-TB). Ten (10) isolates were identified with no mutation (10/89, 11.2%), despite having proven drug resistance to PZA. The 10 PZA-resistant isolates comprised 7 of the 36 XDR-TB strains (19.4%); 2 of the 21 MDR-TB (9.5%); and 1 of the DR-TB. Some mutations revealed in this investigation were not reported previously. These new mutations are shown in Table 3, and the wild-type DNA sequences of *pncA* and the mutation sites including the promoter regions in this study are shown in Figure 3. Mutations in both *rpoB* and *pncA* were found in 28 isolates. Twenty-three (23) out of the 28 isolates (82%), all of which are MDR or XDR-TB, were RIF- and PZA- resistant. Four (4) of these isolates (1 XDR, 1 MDR, 1 MDR Plus and 1 DR) were RIF-resistant, while one isolate was INH-resistant. One MDR isolate (no. 68, resistant to RIF) and two XDR-TB isolates (No. 32 and 55, resistant to RIF and PZA) had new mutations in *rpoB* and *pncA* that had not been previously reported.

Table 1. Drug resistance profiles of 93 *M. tuberculosis* isolates.

No.	Drug resistance	Drug resistance profile	No.	Drug resistance	Drug resistance profile	No.	Drug resistance	Drug resistance profile
1	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	**44**	SM, INH, RFP, CPM, KM, MFX, PZA	XDR	87	CPM	DR
2	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	45	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	88	None Detected	Pan-S
3	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR	**46**	SM, INH, RFP, CPM, KM, OFX, MFX	XDR	**89**	INH, RFP	MDR
4	INH, RFP, CPM, KM, PZA	MDR	47	SM, INH, RFP, OFX, MFX, PZA	MDR	90	INH, RFP, MFX, CPM	XDR
5	SM, INH, RFP, EMB, OFX, MFX, PZA	MDR	48	None Detected	Pan-S	91	INH, RFP, CPM	MDR Plus
6	INH, RFP, OFX, MFX	MDR	49	CPM	DR	92	None Detected	Pan-S
7	SM, INH, RFP, EMB CPM, KM, MFX, PZA	XDR	**50**	INH, RFP, OFX, MFX, PZA	MDR	93	None Detected	Pan-S
8	SM, INH, RFP, MFX, PZA	MDR	51	INH, RFP, KM, OFX, MFX, PZA	XDR			
9	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	52	INH, RFP	MDR			
10	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	**53**	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR			
11	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR	**54**	SM, INH, RFP, EMB, OFX, PZA	XDR			
12	SM, INH, RFP, KM, OFX, MFX, PZA	XDR	55	SM, INH, RFP, OFX, MFX, PZA	XDR			
13	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	56	SM, INH, RFP, CPM, OFX, MFX, PZA	XDR			
14	SM, INH, RFP, EMB, CPM, KM, OFX, MFX	XDR	**57**	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR			
15	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	58	INH, RFP, CPM, OFX, MFX, PZA	XDR			
16	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	59	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR			
17	SM, INH, RFP, CPM, KM, PZA	MDR	60	RFP, CPM	DR			
18	INH, RFP, OFX, MFX, PZA	MDR	61	INH, RFP	MDR			
19	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	62	INH	DR			
20	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	**63**	INH, RFP, LEV, OFX, MFX,	MDR Plus			
21	INH, RFP, CPM, KM, MFX, PZA	XDR	64	INH, RFP, LEV, OFX, MFX, KM, AMK, CPM	XDR			
22	None Detected	Pan-S	**65**	INH, RFP, LEV, OFX, MFX, KM, AMK, CPM	XDR			
23	None Detected	Pan-S	66	INH, RFP, OFX, KM	XDR			

24	None Detected	Pan-S	67	RFP	DR
25	None Detected	Pan-S	68	INH, RFP,	MDR
26	None Detected	Pan-S	69	INH, RFP, CPM	MDR Plus
27	SM, INH, CPM, PZA	DR	70	None Detected	Pan-S
28	INH	DR	**71**	INH, RFP, LEV, OFX	MDR Plus
29	None Detected	Pan-S	72	INH, RFP	MDR
30	None Detected	Pan-S	**73**	INH, RFP	MDR
31	None Detected	Pan-S	**74**	INH, RFP	MDR
32	SM, INH, RFP, EMB, KM, OFX, MFX, PZA	XDR	75	INH, RFP	MDR
33	None Detected	Pan-S	76	RFP, CPM	DR
34	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	77	None Detected	DR
35	None Detected	Pan-S	78	INH, RFP, LEV, OFX, MFX, KM, AMK, CPM	XDR
36	None Detected	Pan-S	**79**	INH, RFP, LEV, OFX	MDR Plus
37	INH	DR	**80**	INH, RFP, LEV, OFX	MDR Plus
38	SM, INH, RFP, EMB CPM, KM, MFX, PZA	XDR	81	CPM	DR
39	INH, RFP, EMB, OFX, MXF, PZA	MDR	82	INH, RFP, LEV, OFX	MDR Plus
40	INH, RFP, CPM, KM, PZA	MDR	83	None Detected	Pan-S
41	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR	84	INH, RFP	MDR
42	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	85	None Detected	Pan-S
43	SM, INH, RFP, EMB, CPM, KM, PZA	MDR	86	CPM	DR

