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Susceptibility of β-Lactam Antibiotics and Genetic Mutation of Drug-Resistant *Mycobacterium tuberculosis* Isolates in Korea

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Abstract

Background: *Mycobacterium tuberculosis* (Mtb) is resistant to the β-lactam antibiotics due to a non-classical transpeptidase in the cell wall with β-lactamase activity. A recent study showed that meropenem combined with clavulanate, a β-lactamase inhibitor, was effective in multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB). However, in Korea, clavulanate can only be used as drugs containing amoxicillin. In this study, we investigated the susceptibility and genetic mutations of drug-resistant Mtb isolates to amoxicillin-clavulanate and meropenem-clavulanate to improve the diagnosis and treatment of drug-resistant TB patients.

Methods: The minimum inhibitory concentration (MIC) of amoxicillin-clavulanate and meropenem-clavulanate was examined by resazurin microtiter assay. We used 82 MDR and 40 XDR strains isolated in Korea and two reference laboratory strains. Mutations of drug targets *blaC*, *blaI*, *ldtA*, *ldtB*, *dacB2*, and *crfA* were analyzed by polymerase chain reaction and DNA sequencing.

Results: The MIC_{90} values of amoxicillin/clavulanate and meropenem/clavulanate in drug-resistant Mtb isolates were 64/2.5 and 16/2.5 mg/L, respectively. Gene mutations related to amoxicillin/clavulanate and meropenem/clavulanate resistance could not be identified, but T448G mutation was found in the *blaC* gene related to β-lactam antibiotics' high susceptibility.

Conclusion: Our results provide clinical consideration of β-lactams in treating drug-resistant TB and potential molecular markers of amoxicillin-clavulanate and meropenem-clavulanate susceptibility.

Keywords: Susceptibility; Amoxicillin; Clavulanate; Meropenem; Mutation; Resistance; Multidrug-Resistant; Extensively Drug-Resistant; Mycobacterium; Tuberculosis

Introduction

Emergence of multi-drug resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) is a public health problem of TB. Treatment of MDR-TB requires a more extended treatment period, higher cost, and more drug adverse events than drug-sensitive TB[1]. Of the two, XDR-TB is more difficult to treat and has a relatively high mortality rate than MDR-TB[2].

The β-lactamase of *Mycobacterium tuberculosis*

(Mtb) rapidly hydrolyzes β-lactam rings in amoxicillin and carbapenem and therefore they are combined with clavulanate, a β-lactam inhibitor, to exert anti-tuberculosis effects. Among the β-lactam antibiotics, meropenem, a carbapenem, in combination with clavulanate showed promising results in treating drug-resistant TB with low rates of adverse events[3,4]. However, in Korea, clavulanate can only be used as drugs containing amoxicillin.

A critical concentration and breakpoint for amoxicillin/clavulanate and meropenem/clavulanate susceptibility testing for Mtb have not been defined by the World Health Organization (WHO)[5], the Clinical & Laboratory Standards Institute (Wayne, PA, USA)[6], and European Committee on Antimicrobial Susceptibility Testing (EUCAST)[7]. However, amoxicillin/clavulanate and meropenem/clavulanate have not been tested for drug susceptibility before being prescript to patients with MDR and XDR-TB in Korea. Consequently, there is no information on the susceptibility distribution of amoxicillin/clavulanate and meropenem/clavulanate against MDR and XDR-TB isolates in Korea.

In this study, we investigated the susceptibility and genetic mutations of drug-resistant Mtb isolates to amoxicillin/clavulanate and meropenem/clavulanate to improve the diagnosis and treatment of drug-resistant TB patients.

Materials and Methods

1. Reference strains and clinical isolates of Mtb

The Mtb H37Rv (ATCC 25618) and K-strain (NCCP 15986) were used as references in the drug susceptibility test. The 82 MDR, and 40 XDR isolates were supplied by the Tuberculosis Specimen Bank of Masan National Hospital. The clinical isolates were collected from November 2009 to January 2016. The isolates used in this study did not have drug susceptibility test results for bedaquiline or linezolid, so the definition of XDR before 2021 was used for drug resistance criteria. The MDR was defined as resistant to both isoniazid and rifampin. The XDR was defined as MDR plus resistance to any fluoroquinolone and any second-line injectable drug (capreomycin, kanamycin, and amikacin)[8]. The clinical isolates of Mtb, Middlebrook 7H9 Broth (BBL MGIT Mycobacteria Growth Indicator Tube, Becton-Dickinson, Sparks, MD, USA), and Ogawa II agar (Asanpharm, Seoul, Korea) were sequentially cultured and used. The bacterial colonies in the log phase were taken and applied to determine the minimum inhibitory concentration (MIC), polymerase chain reaction (PCR), and DNA sequencing.

