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Evaluation of Line Probe Assay in Detecting Rifampicin Resistance of *Mycobacterium tuberculosis*

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The purpose of this study was to evaluate the efficiency of Line Probe Assay (LiPA) in detecting the *rpoB* gene mutation of clinically isolated *Mycobacterium tuberculosis* (MTB) and to compare the level of resistance to the various rifamycins with their mutation sites. The mutation in the *rpoB* gene was found in 84 (97.6%) out of 86 rifampicin (RMP) resistant strains as determined by LiPA. No mutation was observed in 2 RMP resistant strains and in any of 38 RMP susceptible strains tested. Only one of 3 strains with △5/R5, one of 2 strains with △3, and one of 3 strains with △2/R2 LiPA profile showed a slightly lower level of resistance to the rifapentine than the other strains. Although we could not find correlations between mutation sites in the *rpoB* gene and the level of susceptibility to the various rifamycins, the LiPA is recommended as a fast screening tool for detection of RMP resistant MTB.

Key words: *Mycobacterium tuberculosis*, rifampicin, *rpoB* gene, line probe assay

A recent development of molecular biology gives us the hope for replacement of the conventional drug susceptibility test. With molecular technology, the conventional test period required for more than 4 weeks may greaty be shortened. Recently, various mycobacterial genes affecting drug resistance were studied in detail; the *rpoB* to RMP (18, 19), *katG, inhA, ahpC* to isoniazid (1, 22, 23), *gyrA* and *gyrB* to fluoroquinolones (17), *rrs* and *rpsL* to streptomycin (12, 16), and *pncA* to pyrazinamide (15). Any mutations in these genes are known to be related with the resistance of the corresponding antituberculosis drugs (7, 11). Of those, the mutation of the *rpoB* gene, encoding β-subunit of RNA polymerase, has been most well correlated with RMP resistance (18). Almost all of the RMP resistant MTB strains had mutations within 69bp (codon 51-codon 533 in *E. coli*) of the *rpoB* gene (18). The fact has brought the development of techniques to detect RMP resistance in MTB such as single strand conformation polymorphism (18), direct sequencing (8), heminested PCR (21) and LiPA (5). In this study, the RMP resistance of MTB strains isolated from Korean tuberculosis patients were compared with the LiPA patterns to study the relationships between the mutation sites and the level of various rifamycin resistances. The method used was considered to provide information on the presence of mutations in the *rpoB* gene faster and more conveniently than the others.

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Materials and Methods

Strains

Eighty-six RMP resistant and 38 RMP susceptible MTB strains of which drug susceptibility were defined on Lowenstein-Jensen (L-J) medium containing RMP 40 μg/ml by conventional method as previously described (9) were used for the LiPA test.

Polymerase chain reaction (PCR)

The primer sequences which were used to amplify 157 bp fragments of the *rpoB* gene were as follows: IP1: 5'-GGTCGGCATGTCGCGGATGG-3' biotinylated at the 5' end and IP2: 5'-GCACGTCGCGGACCTCCAGC-3' biotinylated at the 5' end. Fifty microliters of PCR mixture contained 10 mM Tris-HCl (pH 8.3), 2.2 mM $MgCl_2$, 200 mM of each dNTP, 0.01% gelatin, 1 Uint of *Taq* polymerase, and 5 μl of autoclaved MTB cell suspension equivalent to the turbidity of MacFarland standard No. 2.

Line probe assay

The Line Probe Assay is based on the reverse hy-

bridization principle. A set of 5 wild type probes and 4 mutant probes were immobilized as parallel lines at known locations on a nitrocellulose strip. The LiPA test was performed using a INNO-LiPA Rif.TB kit (Innogenetics) as previously described (5, 14). Ten microliters of PCR-amplified products were denatured with an equal volume of 0.4 M NaOH for 5 min at room temperature and hybridized with probes on LiPA strip in a water bath for 30 min at 62°C by shaking at 40 rpm. The hybridized strips were visualized with the colorimetric substrates NBT/5-Bromo-4-chloro-3-indolyl -phosphate.

Drug susceptibility test

After detecting strains with mutations in the *rpoB* gene by the LiPA test, three strains which respectively showed distinctive LiPA profiles were selected for drug susceptibility tests. Resistance levels of each MTB isolate to rifampicin (Sigma, USA), rifapentine (Chongkundang, Korea), rifamycin B (Sigma, USA), and rifamycin SV (Sigma, USA) were determined by incubation on the 7H11 media. Drug concentraions in the media were 0.05, 0.1, 0.5, 1, 5, and 25 μg/ml. The susceptibility of the bacteria to drugs was scored after incubation for 4 weeks at 37°C.