CPM = capreomycin; EMB = ethambutol; INH = isoniazid; KM = kanamycin; MFX = moxifloxacin; OFX = ofloxacin; SM = streptomycin; PZA = pyrazinamide; RFP = rifampicin; MDR = multidrug-resistant; MDR plus = ING+RFP+FQ or INH+RFP+Inj.D; XDR = extensively drug-resistant; DR = resistant to any drug, but not MDR or XDR; others = any drug resistance(s) other than MDR or XDR-TB; Pan-S = pan-susceptible.**Bold numbers** indicate isolates with mutations in *rpoC* and resistance to both INH and RFP.

Table 2. Mutations detected in the *rpoB* of 80 isolates.

Nucleotide change (nucleotide no.)	Translational change (codon no.)	Cultured isolates (*n* =80) (100%)	MDR-TB isolates (*n* =18) (22.5%)	XDR-TB isolates (*n*=31) (38.75%)	Others isolates (*n* =5) (6.25%)	Pan-s isolates (*n* =12) (15%)	MDR-Plus isolates (*n* =4) (5%)	DR Isolates (*n* =5) (6.25%)	All S isolate (*n* =5) (6.25%)	Remarks	No.
Substitution											
CAA(1539)->AAA	Gly(513)->Lys	1(1.25)	1(1.25)								75
GAC(1549)->GTC	Asp(516)->Val	8(10)	2(2.5)	5(6.25)			1(1.25)			Substitution	10, 15, 34, 40, 42, 43, 45, **71**
GAC(1549)->TAC	Asp(516)->Tyr	1(1.25)			1(1.25)					Substitution	28
CAC(1578)->GAC,	His(526)->Asp,	3(3.75)	3(3.75)								5, 6, 47
CAC(1578)->CGC	His(526)->Arg	1(1.25)					1(1.25)				**63**
CAC(1578)->TAC	His(526)->Try	1(1.25)	1(1.25)								84
TCG(1594)->TGG	Ser(531)->Try	2(2.5)	1(1.25)	1(1.25)						Substitution	14, **50**
TCG(1594)->CAG	Ser (531)->Glu	1(1.25)		1(1.25)						Substitution	41
TCG(1594)->TTG	Ser (531)->Leu	31(38.7)	6(7.5)	19(23.7)	1(1.25)	1(1.25)	2(2.5)	2(2.5)		Substitution	1, **2**, 3, 7, **8**, **9**, **11**, 12, **13**, **17**, 18, 19, **20**, 21, 30, **38**, 39, **44**, **46**, 52, **53**, **54**, 56, **57**, 59, 60, 67, 69, **74**, 77, **79**
CTG(1635)->TTG	Leu(545)->Leu	1(1.25)			1(1.25)					Substitution	37
Multi-site mutation											
ACC(1441)->ATC TCG(1594)->TTG	Thr (480)->Iso Ser(531)->Leu	1(1.25)		1(1.25)						Substitution	55
TCG(1594)->TTG GGG(1634)->GGC CTG(1635)->TTG	Ser(531)->Leu Gly(544)->Gly Leu(545)->Leu	1(1.25)				1(1.25)				Substitution	22
GAC(1549)->AAC CAC(1578)->AAC	Asp(516)->Asn His(526)->Asn	1(1.25)	1(1.25)							Substitution	68

TCG(1594)->TTG GGG(1634)->GGC CTG(1635)->TTG CAC(1655)->CAT	Ser(531)->Leu Gly(544)->Gly Leu(545)->Leu His(551)->His	1(1.25)		1(1.25)					Substitution	**32**
TCG(1594)->TTG CTG-(1635)->TTG	Ser(531)->Leu Leu-(545)->Leu	1(1.25)				1(1.25)			Substitution	35
GAC(1549)->GGC CTG(1600)->CCG	Asp(516)->Gly Leu(533)->Pro	1(1.25)		1(1.25)					Substitution	78
Wild type	No change	24(30)	3(3.75)	2(2.5)	2(2.5)	9(11.2)	3(3.75)	5(6.25)		4, **16**, 23, 24, 25, 26, 27, 29, 31,33, 36, 48,49 ,51, 61, 62,70, **73**, 76, 85, 86, 88, 92, 93

Number of codon position was counted from the start codon (ATG) of the *rpoB*. **Bold number** indicate isolate with mutation in *rpoC*.