2. Determination of MIC

The MIC of the TB strains was measured using the resazurin microtiter assay[9]. Amoxicillin and meropenem (Sigma-Aldrich, St. Louis, MO, USA) were added to 7H9-S broth with final concentrations ranging from 0.031 to 128 mg/L in 96-well plates. Potassium clavulanate (Sigma-Aldrich) was added to 7H9-S broth with a final concentration of 2.5 mg/L in each well. Growth controls containing no antibiotic and sterility controls without inoculation were also included. The inoculum was adjusted to McFarland 1.0 with DensiCHEK plus instrument (bioMereux, Craponne, France) from fresh colonies on Ogawa II agar, and further diluted at a ratio of 1:10 in 7H9-S broth. The MIC was determined as the lowest concentration where no color change occurred by culturing Mtb in 7H9-S broth containing various concentrations of Amoxicillin and meropenem for up to 9 days, then adding resazurin (Fisher Scientific, Waltham, MA, USA) and culturing for up to 48 hours. The Mtb H37Rv (ATCC 25618) and Mtb K-strain (NCCP 15986) were used as references in the drug susceptibility test. Drug susceptibility tests for the isolates that showed MIC >32/2.5 mg/L in to amoxicillin/clavulanate or meropenem/clavulanate, were repeated twice.

3. PCR and DNA sequencing

Mtb DNA was extracted using a commercial kit, DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany) for PCR of the β-lactam resistance and susceptibility-related genes. PCR amplification of the genes was performed using Maxime PCR premix i-StarTaq (iNtRON, Seongnam, Korea). To identify genes related to high amoxicillin and meropenem susceptibility, seven strains with MIC ≤2 mg/L were selected, and DNA was extracted. Five strains with amoxicillin MIC >32 mg/L or meropenem MIC >16 mg/L were selected, and DNA was extracted to identify genes related to high β-lactam antibiotics resistance. Drug resistance data of the clinical Mtb isolates selected for PCR, and DNA sequencing are shown in Table 1.

The *blaC*, *blaI*, *ldtA*, *ldtB*, *dacB2*, and *crfA* genes were amplified by PCR. Genomic DNA from Mtb isolates and reference strains were subjected to PCR amplification. The primer sets for amplification and sequencing of the β-lactam resistance and susceptibility-related genes are shown in Table 2. The PCR amplification products of each gene were performed using Sanger sequencing with the same primers by Bioneer (Daejeon, Korea). To confirm the mutation site of the genes, each Mtb strain was compared and analyzed using Mega 10.2.5 software[10].

Table 1. Drug resistance data of clinical Mtb isolates to identify genes related to resistance and susceptibility to β-lactam antibiotics

Isolates No.	Type	Drug resistance profile	Year of isolation	MIC (mg/L) with 2.5 mg/L clavulanate	
				Amoxicillin	Meropenem
21873	MDR	INH, RFP, EMB, RBU, PTO	2014	1	1
22549	MDR	INH, RFP, RBU, OFX, LEV, MFX, PTO, CS	2015	2	2
25046	MDR	INH, RFP, EMB, RBU, SM, OFX, LEV, MFX, PAS, PTO, CS	2015	2	2
6631	XDR	INH, RFP, EMB, RBU, SM, KM, AMK, OFX, PAS, PTO, CS	2010	2	2
8002	XDR	INH, RFP, EMB, RBU, SM, KM, AMK, OFX, MFX, PAS, PTO, CS	2010	2	2
13673	XDR	INH, RFP, EMB, RBU, SM, KM, OFX, MFX, PTO	2011	1	1
16081	XDR	INH, RFP, RBU, SM, KM, AMK, OFX, PAS, PTO, CS	2012	2	2
22009	MDR	INH, RFP, EMB, SM, PAS, PTO, CS	2014	128	64
23920	MDR	INH, RFP, EMB, RBU, KM, PAS, PTO, CS	2015	64	32
24605	MDR	INH, RFP, EMB, PTO	2015	128	64
24649	MDR	INH, RFP, EMB, PTO	2015	128	64
23425	XDR	INH, RFP, EMB, PZA, KM, AMK, CPM, OFX, PAS, PTO, CS	2015	>128	32

Mtb: *Mycobacterium tuberculosis*; MIC: minimum inhibitory concentration; MDR: multidrug-resistant; INH: isoniazid; RFP: rifampicin; EMB: ethambutol; RBU: rifabutin; PTO; protionamide; OFX: ofloxacin; LEV: levofloxacin; MFX: moxifloxacin; CS: cycloserine; SM: streptomycin; XDR: extensively drug-resistant; KM: kanamycin; AMK: amikacin; PAS: para-aminosalicylic acid; PZA: pyrazinamide; CPM: capreomycin.

