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국내에서 *katG*, *inhA*, *rpoB* 유전자 염기서열분석을 통한 아이소니아지드 및 리팜피신 내성의 검출

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Detection of Isoniazid and Rifampicin Resistance by Sequencing of *katG*, *inhA*, and *rpoB* Genes in Korea

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Background : In Korea, tuberculosis is resistant to isoniazid (INH) and/or rifampicin (RIF) in more than 10% of cases. To prevent the spread of resistant *Mycobacterium tuberculosis* strains, it is crucial to develop more rapid resistance detection methods.

Methods : To determine the feasibility of using direct sequencing for detecting INH- and RIF-resistant strains, the *katG* gene, the regulatory region of the *inhA* gene, and the 81-bp hot-spot region of the *rpoB* gene from 95 culture isolates and 46 respiratory specimens were sequenced. Total 141 culture isolates were classified by conventional drug susceptibility testing (DST) as INH^R/RIF^R (N=30), INH^R/RIF^S (N=23), INH^S/RIF^R (N=15), and INH^S/RIF^S (N=73).

Results : Compared with phenotypic DST, the overall sensitivity and specificity of sequencing were 83.0% (44/53) and 96.6% (85/88), respectively, for INH resistance, and 93.3% (42/45) and 100% (96/96), respectively, for RIF resistance. The rates were similar between culture isolates and respiratory specimens. Interestingly, three specimens with *inhA* -15C>T mutation were susceptible to INH by conventional DST.

Conclusions : Detection of mutations in the *katG* codon 315, the *inhA* regulatory region, and the hot-spot region of *rpoB* would be useful for rapid detection of INH and RIF resistance in Korea. *(Korean J Lab Med 2009;29:455-60)*

Key Words : *Mycobacterium tuberculosis, inhA, katG, rpoB*

INTRODUCTION

Isoniazid (INH) and rifampicin (RIF) are the most important first-line antituberculosis drugs, and development of resistance to these drugs results in high rates of treatment failure and death [1]. In a 2004 survey, the Tuberculosis Laboratory Network of the Korean National Tuberculosis Association reported that, of the 2,636 new smear-positive patients, 12.8% were resistant to at least one of the first-line drugs, with 9.9% resistant to INH, 3.7% resistant to RIF, and 2.7% resistant to both (multidrug resistant *Mycobacterium tuberculosis*, MDR-TB) [2]. Rapid detection of resistance

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to these drugs is important for effective disease management and for prevention of further spread of drug resistant strains.

Conventional laboratory testing of drug resistance is a multi-step process that includes the isolation of strains, culture growth, and drug susceptibility testing (DST), a process that can require more than 2-3 months. More rapid results could be obtained by direct molecular testing of respiratory specimens. INH resistance is mainly caused by mutations in the *katG* gene, which occur most frequently at codon 315, and in the regulatory region of the *inhA* gene. RIF resistance is mostly caused by mutations in an 81-bp hot-spot region of the *rpoB* gene. The type and frequency of these mutations vary across countries [3-5]. The molecular methods used for rapid detection of INH and RIF resistance are reverse membrane hybridization, line probe assay, DNA microarrays, and PCR-single nucleotide conformation polymorphism (SSCP) [6-9]. However, direct sequencing is required to confirm the presence of a mutation [3].

To determine the feasibility of using direct sequencing for detecting INH- and RIF-resistant strains, we sequenced the *katG* gene, the regulatory region of the *inhA* gene, and the 81-bp hot-spot region of the *rpoB* gene in 95 culture isolates and 46 respiratory specimens. The results of the molecular method were compared with those of the conventional DST method.

MATERIALS AND METHODS

1. Materials

A total of 141 samples that had been referred to the Green-cross Reference Laboratory for DST between April 2007 and May 2008 were selected. Among them, 46 respiratory specimens, which were confirmed to be TB positive by the COBAS Amplicor system (Roche Diagnostic Systems, Branchburg, NJ, USA), were randomly obtained, and 95 culture isolates were selected on the basis of the conventional DST result (23 INH^R/RIF^R, 15 INH^R/RIF^S, 10 INH^S/RIF^R, and 47 INH^S/RIF^S). Of the 46 respiratory specimens, 31 were sputum specimens and 15 were bronchial or bronchoalveolar wash specimens.