Table 3. Mutations detected in *rpoC* of 93 *M. tuberculosis* isolates.

Nucleotide change (s) (Nucleotide no.)	Translational change (codon no.)	Cultured Isolates (*n* = 93) *n* (%)	MDR-TB Isolates (*n* = 20) *n* (%)	XDR-TB isolates (*n* = 36) *n* (%)	Others Isolates (*n* = 5) *n* (%)	Pan-S Isolates (*n* = 12) *n* (%)	MDR-Plus isolates (*n* =7) *n* (%)	DR Isolates (*n* =7) *n* (%)	All S Isolate (*n* =6) *n* (%)	NO.	Remarks
Substitution											
ATC (843)->GTC	Iso (281)->Val[a]	3 (3.2)	2 (2.2)				1 (1.1)			**71**, **73**, **89**	Substitution
AAC (1249)->AGC	Asn (416)->Ser[a]	1 (1.1)		1 (1.1)						**20**	Substitution
CCG(1302)->ACG	Pro (434)->Thr[a]	1 (1.1)					1 (1.1)			**79**	Substitution
CTG (1338)->ATG	Leu (446)->Met[a]	1 (1.1)					1 (1.1)			**63**	Substitution
TTC (1356)->CTC	Phe (452)->Leu	7 (7.5)	1 (1.1)	6 (6.4)						**2**, **11**, **13**, **17**, **44**, **53**, **57**	Substitution
GTG (1450)->GCG	Val (483)->Ala	1 (1.1)		1 (1.1)						**38**	Substitution
GTG (1450)->GGG	Val (483)->Gly	1 (1.1)		1 (1.1)						**46**	Substitution
TCC (1683)->CCC	Ser (561)->Pro[a]	1 (1.1)		1 (1.1)						**54**	Substitution
GCC (1726)->GTC	Ala (575)->Val[a]	1 (1.1)		1 (1.1)						**16**	Substitution
GGC(2186)->GGT	Gly (728)->Gly[a]	1 (1.1)	1 (1.1)							**74**	Substitution
GAC (2242)->GGC	Asp (747)->Gly[a]	2 (2.2)	1 (1.1)	1 (1.1)						**8**, **65**	Substitution
ACC (2437)->ATC	Thr (812)->Iso[a]	1 (1.1)	1 (1.1)							**50**	Substitution
CAG (2441)->CAC	Glu (813)->His[a]	1 (1.1)		1 (1.1)						**32**	Substitution
Multi-site mutation[a]											
TCC (1683)->CCC, ATG (1745)->ATA	Ser (561)->Pro[a], Met (581)->Iso[a]	1 (1.1)		1 (1.1)						**9**	Substitution
CCG(1302)->GTG, ACC(2403)->TCC	Pro (434)->Val, Thr (801)->Ser[a]	1 (1.1)					1 (1.1)			**80**	Substitution
Wild type	No change	69 (74.2)	14 (15.1)	22 (23.6)	5 (5.3)	12 (12.9)	3 (3.2)	7 (7.5)	6 (6.4)	1, 3, 4, 5, 6, 7, 10, 12, 14, 15, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 33,	

	34, 35, 36, 37, 39, 40, 41, 42, 43, 45, 47, 48, 49, 51, 52, 55, 56, 58, 59, 60, 61, 62, 64, 66, 67, 68, 69, 70, 72, 75, 76, 77, 78, 81, 82, 83, 84, 85, 86, 87, 88, 90, 91, 92, 93

[a]New mutation not reported in previous studies. **Bold numbers** indicate isolates with mutations in *rpoC* and resistance to both INH and RFP.

Table 4. Mutations detected in the *pncA* gene of 89 cultured isolates.