Table 2. Primers used for amplification and sequencing of the β-lactam resistance and susceptibility-related genes

Primer	Sequence (5'-3')	Product length (bp)	Reference
BlaCF	ATGCGCAACAGAGGATTCGGTC	924	Li et al.[11]
BlaCR	CTATGCAAGCACACCGGCAACG		
crfAF	ACCCGGCTCACAGAGAATCG	457	
crfAR	TATCACCGGTAGGCCATGC		
dacB2F	ACCAGCAACTGCTGGATTTC	1,196	
dacB2R	CGTTGATGACCAACGTCTTC		
LdtBF	ATGCCAAAGGTGGGGATTGC	1,227	
LdtBR	TTACGCCTTGGCGTTACCGGC		
LdtAF	ATGCGTCGAGTGGTTCGTTATC	756	
LdtAR	CTAGCCGACCACCTCAATGG		
BlaIF	ATGGCCAAGCTGACACGG	417	In this study
BlaIR	TCAAGTCTCCGTTGCCGC		

4. Statistical analysis

The susceptibility rates of Mtb isolates to amoxicillin and meropenem with clavulanate were analyzed using the Chi-square test. A cross-tabulation analysis was used to assess meropenem and amoxicillin susceptibility between MDR and XDR isolates. We used the nonparametric test because the MICs were recorded as discrete ordinal values and were not continuous. Fisher's exact test and Paired sample t-test were used to compare the susceptible rates of MDR and XDR isolates to meropenem and amoxicillin with clavulanate. A p-value of <0.05 was considered statistically significant.

5. Ethics

This study protocol was approved by the Institutional Biosafety Committee (MTHIBC-18-11) and Institutional Review Board (IRB-398837-2018-E-26) of the Masan National Tuberculosis Hospital. The clinical information was retrieved by the medical staff from electronic medical records, to analyze the relationship between the treatment history and progress in patients with β-lactam resistance.

Results

1. MIC to Mtb

Amoxicillin/clavulanate MIC values of H37Rv were from 32 to 64/2.5 mg/L, and K-strain was from 64 to 128/2.5 mg/L. Meropenem/clavulanate MIC values of H37Rv were from 8 to 16/2.5 mg/L, and K-strain was from 16 to 64/2.5 mg/L. The WHO does not define a critical concentration and breakpoint for amoxicillin/clavulanate and meropenem/clavulanate susceptibility testing for Mtb. Based on a previously described study[11], the tentative susceptible criterion of amoxicillin/clavulanate and meropenem/clavulanate was ≤4/2.5 mg/L and ≤8/2.5 mg/L, respectively. The susceptibilities of drug-resistant isolates to different concentrations of amoxicillin and meropenem with clavulanate are shown in Table 3. Clinical Mtb isolates to 4 mg amoxicillin and 8 mg meropenem with potassium clavulanate at 2.5 mg/L, showed 24.6% (30/122) and 94.3% (115/122), respectively. Among these, MDR and XDR strains were 28.0% (23/82) and 17.5% (7/40) susceptible to amoxicillin-clavulanate, respectively. The susceptibility rates of clinical Mtb isolates were increased on two additional concentrations of amoxicillin and meropenem. MDR and XDR strains were 85.4% (70/82) and 87.5% (35/40) susceptible to meropenem-clavulanate, respectively. The amoxicillin-clavulanate susceptibilities were higher in MDR than XDR, but the difference was not statistically significant (p=0.38). The meropenem-clavulanate susceptibilities were higher in XDR than MDR, but the difference was not statistically significant (p=0.12).