Results

Eighty six RMP resistant strains and 38 RMP susceptible strains priviously determined by conventional drug susceptibility test were screened by LiPA test. The LiPA test detected *rpoB* gene mutations in 84 (97.7%) out of 86 RMP resistant strains. Two (2.3%) out of 86 RMP rifampicin resistant strains and all of 38 RMP susceptible strains (100%) did not show any reactivity on the LiPA strips. The "R" probes of LiPA were designed to detect point mutations without sequencing. A positive reaction to R2 indicates mutations of Asp at codon 516 to Val; R4A, His at 526 to Tyr; R4B, His at codon 526 to Asp; and R5, Ser at codon 531 to Leu. Sixty nine (80.2%) RMP resistant strains containing point mutation were detected by LiPA without sequencing ; 53.5% in R5, 11.6% in R4A, 9. 3% in R2, and 5.8% in R4B (Table 1). There were also strains mutated at two codon sites simultaneously such as KIT10796 (△S1/△S4), KIT10812 (△S1/△S4, deletion of the S1 band and S4 band), KIT10792 (△S1/△S3), and KIT10449 (△S2/△S5). KIT10786 had a distinctive band at S2 and S4 as well as a weak band at R2 and R4A probes which was regarded as a false reaction. LiPA test was shown to be sensitive to temperature during hybridization, especially with R2 or R4A probe which tended to reveal a weak false band.

Table 1. Mutation sites of RMP resistant isolates of *M. tuberculosis* on the LiPA strips

LiPA profiles	Number of strains (%)	Mutation sites
△S5, R5	46 (53.5)	Codon 531: Ser to Leu
△S4, R4A	10 (11.6)	
△S4, R4B	5 (5.8)	Codon 526: His to Tyr
△S3	2 (2.3)	
△S2, R2	8 (9.3)	Codon 526: His to Asp
△S5	3 (3.5)	
△S4	4 (4.7)	ND[a]
△S2	2 (2.3)	Codon 516: Asp to Val
Susceptible	2 (2.3)	
△S1, △S4, R4B	1 (1.2)	ND
△S1, △S4	1 (1.2)	ND
△S1, △S3	1 (1.2)	ND
△S2, △S5	1 (1.2)	ND
Total	86 (100.0)	

[a]Mutation sites could not be determined by the LiPA test.

RMP resistant strains which represent each LiPA profile were inoculated on the 7H11 media containing various rifamycins to observe the correlation between mutation sites in *rpoB* gene and drug resistance. The drug susceptibility patterns of tested strains are summarized in Table 2. KIT10786 and KIT10519 with △S3 LiPA profile tended towards a sightly lower MIC (≤25 μg/ml), while strains with △S4/R4 pattern consistently showed much higher MIC (≥25 μg/ml) comparitively than other strains with different LiPA profile. KIT10786 of 2 strains with △3 LiPA profile, KIT10791 of 3 strains with △S5/R5 LiPA profile, and KIT10433 of 3 strains with △S2/R2 LiPA profile showed an apparent lower MIC (5 μg/ml) in rifapentine, while the remainders had higher MIC greater than 25 μg/ml. There was no cotrelation between the LiPA profile and the level of resistance. The KIT10809, KIT19811, and KIT 10218, which were defined to be susceptible to rifampicin by previous conventional proportion method, showed susceptible LiPA profile showing 5 S bands and none of 4 R bands. The MICs of these strains were in the range of 0.1~1.0 μg/ml to all the rifamycin derivatives. The strain KIT10790 and KIT10802, which were defined to be resistant to rifampicin by the conventional method, showed susceptible LiPA profiles. Yet, these two strains with susceptible LiPA profile did not contain mutations in the 69bp region according to sequencing.

Discussion

Rifampicin is an essential component of antituberculous drugs. An earlier detection of RMP

Table 2. The minimal inhibitory concentration MIC of the drugs for the strains which showed distinctive LiPA profile[a]

Strain	LiPA profile	Drug (μg/ml)			
		Rifampicin	Rifapentine	Rifamycin B	Rifamycin SV
10809	Susceptible	0.5	0.1	1.0	1.0
10811	Susceptible	0.5	0.1	0.5	0.5
10218	Susceptible	1.0	0.5	1.0	1.0
10787	△S5, R5	>25	>25	>25	>25
10791	△S5, R5	25	5	>25	25
10794	△S5, R5	>25	25	>25	25
10784	△S4, R4A	>25	>25	>25	>25
10789	△S4, R4A	>25	>25	>25	>25
10798	△S4, R4B	>25	>25	>25	>25
10813	△S4, R4B	>25	>25	>25	>25
10453	△S4, R4B	>25	>25	>25	>25
10815	△S4, R4B	>25	>25	>25	>25
10786	△S3	25	5	25	25
10519	△S3	25	25	25	25
10797	△S2, R2	>25	>25	>25	25
10807	△S2, R2	>25	25	>25	25
10433	△S2, R2	25	5	5	5
70043	△S5	25	25	25	25
10803	△S5	>25	>25	>25	>25
10427	△S4	25	25	25	25
10451	△S4	>25	>25	>25	>25
10790	Susceptible	>25	>25	>25	>25
10802	Susceptible	>25	>25	>25	25
10796	△S1, △S4, R4B	>25	>25	>25	>25
10812	△S1, △S4	>25	>25	>25	25
10792	△S1, △S3	>25	>25	>25	25