Nucleotide change (s) (nucleotide no.)	Translational change (codon no.)	Cultured Isolates (*n* = 89) (99%)	MDR-TB Isolates (*n* =19) (21.3%)	XDR-TB isolates (*n* =33) (37.0%)	Others isolates (*n* =5) (5.61%)	Pan-S Isolates (*n* =12) (13.4%)	MDR-Plus isolates (*n* =7) (7.86%)	DR Isolates (*n* =7) (7.86%)	All S isolate (*n* =6) (6.74%)	Remarks	No.
Substitution											
TAT->TGT(-11)	Tyr(-4)->Cys	3(3.3)	2(2.2)	1(1.1)						Substitution	8, 6, 90
TTG->TGG(11)	Leu(4)->Try	1(1.1)		1(1.1)						Substitution	20
CAG->CGG(29)	Gly(10)->Arg	1(1.1)					1(1.1)			Substitution	71
CAG->CCG(29)	Gly(10)->Pro	1(1.1)	1(1.1)							Substitution	43
*CTG->CCG(104)	Leu(35)->Pro	1(1.1)	1(1.1)							Substitution	4
*GTG->GAG(134)	Val(44)->Met	1(1.1)		1(1.1)						Substitution	78
AAG->GAG(142)	Lys(48)->Glu	1(1.1)		1(1.1)						Substitution	46
*CAC->CCC(170)	His(57)->Pro	1(1.1)		1(1.1)						Substitution	38
TCG->CCG(199)	Ser(66)->pro	1(1.1)		1(1.1)						Substitution	45
TGG->TCG(203)	Try(68)->Ser	1(1.1)		1(1.1)						Substitution	9
TGG->TAG(203)	Try(68)->Stpo	1(1.1)		1(1.1)						Substitution	65
*TGG->TGT(204)	Try(68)->Cys	1(1.1)		1(1.1)						Substitution	64
*CAT->TAT(211)	His(70)->Tyr	1(1.1)			1(1.1)					Substitution	60
ACT->CCT(226)	Try(76)->Pro	1(1.1)		1(1.1)						Substitution	51
*TTC->GTC(241)	Phe->(80)Val	1(1.1)						1(1.1)		Substitution	67
*CTG->CGG(254)	Leu->(87)->Arg	1(1.1)	1(1.1)							Substitution	72
*GGT->AGT(289)	Gly(97)->Ser	1(1.1)	1(1.1)							Substitution	5
*ACC->CCC(298)	Thr(99)->Pro	1(1.1)		1(1.1)						Substitution	41

*TAC->CAC(307)	Thr(102)->His	1(1.1)		1(1.1)				Substitution	54
AGC->AGA(312)	Ser(104)->Arg	1(1.1)	1(1.1)					Substitution	39
ACC->CCC(403)	Thr(134)->Pro	8(8.8)	1(1.1)	7(7.7)				Substitution	1, 2, 13, 11,17, 44, 53, 57
*GAT->GGT(407)	Asp(136)->Gly	1(1.1)	1(1.1)					Substitution	68
CAG->CTG(422)	Glu(140)->Leu	1(1.1)				1(1.1)		Substitution	24
CAG->CCG(422)	Glu-(140)>Pro	1(1.1)		1(1.1)				Substitution	15
*GAC->GAA(435)	ASP(145)->Glu	1(1.1)			1(1.1)			Substitution	28
GCG->GTG(437)	Ala(146)Val	1(1.1)	1(1.1)					Substitution	50
AGG->GGG(460)	Arg(153)->Gly	1(1.1)	1(1.1)					Substitution	47
Multi-site mutation									
TAT->TGT(-11) ACC->CCC(403)	Tyr(-4)->Cys Thr(143)->Pro	1(1.1)		1(1.1)				Substitution	7
*TCG->CCG(199) *AGC->GGC(535)	Ser(66)->pro Ser(178)->Gly	1(1.1)		1(1.1)				Substitution	42
*TAT->TGT(-11) *GAC->GAA(189)	Tyr(-4)->Cys Asp(85)->Glu	1(1.1)		1(1.1)				Substitution	66,
*GTC->GGC(392) ->T(392)	Gly(131)->Gly Frameshift	1(1.1)					1(1.1)	Substitution	79
Insertion									
*->A(192)	Frameshift	1(1.1)		1(1.1)				Insertion at 192	10
*->G(417)	Frameshift	2(2.2)		2(2.2)				Insertion at 417	16, 32
*->C(392)	Frameshift	1(1.1)					1(1.1)	Insertion at 392	82

Deletion											
*CAT->—(211,212,213)	Frameshift	1(1.1)		1(1.1)						Deletion from 211 to 212	12
*C->—(393)	Frameshift	1(1.1)		1(1.1)						Deletion at 393	55
Wild type	No change										
		43(48.3)	8(8.98)	5(5.61)	3(3.37)	11(12.3)	4(4.49)	6(6.74)	6(6.74)		14, 18, 19, 21, 22, 23, 25, 26, 27, 29, 30, 31, 33, 34, 35, 36, 37, 40, 48, 49, 52, 59, 62, 63, 69, 70 73, 74, 75, 76, 77, 80, 81, 83, 84, 85, 86, 87, 88 89, 91, 92, 93

Number of codon position was counted from the start codon (ATG) of the *pncA*. * New mutation not reported in previous report.

Figure. 1. Genomic DNA sequences of *rpoB* encoding the β subunit of RNA polymerase in *M. tuberculosis*.