The MICs of the amoxicillin and meropenem with clavulanate for MDR and XDR Mtb isolates are shown in Figure 1. MIC_{50} and MIC_{90} values of amoxicillin/clavulanate, which mean 50% and more than 90% inhibition of the target Mtb, were 16/2.5 mg/L and 64/2.5 mg/L, respectively. The MIC_{50} and MIC_{90} values of meropenem/clavulanate were 8/2.5 mg/L and 16/2.5 mg/L, respectively. The MIC_{50} and MIC_{90} values of amoxicillin/clavulanate were higher than those of the meropenem/clavulanate (p=0.001). XDR-TB is more difficult to treat than MDR-TB. Therefore, we included XDR Mtb, since *in vitro* susceptibility to amoxicillin/clavulanate and meropenem/clavulanate may provide effective treatment options for patients with XDR-TB. The MIC_{50} and MIC_{90} values of amoxicillin/clavulanate, which mean 50% and more than 90% inhibition of the target Mtb, for MDR were 8/2.5 mg/L and 64/2.5 mg/L, respectively. The MIC_{50} and MIC_{90} values of amoxicillin/clavulanate for XDR were 16/2.5 mg/L and 32/2.5 mg/L, respectively. The MIC_{50} values of amoxicillin/clavulanate were higher in XDR than MDR (p<0.001), and The MIC_{90} values of amoxicillin/clavulanate were higher in MDR than XDR (p<0.001). The MIC_{50} and MIC_{90} values of meropenem/clavulanate for MDR were 4/2.5 mg/L and 16/2.5 mg/L, respectively. The MIC_{50} and MIC_{90} values of meropenem/clavulanate

Table 3. Susceptibilities of MDR and XDR-TB isolates to different concentrations of amoxicillin and meropenem with clavulanate (2.5 mg/mL)

Critical concentration (mg/L)	No. of susceptible isolates (susceptible percentage)		
	MDR (n=82)	XDR (n=40)	Total (n=122)
Amoxicillin			
4.0	23 (28.0)	7 (17.5)	30 (24.6)
8.0	42 (51.2)	18 (45.0)	60 (49.2)
16.0	61 (74.4)	28 (70.0)	89 (73.0)
Meropenem			
4.0	32 (39.0)	22 (55.0)	54 (44.3)
8.0	70 (85.4)	35 (87.5)	115 (94.3)
16.0	78 (95.1)	39 (97.5)	117 (96.0)

Values are presented as number (%).
MDR: multidrug-resistant; XDR: extensively drug-resistant; TB: tuberculosis.

Figure 1. Distribution of the MIC (mg/L) of amoxicillin (A) and meropenem (B) with clavulanate for MDR and XDR *Mycobacterium tuberculosis* isolates. MIC: minimum inhibitory concentration; MDR: multidrug-resistant; XDR: extensively drug-resistant.

