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Performance of PCR-reverse blot hybridization assay for detection of rifampicin-resistant *Mycobacterium leprae*

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Drug resistance in *Mycobacterium leprae* is a significant problem in countries where leprosy is endemic. A sensitive, specific, and high-throughput reverse blot hybridization assay (REBA) for the detection of genotypic resistance to rifampicin (RIF) was designed and evaluated. It has been shown that resistance to RIF in *M. leprae* involves mutations in the *rpoB* gene encoding the β-subunit of the RNA polymerase. The PCR-REBA simultaneously detects both 6 wild-type regions and 5 different mutations (507AGC, 513GTG, 516TAT, 531ATG, and 531TTC) including the most prevalent mutations at positions 507 and 531. Thirty-one clinical isolates provided by Korea Institute of Hansen's Disease were analyzed by PCR-REBA with RIF resistance of *rpoB* gene. As a result, missense mutations at codons 507 AGC and 531ATG with 2-nucleotide substitutions were found in one sample, and a missense mutation at codon 516 TAT and ΔWT6 (deletion of 530-534) was found in another sample. These cases were confirmed by DNA sequence analysis. This rapid, simple, and highly sensitive assay provides a practical alternative to sequencing for genotypic evaluation of RIF resistance in *M. leprae*.

Keywords: *Mycobacterium leprae*, rifampicin, PCR-REBA (Reverse blot hybridization assay), molecular diagnosis

Introduction

Leprosy, the second most common communicable disease due to mycobacteria after tuberculosis, is still a significant disease as 230,000 new cases were reported in 2010 alone (www.who.int/lep/) (Cambau *et al*., 2012). Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* and mainly affects the skin and peripheral nerves. In addition, Leprosy is a major global health problem in several countries of Asia, Latin America, and Africa (Ji *et al*., 1998; Maeda *et al*., 2001). Leprosy is effectively controlled by a multidrug therapy (MDT) regimen of dapsone, rifampicin, and clofazimine. Global efforts to control leprosy using intensive chemotherapy have led to a significant decrease in the number of registered patients. Although MDT, introduced by the World Health Organization (WHO) in 2013 (WHO reports, 2013), has been very successful in reducing the prevalence of the disease, widespread use has led to the emergence of drug-resistant *M. leprae*, threatening its usefulness in treating leprosy (Suzuki and Matsuoka, 2006; Sekar *et al*., 2011). Annual reports indicate the emergence of drug-resistant *M. leprae* to be more than 500,000 new cases each year (Matsuoka *et al*., 2010). To prevent the emergence and transmission of multidrug resistant (MDR) leprosy and to identify and treat existing cases, it is necessary to establish rapid methods for the detection of drug resistance in *M. leprae* (Ji *et al*., 1992).

Rifampicin (RIF) resistance is caused by mutations in the β-subunit of RNA polymerase, a target of RIF encoded by the *rpoB* gene (Zhang *et al*., 2004). More than 95% of the resistant strains harbor mutations in an 81-bp hot-spot region (codons 507 to 533) of the *rpoB* gene, named the RIF-resistant determining region (RDR). The same region has been shown to be associated with rifampicin resistance in almost all resistant isolates of *Mycobacterium tuberculosis* and in a range of other bacteria (Matsuoka *et al*., 2007; Choi *et al*., 2010).

Due to practical difficulties culturing *M. leprae in vitro*, the antibiotic susceptibility test of *M. leprae* still relies on time-consuming methods based on the growth of *M. leprae* in the mouse footpad (Shetty *et al*., 2003). This *in vivo* method requires at least 6 months and relatively large numbers of bacteria (Shetty *et al*., 2003). The rapid determination of drug susceptibility of this microbe using clinical specimens has been problematic. Therefore, the establishment of a rapid, simple, and reliable method for the detection of drug-resistant *M. leprae* is one of the most urgent subjects in the treatment of leprosy and is expected to lead to better strategies for more rapid initiation of treatment.

In recent years, studies have been performed to develop a rapid and effective molecular method to serve as a replacement for the conventional antibiotic susceptibility test (Sapkota *et al*., 2008; Gupta *et al*., 2009). In these studies, different typing methods were used to measure the associations be-

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tween genotype and gene mutations. There have also been studies done to evaluate the role of the *rpoB* gene in RIF resistance using methods including DNA sequence analysis (Cavusoglu *et al*., 2002), line probe assay (Mokrousov *et al*., 2004; Morgan *et al*., 2005), and analysis with DNA microarrays (Matsuoka *et al*., 2008, 2010).

In the present study, mutations in the *rpoB* gene of rifampicin-resistant *M. leprae* isolates were analyzed in the search for a useful marker to predict drug resistance, and rapid detection of the mutation was evaluated by using a recently described PCR-reverse blot hybridization assay (REBA).

Materials and Methods

Isolation of *M. leprae* and DNA extraction from biopsy tissues

Clinical isolates of *M. leprae* strain 4264, which was used as the standard wild-type strain, were provided by Colorado State University in the form of infected livers and spleens (Groathouse *et al*., 2004). About 20 g of tissue was processed to obtain approximately 100 mg of purified *M. leprae* cells (containing, on average, 2.9×10^9 cells/mg). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH) and used at a final concentration of 50 ng/μl. DNA used as clinical specimens was provided by Korea Institute of Hansen's Disease and extracted from skin biopsy samples using the QIAamp DNA Mini Kit (Qiagen GmbH) according to the instructions provided by the manufacturer (Ahmed *et al*., 2009).