1335																			
	GAC	CAC	TTC	GGA	AAC	CGC	CGC	CTG	CGT	ACG	GTC	GGC	GAG	CAG	CTG	ATC	CAA	AAC	CAG	ATC
1395																			
	CGG	GTC	GGC	ATG	TCG	CGG	ATG	GAG	CGG	GTG	GTC	CGG	GAG	CGG	ATG	ACC	ACC	AGG	ACC	GTG
1455																			
	GAG	GCG	ATC	ACA	CCG	CAG	ACG	TTG	ATC	AAC	ATC	CGG	CCG	GTG	GTC	GCC	GCG	ATC	AAG	GAG
1515																			
	TTC	TTC	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG
1575																			
	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG	GGG	CCC	GGC	GGT	CTG	TCA	CGT	GAG	CGT	GCC	GGG
1635																			
	CTG	GAG	GTC	CGC	GAC	GTG	CAC	CCG	GCC	GGG	CTG	GAG	GTC	CGC	GAC	GTG	CAC	CCG	TCG	CAC
1695																			
	TAC	GGC	CGG	ATG	TGC	CCG	ATC	GA											

Mutations spot in 80 isolates were marked (bold), and the underlined sequence has not been previously reported. Nucleotides were numbered from the start codon (ATG) of *rpoB*.

Figure 2. Genomic DNA sequences of *rpoC* in *M. tuberculosis*.

795
ATC GAG AAC TTC GAC ATC GAC GCC GAA GCC GAG TCG CTG CGG GAT GTC ATC CGA AAC GGC
855
AAG GGG CAG AAG AAG CTT CGC GCC CTC AAG CGG CTG AAG GTG GTT GCG GCG TTC CAA CAG
915
TCG GGC AAC TCG CCG ATG GGC ATG GTG CTC GAC GCC GTC CCG GTG ATC CCG CCG GAG CTG
975
CGC CCG ATG GTG CTG CTC GAC GGC GGC CGG TTC GCC ACG TCC GAC TTG AAC GAC CTG TAC
1035
CGC AGG GTG ATC AAC CGC AAC AAC CGG CTG AAA AGG CTG ATC GAT CTG GGT GCG CCG GAA
1095
ATC ATC GTC AAC AAC GAG AAG CGG ATG CTG CAG GAA TCC GTG GAC GCG CTG TTC GAC AAT
1155
GGC CGC CGC GGC CGG CCC GTC ACC GGG CCG GGC AAC CGT CCG CTC AAG TCG CTT TCC GAT
1215
CTG CTC AAG GGC AAG CAG GGC CGG TTC CGG CAG AAC CTG CTC GGC AAG CGT GTC GAC TAC
1275
TCG GGC CGG TCG GTC ATC GTG GTC GGC CCG CAG CTC AAG CTG CAC CAG TGC GGT CTG CCC
1335
AAG CTG ATG GCG CTG GAG CTG TTC AAG CCG TTC GTG ATG AAG CGG CTG GTG GAC CTC AAC
1395
CAT GCG CAG AAC ATC AAG AGC GCG AAG CGC ATG GTG GAG CGC CAG CGC CCC CAA GTG TGG
1455
GAT GTG CTC GAA GAG GTC ATC GCC GAG CAC CCG GTG TTG CTG AAC CGC GCA CCC ACC CTG
1515
CAC CGG TTG GGT ATC CAG GCC TTC GAG CCA ATG CTG GTG GAA GGC AAG GCC ATT CAG CTG
1575
CAC CCG TTG GTG TGT GAG GCG TTC AAT GCC GAC TTC GAC GGT GAC CAG ATG GCC GTG CAC
1635
CTG CCT TTG AGC GCC GAA GCG CAG GCC GAG GCT CGC ATT TTG ATG TTG TCC TCC AAC AAC
1695
ATC CTG TCG CCG GCA TCT GGG CGT CCG TTG GCC ATG CCG CGG CTG GAC ATG GTG ACC GGG
1755
CTG TAC TAC CTG ACC ACC GAG GTC CCC GGG GAC ACC GGC GAA TAC CAG CCG GCC AGC GGG
1815

GAT CAC CCG GAG ACT GGT GTC TAC TCT TCG CCG GCC GAA GCG ATC ATG GCG GCC GAC CGC
1875
GGT GTC TTG AGC GTG CGG GCC AAG ATC AAG GTG CGG CTG ACC CAG CTG CGG CCG CCG GTC
1935
GAG ATC GAG GCC GAG CTA TTC GGC CAC AGC GGC TGG CAG CCG GGC GAT GCG TGG ATG GCC
1995
GAG ACC ACG CTG GGC CGG GTG ATG TTC AAC GAG CTG CTG CCG CTG GGT TAT CCG TTC GTC
2055
AAC AAG CAG ATG CAC AAG AAG GTG CAG GCC GCC ATC ATC AAC GAC CTG GCC GAG CGT TAC
2115
CCG ATG ATC GTG GTC GCC CAG ACC GTC GAC AAG CTC AAG GAC GCC GGC TTC TAC TGG GCC
2175
ACC GCG AGC GG**C** GTG ACG GTG TCG ATG GCC GAC GTG CTG GTG CCG CCG CGC AAG AAG GAG
2235
ATC CTC G**A**C CAC TAC GAG GAG CGC GCG GAC AAG GTC GAA AAG CAG TTC CAG CGT GGC GCT
2295
TTG AAC CAC GAC GAG CGC AAC GAG GCG CTG GTG GAG ATT TGG AAG GAA GCC ACC GAC GAG
2355
GTC GGT CAG GCG TTG CGG GAG CAC TAC CCC GAC GAC AAC CCG ATC ATC **A**CC ATC GTC GAC
2415
TCC GGC GCC ACC GGC AAC TTC A**C**C CA**G** ACT CGA ACG CTG GCC GGT ATG AAG GGC CTG GTG
2475
ACC AAC CCG A