2. Methods

1) DNA extraction and TB identification

For DNA extraction, 1 N liquefied NaOH, decontaminated and concentrated human respiratory specimens, and culture isolates in 400 μL of distilled water were processed using the Roche COBAS Amplicor extraction kit (Roche Diagnostic Systems, Branchburg, NJ, USA). TB detection was performed using the COBAS Amplicor Mycobacterium PCR system according to the manufacturer's instructions. All the specimens were confirmed as positive for TB by the COBAS Am-

Table 1. Oligonucleotide primers used in this study

Target gene*	Primer set (direction)	Nucleotide sequence (5′-3′)	Nucleotide positions†	Product size (bp)
rpoB	1st forward	AGGACGTGGAGGCGATCA	1,445-1,462	245
	1st reverse	GGTTTCGATCGGGCACAT	1672-1,689	
	2nd forward	ACCGCAGACGTTGATCAACAT	1,464-1,484	166‡
	2nd reverse	GGCACGCTCACGTGACAG	1,612-1,629	
katG	1st forward	TGGCCGCGGCGGTCGACATT	725-744	420‡
	1st reverse	GGTCAGTGGCCAGCATCGTC	1,125-1,144	
	2nd forward	GGTGTTCGTCCATACGACCT	768-787	205
	2nd reverse	CATGAACGACGTCGAAACAG	953-972	
inhA regulatory region	1st forward	CCTCGCTGCCCAGAAAGGGA	-168 to -149	249‡
	1st reverse	ATCCCCCGGTTTCCTCCGGT	61-81	
	2nd forward	GTCACACCGACAAACGTCAC	-124 to -105	190
	2nd reverse	TCCGGTAACCAGGACTGAAC	47-66	

*The complete sequences of target genes in *M. tuberculosis* H37Rv are reported in the GenBank database under accession number NC_000962; †Numbering based on nucleotide position relative to the initiation codon of each gene; ‡Primers that were reported in other studies.

plicor PCR systems.

2) Genotypic characterization

All 95 culture isolates and 45 clinical specimens were sequenced. The 205-bp long *katG* gene fragment; a 190-bp fragment, including the regulatory region, of the *inhA* gene; and the 166-bp long *rpoB* gene fragment were amplified by nested PCR using the DNA extracted from respiratory specimens. Previously reported primers [10] and designed primers were used (Table 1). Each standard PCR mixture (50 μL) contained 2 U *Taq* polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, 0.4 μM each forward and reverse primers, 200 μM each dNTP, and 5 μL DNA template. PCR was performed using an ABI 9600 thermocycler (Applied Biosystems, Foster city, CA, USA) with the following cycle conditions: initial denaturation at 94℃ for 10 min, followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ for 40 sec, and extension at 72℃ for 60 sec, with a final extension at 72℃ for 10 min. The PCR products were electrophoresed on 2.0% agarose gels, followed by staining with ethidium bromide. PCR products were purified using a Qiaquick purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and sequenced using an ABI 3130xl automated DNA sequencer (Applied Biosystems, Foster city, CA, USA).

For culture isolates, the extracted DNA was amplified by one round of PCR, and the products were sequenced as described above.

3) Conventional DST

For conventional DST, 46 clinical specimens were cultured on Löwenstein-Jensen solid medium. In all, 141 TB isolates, including the culture isolates from respiratory specimens, were tested. DST was performed as previously described, using 0.2 μg/mL INH, 40 μg/mL RIF, 10 μg/mL streptomycin, 2 μg/mL ethambutol, 40 μg/mL kanamycin, 40 μg/mL amikacin, 40 μg/mL ethionamide, 30 μg/mL cycloserine, 1 μg/mL para-aminosalicylic acid, and 2 μg/mL ofloxacin [11]. Resistance to each drug was defined as 1% bacterial growth on Löwenstein-Jensen media using the proportion method.

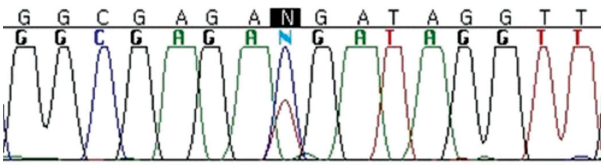

Fig. 1. A mixed pattern of the *inhA*-15C and T mutation.