[a]MICs were determined by incubation on the 7H11 agar medium. The rifampicin susceptible strains 10809, 10811 and 10218 as determined by the conventional method were used as control strains.

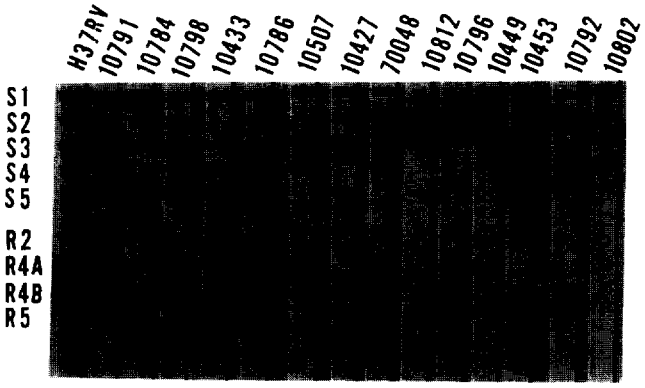

Fig. 1. Line probe assay profiles of rifampicin resistant strains. Wild strains showed five S bands (S1-S5), while mutant strains were devoid of at least one of those bands. A positive reaction to R2 is related with mutations at codon 516, Asp to Val; R4A, His at 526 to Tyr; R4B, His at codon 526 to Asp; and R5, Ser at codon 531 to Leu. Wild *M. tuberculosis* H37Rv showed five S bands. The remaining RMP resistant strains as determined by the conventional susceptibility test were devoid of at least one band out of five S bands except KIT10802 strain.

resistance will be helpful for selecting more effective regimens. RMP belongs to a structurally unique class of ansa molecules with an aromatic center bridged on both ends by an aliphatic chain. This structure is very important for microbiological activity, probably because of the interaction of the drug with its target. RMP's target is RNA polymerase (6). RMP binds to the beta subunit of this complex. Initiation of RNA transcripts is blocked by the RMP but their elongation is not. The mechanism of rifampin resistance is related with the mutations in the central region of the RNA polymerase beta subunit, which lead to decreased enzyme affinity for RMP (20). Most RMP resistant strains accompany the resistance of isoniazid which is also one of the essential drugs. Thus RMP resistance may also be used as an indicator of multi-drug resistance (3). We evaluated the efficiency of LiPA in detecting the RMP resistance and compared the distinctive patterns of LiPA with the level of resistance in various rifamycin derivatives. As shown in Table 2, KIT10790 and KIT10802, which were defined as RMP resistant strains by previous conventional drug susceptibility tests, showed no mutation profiles within 69bp *rpoB* gene on the LiPA strips. Telenti *et al.* (18) suggested that it is probably due to a possible permeability barrier in strains. Dabbs *et al.* (4) demonstrated that ribosylation inactivates RMP in fast growing mycobacterial strains, suggesting that may what's for RMP resistance of MTB. This study revealed that the most frequent mutation was the replacement of Ser at codon 531 with Leu (52.8% of RMP resistant strains) as previously observed by other investigators (7, 18). Unlike what is presumed, most of the mutation profiles did not correlate with the level of resistance to rifamycins. In a few strains (KIT 10791, KIT10786, KIT10433) varying resistance levels in different rifamycins were observed. Previous studies showed that varying degrees of drug resistance to rifamycins are related with the mutations in *rpoB* gene; mutations at codon 516 (2) or in adjacent codons 511 and 512, resulting in different level of RMP and rifapentine (10), and mutation at codon 533 or 515 relating with RMP resistant phenotype (13). In this study the mutation at the S 3 region (codon 519-codon 525) of the LiPA strip might be considered to affect the resistance level of all rifamycins toward lower levels. Further studies should be performed to define the correlation between the mutation sites and their resulting level of drug resistance. This proposed LiPA method need to be further improved, since R2 or R4A

probes often show a weak false band. This method could be used as an easy and fast screening tool for MTB resistance to RMP.

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