Mutations spot in 93 isolates were marked (bold).

Figure 3. Genomic DNA sequences of *pncA* encoding pyrazinamidase in *M. tuberculosis*.

-80

GG CGT CAT CGA CCC TAT ATC TGT GGC TGC CGC GTC GGT AGG CAA ACT GCC CGG GCA GTC

-21

GCC CGA ACG TAT GGT GGA CGT ATG CGG GCG TTG ATC ATC GTC GAC GTG CAG AAC GAC TTC

40

TGC GAG GGT GGC TCG CTG GCG GTA ACC GGT GGC GCC GCG CTG GCC CGC GCC ATC AGC GAC

100

TAC CTG GCC GAA GCG GCG GAC TAC CAT CAC GTC GTG GCA ACC AAG GAC TTC CAC ATC GAC

160

CCG GGT GAC CAC TTC TCC GGC ACA CCG GAC TAT TCC TCG TCG TGG CCA CCG CAT TGC GTC

220

AGC GGT ACT CCC GGC GCG GAC TTC CAT CCC AGT CTG GAC ACG TCG GCA ATC GAG GCG GTG

280

TTC TAC AAG GGT GCC TAC ACC GGA GCG TAC AGC GGC TTC GAA GGA GTC GAC GAG AAC GGC

340

ACG CCA CTG CTG AAT TGG CTG CGG CAA CGC GGC GTC GAT GAG GTC GAT GTG GTC GGT ATT

400

GCC ACC GAT CAT TGT GTG CGC CAG ACG GCC GAG GAC GCG GTA CGC AAT GGC TTG GCC ACC

460

AGG GTG CTG GTG GAC CTG ACA GCG GGT GTG TCG GCC GAT ACC ACC GTC GCC GCG CTG GAG

520

GAG ATG CGC ACC GCC AGC GTC GAG TTG GTT TGC AGC TCC TGA TGG CAC CGC CGA ACC GGG

580

ATG AAC TGT TG

Mutations spot in 89 isolates were marked (bold), and the underlined sequence has not been previously reported. Nucleotides were numbered from the start codon (ATG) of *pncA*.

4. DISCUSSION

RIF is one of the primary first-line combination anti-tubercular agents indicated for *Mycobacterium tuberculosis*, and RIF resistance is a valuable surrogate marker of MDR-TB. Over 90% of RIF resistance in clinical isolates of *M. tuberculosis* is identified with genetic alterations within the *rpoB* gene (Yue, et al., 2003, World Health Organization. 2015). RIF resistance is a valuable surrogate marker of drug-resistant tuberculosis, and detection of drug resistance to RIF is important in the treatment of tuberculosis (Cavusoglu, et al., 2002, Yue, et al., 2003, Yun, et al., 2005). *M. tuberculosis* can acquire resistance to RIF through mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase (Cavusoglu, et al., 2002, Yue, et al., 2003, Yun, et al., 2005). The ß' subunit of RNA polymerase is encoded by the rpoC gene. Iñaki Comas *et al.* suggested that the acquisition of particular mutations in *rpoC* in RIF-resistant *M. tuberculosis* strains over time leads to the emergence of MDR strains with a high fitness (Comas,et al., 2011). Moreover, de Vos M *et al.* showed that nonsynonymous mutations in the *rpoC* region are prevalent among RIF-resistant isolates in a highly-burdened setting in South Africa, and that these mutations are strongly associated with transmissions of RIF-resistant strains (de Vos, et al., 2013). Mutations of the *rpoC* gene have not been studied in South Korea yet, and this study investigated the patterns of *rpoC* mutations in drug-resistant and susceptible *M. tuberculosis* among patients in South Korea. Nucleotide substitutions and multiple-site mutations in *rpoB* were identified, and neither deletion nor insertion mutation was detected. Substitutions at codon 531 (nucleotide 1594) were the most commonly found variations (60.7%), while new mutations in rpoB, which had not been previously reported, were found (Table 2). Fifteen (15) different types of mutations in *rpoC* were identified, and 12 of these 15 mutation variations were first reported in this study (marked in Table 2) (Comas, et al., 2011, de Vos, et al., 2013). A mutation at codon