RESULTS

Conventional DST of the 141 isolates showed that 30 (21.2%) were INH^R/RIF^R, 23 (16.3%) were INH^R/RIF^S, 15 (10.6%) were INH^S/RIF^R, and 73 (51.8%) were INH^S/RIF^S.

1. Results of INH resistance

Sequencing of the 53 INH-resistant isolates classified by conventional DST showed that 44 isolates (83.0%) contained resistance-associated mutations in the *katG* gene and/or the *inhA* regulatory region. Mutations in codon 315 of the *katG* gene were most frequently observed, with 30 of the 53 (56.6%) INH-resistant isolates showing mutations in this region. Of the 53 INH-resistant isolates, 17 (32.1%) had mutations in the *inhA* regulatory region with 16 having the -15C>T mutation. Of the 16 samples with *inhA* C>T mutation, 3 had a mixed peak of *inhA* -15C and T (Fig. 1). Further, three samples (5.7%) had both the mutations, i.e., *katG* Ser315Thr and *inhA* -15C>T (Table 2). Sequencing of the 88 INH-susceptible isolates classified by conventional DST showed that 85 isolates had no mutations. However, three of the 88 INH-sus-

Table 2. *inhA* and *katG* genotypic analysis of 95 culture isolates and 46 respiratory specimens by conventional drug susceptibility testing (DST) of isoniazid (INH)

INH result by conventional DST	Genotype	Number (%)
Resistant	Total	53 (100)
	katG Ser315Thr	26 (49.1)
	katG Ser315Gly	1 (1.9)
	inhA -15C>T	13 (24.5)
	inhA -8T>A	1 (1.9)
	katG Ser315Thr and *inhA* -15C>T	3 (5.7)
	Wild type	9 (17.0)
Susceptible	Total	88 (100)
	inhA -15C>T	3 (3.4)
	Wild type	85 (96.6)

Table 3. *rpoB* genotypic analysis of 95 culture isolates and 46 respiratory specimens by conventional drug susceptibility testing (DST) of rifampicin (RIF)

RIF result by conventional DST	Genotype	Number (%)
Resistant	Total	45 (100)
	S531L	29 (64.4)
	H526Y	4 (8.9)
	H526N	1 (2.2)
	Q513L	2 (4.4)
	Q513P	2 (4.4)
	D516Y	2 (4.4)
	D516N	1 (2.2)
	L533P	1 (2.2)
	Wild type	3 (6.6)
Susceptible	Wild type	96 (100)

ceptible isolates had the *inhA* −15C>T mutant peaks; two sputum specimens had a mixed peak of *inhA* −15C and T and one isolate from the culture showed only the mutant peak.

2. Results of RIF resistance

Sequencing of the 45 RIF-resistant isolates classified by conventional DST showed that 42 (93.3%) had mutations in the 81-bp hot-spot region of the *rpoB* gene. The most common mutation was the Ser531Leu mutation, which was present in 29 of the 45 (64.4%) isolates, followed by the His-526Tyr mutation (Table 3). Sequencing of the 96 RIF-susceptible isolates classified by conventional DST showed that none had mutations in the 81-bp hot-spot region of the *rpoB* gene.

3. Performance of MDR-TB detection

Sequencing of the 30 MDR isolates classified by conventional DST showed that 25 (83.3%) isolates had mutations in *katG* or *inhA* or both, and 28 (93.3%) had mutation in the *rpoB* gene. Surprisingly, two of the MDR samples contained no mutations in the three tested genes.

4. Sensitivity and specificity by specimen sources

Compared with phenotypic DST, the overall sensitivity and specificity of sequencing were 83.0% and 96.6%, respectively, for INH resistance, and 93.3% and 100%, respectively, for RIF resistance. The rates were similar between culture isolates and respiratory specimens (Table 4).