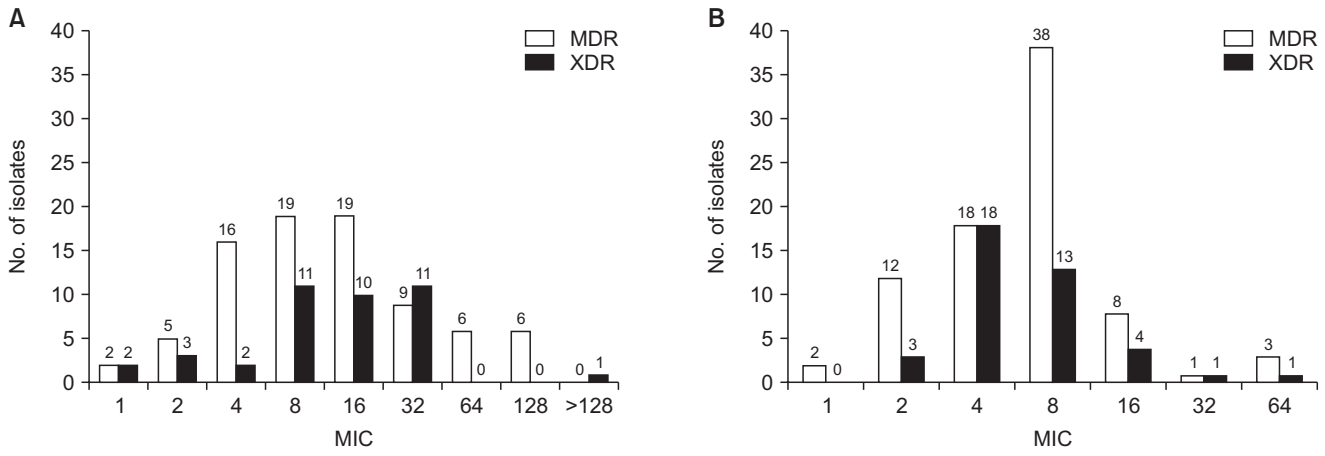

Table 4. The isolates and treatment history of the patients

Patient No.	Isolates No.	Type	Treatment regimen	Duration (mo)
1	23425	XDR	PZA, CS, CFZ, LZD/PZA, CS, LZD	6/18
2	24605, 24649	MDR	PZA, PTO, CS, MFX, KM/PZA, PTO, CS, MFX	8/16
3	22009	MDR	PZA, PTO, CS, MFX, KM/PZA, PTO, CS, MFX	8/16
4	23920	MDR	PZA, PTO, MFX, CS, KM/PZA, MFX, AMX-C, MER, AMK/DEL, MFX, PTO, CLA, AMX-C, MER, AMK	5/11/1

XDR: extensively drug-resistant; PZA: pyrazinamide; CS: cycloserine; CFZ: clofazimine; LZD: linezolid; MDR: multidrug-resistant; PTO: protionamide; MFX: moxifloxacin; KM: kanamycin; AMX-C: amoxicillin-clavulanate; MER: meropenem; AMK: amikacin; DEL: delamanid; CLA: clarithromycin.

for XDR were 8/2.5 mg/L and 16/2.5 mg/L, respectively. The MIC_{50} values of meropenem/clavulanate were higher in XDR than MDR ($p<0.001$).

2. Clinical data analysis of β-lactam resistant isolates

The treatment history of the patients and bacterial culture test of isolates are shown in Table 4. The five strains confirmed to be highly resistant to amoxicillin MIC >32 mg/L or meropenem MIC >16 mg/L, were isolated from four patients. Only a patient from whom 23920 strain was isolated, had a history of treatment with amoxicillin-clavulanate for 12 months during inpatient treatment. However, the isolate was cultured from the patient before administering amoxicillin-clavulanate and meropenem. The other patients had no history of treatment with amoxicillin-clavulanate and meropenem. The relationship between drug discontinuation and resistance was low in the β-lactam resistant isolates.

3. Relationship between drug target mutation and resistance and susceptibility

The relationship between mutations in the β-lactam target genes and the drug susceptibility and resistance was investigated. A total of 12 Mtb isolates with *blaC*, *blaI*, *crfA*, *ldtA*, *ldtB*, and *dacB2* genes were sequenced and analyzed. Gene mutations related to amoxicillin and meropenem resistance could not be identified in five resistant Mtb isolates.

The G514A a sensitivity-related mutation in the *blaC* gene previously reported concerning β-lactam drug-susceptibility[11], could not identify the same positional mutation in the seven susceptible Mtb isolates in this study. However, the T448G common mutation of the *blaC* gene was identified in susceptible Mtb isolates, as shown in Figure 2. The T448G mutation of the *blaC* was observed in four isolates among the seven β-lactam susceptible Mtb isolates. The mutation was detected with a higher frequency in XDR than in MDR among the β-lactam drug-susceptible Mtb isolates.

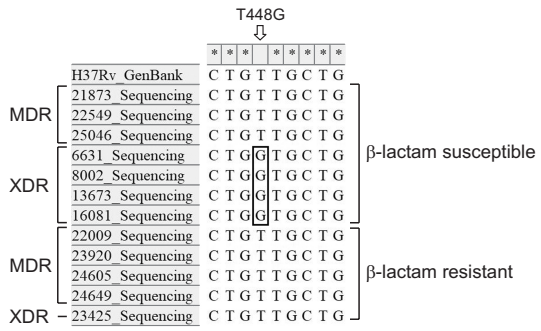

Figure 2. The *blaC* gene mutation site in β-lactam susceptible and resistant isolates. The mutation site of single nucleotide variants is shown with white arrows and squares. MDR: multidrug-resistant; XDR: extensively drug-resistant.

Discussion

In our results, amoxicillin-clavulanate and meropenem-clavulanate of H37Rv were 2 to 4 times higher than those of each drug compared to previous research reports[12]. The clavulanate concentration of the previous MIC investing studies ranged from 2.5 mg to 10 mg[11-13]. As the clavulanate concentration increased, the MICs of amoxicillin and meropenem gradually decreased using the H37Rv strain. This difference in the reference strains' MIC of amoxicillin-clavulanate and meropenem-clavulanate could be due to the difference in susceptibility test methods and characteristics of the reference strains. Therefore, the drug susceptibility test should be standardized.