452 was the most common transformation (7/24, 29.2%). Mutations at codon 531, a nucleotide most frequently involved in *rpoB* mutation, were also detected in these isolates (Table 1) (Cavusoglu, et al., 2002, Yue, et al., 2003, Yun, et al., 2005). Mutations were only found among MDR-TB strains, all of which were resistant to both INH and RIF, while no mutation was identified in the *rpoC* region of any drug-susceptible strains (marked in Table 1 and Table 2). Therefore, *M. tuberculosis* can acquire resistance to RIF through mutations in the *rpoB* and *rpoC,* suggesting that mutations of *rpoB* and *rpoC* maybe used as a marker of MDR-TB and DNA-based diagnostic confirmation for the detection of INH and RIF resistance. Nonetheless, further extensive studies with a larger collection of isolates are necessary. PZA is also one of the most effective pharmacologic agents indicated for tuberculosis. When PZA is combined with the first-line drugs of INH and RIF, it shortens the duration of anti-tubercular treatment (Gandhi, et al., 2010, Mphahlele, et al., 2008, World Health Organization, 2015). In cases of MDR- and XDR-TB, where tubercular bacilli are resistance to at least both INH and RIF, PZA becomes an important treatment option (Gandhi, et al., 2010, Mphahlele, et al., 2008, World Health Organization, 2015). Thus, the detection of drug resistance to PZA is important in the treatment of tuberculosis, and is especially an urgent issue when there are tubercular resistance to INH and/or RIF (Gandhi, et al., 2010, World Health Organization, 2015). In this study, we investigated the patterns of *pncA* mutations of *M. tuberculosis* isolates that identified MDR- and XDR-TB strains among patients in South Korea. This study identified nucleotide substitutions, multiple-site mutations, as well as insertion and deletion (frameshift) mutations in *pncA* (Table 3 and Figure 3). We observed newly-developed mutations in the *pncA* gene that had not been previously reported, and also discovered that *pncA* mutations were more scattered and diverse than *rpoB* mutations (Tables 3 and Figure 3). In summary, *M. tuberculosis* can acquire resistance to RIF and

PZA through mutations in *rpoB*, *rpoC* and *pncA,* respectively (Hirano, et al., 1998, Kim et al., 1999, Kim, et al., 2012, Mphahlele, et al., 2008, Yun, et al., 2005, Scorpio, et al., 1996). Explicitly, mutations of *rpoB*, *rpoC* and *pncA* in *M. tuberculosis* are important mechanisms of RIF and PZA resistance. There is a strong correlation between mutations of *rpoB*, *rpoC* and *pncA* and RIF and PZA resistance to *M. tuberculosis* among drug-resistant isolates especially with MDR- and XDR-TB strains among patients in South Korea (Hirano, et al., 1998, Kim, et al., 2012, Kim et al., 1999, Mphahlele, et al., 2008, Yun, et al., 2005,). Therefore, the detection of *rpoB, rpoC* and *pncA* mutations, which complement the results of in vitro DST and DNA-based diagnosis of RIF and PZA resistance, would be a promising approach for the speedy detection of drug resistance (Kim, et al., 2012, Kim et al., 1999, Mphahlele, et al., 2008).

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6. ABSTRACT IN KOREAN

Rifampicin (RFP)은 결핵치료에 있어서 1 차 항결핵제로 많이 사용되는 약제이다. Pyrazinamide (PZA)은 INH 와 RFP 에 대해서 내성을 가지는 다제약제내성결핵균 (MDR-TB)에 효과적인 항결핵제이다. 본연구에서는 한국인 환자에서 분리된 93 주의 결핵균 (MDR-TB 포함)에 대해서 RFP 와 PZA 내성과 관련이 있는 유전자인 *rpoB*, *rpoC* 그리고 *pncA* 의 유전자 분석을 통하여 이들 유전자에서의 돌연변이 (mutation)와 이들 약제내성과의 관련성에 대해서 연구하였다. 93 개의 임상분리균주는 실험실에서 성공적으로 배양이 되었으며 약제내성검사 (drug susceptibility testing, DST)를 통하여 INH, PZA, RFP 를 포함한 항결핵제에 대한 내성 및 감수성을 확인하였다. 확인결과 75 개 분리주가 항결핵제에 대해서 내성을 갖는 것으로 확인되었다. 이중 20 주는 MDR-TB, 7 주는 MDR-Plus, 36 주는 XDR-TB 그리고 12 주는 DR-TB 로 확인이 되었으며 66 주에서 RFP 내성, 40 주는 PZA 에 대해서 내성 그리고 39 주는 RFP 와 PZA 에 대해서 내성을 가지는 것으로 확인되었으며 18 주는 pan S 로 확인되었다. 56 주에서 *rpoB* 유전자에서의 substitutions 또는 multiple-site mutations 이 확인되었으며 (56/80, 70.0%) 이중 51 주가 RFP 에 대해서 내성을 가지는 것으로 확인되었으며 (51/56, 91.1%), 9 개의 다른 변이가 확인되었다. 24 주에서 *rpoC* 에서 15 개의 다른 변이가 확인되었으며 (24/93, 25.8%), 24 주 모두 INH 와 RFP 에 대해서 내성을 가지며 MDR-TB 에서 37.0% (10/27), XDR-TB 에서 38.9% (14/36)에서 내성을 가지는 것을 확인했다. Single nucleotide 의 substitutions (22/24, 91.7%) 또는 multiple-site 의 substitutions (2/24, 8.3%)이 확인되었으며 deletion 이나 insertion 은 확인되지