Table 4. Sensitivity and specificity of sequencing for the detection of resistance in culture isolates and respiratory specimens

	N of positive strains/N of strains tested (%)			
	Sensitivity		Specificity	
	Culture isolates	Respiratory specimens	Culture isolates	Respiratory specimens
Isoniazid	32/39 (82.0%)	12/14 (85.7%)	55/56 (98.2%)	30/32 (93.8%)
Rifampicin	31/33 (93.9%)	11/12 (91.7%)	60/60 (100%)	34/34 (100%)

DISCUSSION

MDR-TB has become a serious public health problem worldwide. For example, between 2002 and 2004, 3.9% of the newly diagnosed patients and 27.2% of the previously treated patients at a private referral center in South Korea had MDR-TB [12]. It is difficult to treat MDR-TB because second-line drugs are not as potent or as well tolerated as first-line drugs. Thus, to prevent the spread of MDR-TB in Korea, it is essential to develop a rapid drug resistance test. In Korea, Joh et al. [13] reported that the median interval from the initiation of tuberculosis treatment to obtaining mycobacterial culture results was 37 days, and the median interval from the initiation of treatment to the confirmation of the DST result was 80.5 days. Therefore, the direct testing for resistance in respiratory samples could advance the initiation of appropriate therapy by about 3 months compared to the time required for treatment initiation at present, thereby preventing the spread of MDR-TB.

Sequencing of nested PCR products was successful in all the tested respiratory specimens positive for TB. The sensitivity and specificity of this method were similar for culture isolates and clinical specimens. Recently, commercial kits employing reverse hybridization methods are also available for testing clinical specimens as well as culture isolates [14]. However, to apply the reverse hybridization method, it is essential to have the information about the mutation pattern of the genes tested in this method. In addition, re-

verse hybridization method may lead to a false result because of the sequence variations in the genomic regions, from which primers and probes are selected. Therefore, sequencing remains the most reliable method for the detection of both known and novel mutations. However, sequencing is relatively expensive and involves a rather complicated procedure for routine use at clinical laboratories.

Although the number of strains analyzed was comparatively small, the findings of this study are similar to those of other studies conducted in Korea [15, 16]. For example, the results of a study that used the reverse hybridization method showed that, of the INH-resistant strains, 65.1% had the *katG* Ser315Thr mutation and 19.3% of had mutations in the regulatory region of the *inhA* gene, while 92.4% of the RIF-resistant strains had an *rpoB* mutation [16]. Molecular diagnostic tests that are designed to target these mutations could be useful for the rapid detection of INH- and RIF-resistant strains in Korea. In this study, sequencing of the 81-bp hot-spot region of the *rpoB* gene led to the identification of 28 of the 30 MDR-TB strains.

Five respiratory specimens with the *inhA* -15C>T mutation showed a mixed peak of *inhA* -15C and T, as revealed by sequencing, which might be because of the presence of a mixed population of INH-resistant and INH-susceptible strains. Conventional DST showed that two of these isolates were INH resistant and three were INH susceptible. In addition, three isolates with *inhA* -15C>T mutation were detected, which were found to be susceptible to INH by conventional DST. A comparative study on the MGIT 960 antimicrobial susceptibility test (AST) and the proportion method using Ogawa medium, which was performed using 1,112 clinical TB isolates from Japan, identified 30 strains that showed discrepant results between the two methods for testing INH resistance [17]. This study results revealed that the strains with low-level INH resistance may show resistance by the MGIT 960 AST, while they may show susceptibility to INH by the method using Ogawa medium and probably also by the proportion method using the Löwenstein-Jensen medium, as was used in our study. Of the aforementioned 30 discrepant strains, 13 had the *inhA* -15C>T mutation. Therefore, our three isolates with discrepant results indicate that the proportion method using Löwenstein-Jensen medium may fail to detect isolates with low-level resistance to INH. The relationship between resistance level and mutation type could not be confirmed in our study because high-level INH resistance was not tested by conventional DST. TB patients usually receive multiple drug therapy; hence, it is not clear whether low-level INH resistance has an important role to play in the development of INH resistance, or whether INH would be effective in the treatment of these patients. Mutations in the *inhA* gene are also known to confer resistance to ethionamide (ETH); therefore, the ETH susceptibility of the INH-resistant strains was examined in this study [18]. Only three of the 16 isolates carrying the *inhA* -15C>T mutation were found to be resistant to both INH and ETH in our study. Further studies are needed to determine the mechanism underlying ETH resistance.

In conclusion, our DNA sequencing results could be used for the development of more rapid and simple molecular INH and RIF susceptibility testing methods in Korea. These methods would be helpful in more rapid detection of MDR-TB in respiratory specimens. At present, however, this molecular method cannot be considered as an alternative to conventional DST for INH and RIF. In addition, it is necessary to analyze the strains with the *inhA* -15C>T mutation for determining their susceptibility or resistance to INH.

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