Amoxicillin-clavulanate showed variable effectiveness against Mtb in the previous susceptibility studies[3,11,13]. The *C*max and AUC values of amoxicillin were 11.2 mg/L and 30.1 mg hr/L, respectively, after a single oral dose of 1,000 mg of AUGMENTIN, an amoxicillin-clavulanate combination[14]. The *C*max and AUC values of clavulanate in serum were 2.6 mg/L and 4.6 mg hr/L, respectively, after a single oral dose of 1,000 mg of AUGMENTIN. Our data, documenting an MIC_{90} of 32 mg/L of amoxicillin, suggests that this dosage of amoxicillin-clavulanate will not be effective against drug-resistant TB.

Meropenem has shown good efficacy against drug-resistant isolates of Mtb in the previous studies[3,13,15]. Meropenem with clavulanate exhibited anti-Mtb activity with MIC_{50} and MIC_{90} of 2 mg/L and 8 mg/L, respectively[11]. The *C*max and AUC values of meropenem were 26 mg/L and 27.2–32.4 mg hr/L, respectively, with an intravenous infusion of 500 mg of meropenem[16]. Our meropenem-clavulanate results with an MIC_{90} of 16 mg/L are comparable to previous investigations which showed potent activity against drug-resistant Mtb.

The drug-resistant Mtb were 58% and 94% susceptible to amoxicillin-clavulanate and meropenem-clavulanate, respectively[12]. A specific clade LAM4 type of XDR strains from South Africa exhibited high susceptibility to certain β-lactam antibiotics. The resistance criterion of amoxicillin and meropenem with clavulanate (2.5 mg/L) was MIC >4 mg/L and MIC >12 mg/L, respectively. Our drug susceptibility rate data of amoxicillin-clavulanate and meropenem-clavulanate, as in the same resistance criteria, were 60/122 (49.2%) and 115/122 (94.3%), respectively. The lower susceptibility rates of amoxicillin could be due to the different clade types from other areas, so it is necessary to conduct a drug susceptibility test before treating drug-resistant patients in Korea with β-lactam antibiotics.

The relationship between drug discontinuation and resistance was low in this study. The cause of the resistance was not related to treatment with amoxicillin and meropenem. It is expected that the main mechanism of acquired resistance to drugs such as amoxicillin and meropenem is infection by bacteria that have already developed drug resistance rather than acquiring resistance to Mtb through discontinuation of related medications. It is necessary to analyze a higher number of drug-resistant Mtb and the gene that changes during drug treatment, for more accurate results.

The study on the mechanism of Mtb resistance to β-lactam antibiotic-clavulanate focused on β-lactamases expressed by *blaC* gene. Structural changes of β-lactamases due to gene mutation may cause drug resistance by interfering with the action of clavulanate, a β-lactamase inhibitor. Through structural analysis of the β-lactamase proteome, N132, R164, R244, R276, R220, A244, S130, and T237 sites were predicted to be related to drug resistance[17,18]. It was reported that the mutation sites of the *blaC* gene T333G and G514A are related to sensitivity rather than resistance[11,19]. The β-lactam antibiotics inhibit cell wall synthesis by binding to transpeptidases that catalyze peptidoglycan crosslinking. An alternative form of peptidoglycan cross-linking, called L,D-transpeptidation, is predominant in Mtb[20]. The transpeptidase encoding related genes, *ldtA*, *ldtB*, *dacB2*, *crfA*, and *blaI* were analyzed to determine the relationship between drug target mutations and β-lactam resistance and susceptibility. We could not observe the exact position of these gene mutations of previous studies in the β-lactam resistance and susceptibility isolates in this study. However, common mutations of the *blaC* gene T448G were observed in β-lactam high susceptible isolates. The mutation T448G may affect the interaction of *blaC* and β-lactams

and the amino change Leu150Val leads to a conformation change in *blaC*, which influences its activity.

The T448G mutation was detected with a higher frequency in XDR than in MDR among the β-lactam drug-susceptible Mtb isolates. This is considered to have important clinical implications on the treatment of XDR TB, limited to the drug of choice. Furthermore, it is expected that the gene mutations related to drug susceptibility, such as β-lactam antibiotics, revealed through this study can be used as a marker for rapid diagnosis of TB drug susceptibility in the future.

Authors' Contributions

Conceptualization: Park S. Methodology: Jung J, Park S. Formal analysis: Kim J, Han SB, Park S. Writing - original draft preparation: Park S. Writing - review and editing: Park S, Ryoo S. Approval of final manuscript: all authors.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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