않았으며, 항결핵제에 대해서 감수성인 균주는 *rpoC* 에서 변이가 확인되지 않았다. *pncA* 유전자의 경우는 46 주에서 다양한 변이가 확인되었으며 (nucleotide substitutions, deletions, insertion 그리고 multiple-site mutations) 25 개의 다른 변이가 확인이 되었다. Single nucleotide 의 substitution 이 가장 많았으며 (27/46, 58.7%), 그다음으로는 multiple-site mutation (4/46, 8.7%) 그리고 insertion (4/46, 8.7%) 순으로 많았다. 다양한 부위에서 insertion 또는 single 또는 multiple nucleotides 에서 deletion 에 의한 frameshifts 가 있었다 (7/46, 15.2%). 본 연구를 통해서 결핵균 (*M. tuberculosis*)의 *rpoB, rpoC* 그리고 *pncA* 유전자의 변이는 RFP 와 PZA 내성과 연관이 있는 것으로 확인되었으며 *rpoB, rpoC* 그리고 *pncA* 유전자의 변이를 확인하는 것은 RFP 와 PZA 약제에 대해서 내성을 가지는 다제약제내성결핵균을 신속분자진단법 개발에 중요한 자료로 사용이 될 것으로 생각된다.

감사의 글

10 년 전으로 기억합니다. 2007 년 대구가톨릭대학병원 신경외과에서 전임의를 시작하면서 대학에 남을 계획을 하고 석사를 시작한 후 2009 년에 석사학위를 받았습니다. 대학교수의 꿈을 접고 제주도에 내려오면서 크게 학위에 대한 욕심을 버렸었는데 2012 년 제주한라병원에서 신경외과가 전공의 수련병원으로 지정되면서 제자를 키우려면 박사학위가 있는 것이 나을 수 있다는 이상평 과장님의 조언을 받아 박사과정 및 학위를 받을 과정을 밟기로 하였습니다. 먼저 이 학위를 받을 수 있도록 시작점이 되어 주신 대구가톨릭대학병원 여형태 교수님, 최기환 교수님, 김종기 교수님께 감사의 말씀을 먼저 드립니다.

제가 수련의과정을 할 때나 한라병원신경외과에 제직을 하면서나, 지금 서귀포유신경외과로 개원해 있을 때나 한결같이 지켜봐 주시고 든든한 버팀목이 되어 주시는 이상평 과장님께 말로는 부족한 감사를 드립니다. 아울러 사모님이신 한라대학교 박신영 교수님께도 감사의 말씀을 드립니다. 대학원과정을 시작하면서부터 지도교수님이 되어 주시고 논문작성과정에 꼼꼼히 지도 편달해 주시고 박사과정 전반에 많은 도움을 주신 이근화 교수님께 늘 감사드립니다. 박사학위를 시작할 때부터 늘 힘이 되어준 사랑하는 저의 부인께도 감사드립니다. 저를 제주대학교와 인연을 맺게 해주시고 늘 격려해주신 김성엽 교수님, 이근화 교수님의 해외출장기간 동안 지도교수님으로 수고해 주신 이창섭 교수님께도 감사의 말씀을 전합니다. 교육과정을 논문작성과정에 여러 모로 도와주신 이범석 선생님, 정재훈 선생님께도 감사의 말씀을 드립니다. 실험실에 도움을 주신 양미해 선생님, 부상언 선생님께도 감사드립니다.

고등학교를 졸업하고 대구에서만 살다가 제주도에 내려 온지 벌써 만 9 년이 다 되어 가는데 크고 좋은 결실을 맺는 것 같습니다. 이 과정에서 여기에 언급하지 못했지만 많은 분들께 감사드립니다.

2017 년 유재철 드림