Site-directed mutagenesis for mutant probe manufacture

The wild-type *rpoB* gene of *M. leprae* was amplified by PCR from *M. leprae* strain 4264 and cloned into pTOPO 2.1 (Invitrogen). The primer sets used to amplify target *rpoB* gene are presented in Table 1. PCR products were purified and phosphorylated with T4 kinase and ATP and were then ligated to produce a circular form. The ligation mixture was used to transform *Escherichia coli* DH5α, and ampicillin-resistant colonies were isolated. The 5 different mutagenic oligonucloeotides for mutant-type *rpoB* gene were synthesized (Bioneer) with PCR primers (F-5′-GTCGGTA TGTCGCGGATGGAGC-3′ and R-5′-CAGCGGTCAAGTA TTCGATCTCG-3′, which resulted in a 430-bp PCR product) and mutagenized using the pBHA vector. Five mutant plasmids were extracted from the transformants, and the mutated sequences were confirmed by sequence analysis using an ABI 3730 automated DNA sequencer and the ABI Prism BigDye Terminator kit (CosmoGenetech).

Membrane preparation for PCR-REBA

Oligonucleotide primers corresponding to both strands of the rifampicin resistance region of *M. leprae* (Fig. 1) were designed by Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primers were made as probes corresponding to the complementary strand and were used exclusively thereafter. Probes were synthesized with a 5′-amino group to facilitate a covalent linkage to Biodyne C membranes (PALL) and were diluted to the optimized concentrations in 145 μl of 500 mM $NaHCO_3$ at pH 8.4. Membranes (14.5 × 14.5 cm) were activated by incubation in 10 ml of freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, Sigma) with shaking at room temperature for 15 min, rinsed with water, and placed in a clean miniblotter system. Diluted oligonucleo-

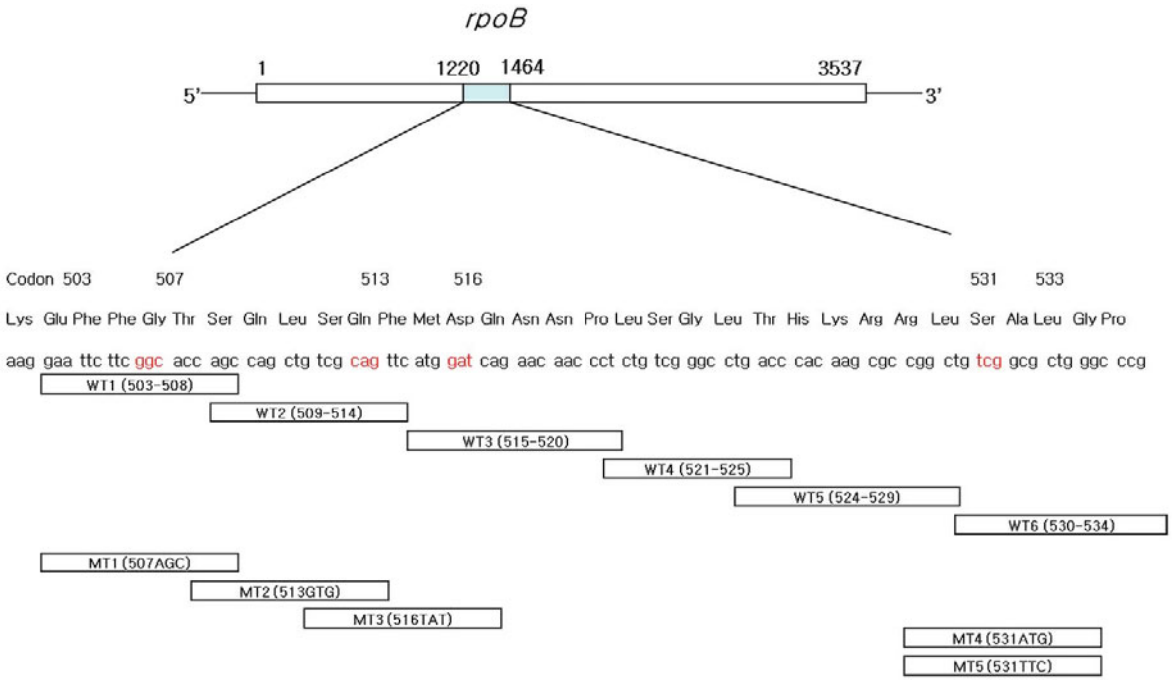

Fig. 1. Schematic representation of the *rpoB* gene from positions 1220 to 1464 and positions of primers and probes used in this study; 6 wild-type probes (WT1 to WT6) and 5 additional probes (MT1 to MT5).

Table 1. Oligonucleotides used in this study

Gene			
	Primer	Sequence	
rpoB	1220-F	5′-AGGACGTCGAGGCGATCA-3′	1st PCR
	1464-R	5′-TAGTGCGAAGGGTGCACGTC-3′	
	1240-F	5′-CCGCAGACGCTGATCAATA-3′	2nd PCR
	1424-R	5′-ACGTCACGGACCTCTAGCC-3′	
	Probe	Sequence	
	WT1	GAATTCTTCGGCACCAGC	503–508
	WT2	AACCAGCTGTCGCAGTTC	509–514
	WT3	ATGGATCAGAACAACCCT	515–520
	WT4	CTCTGTCGGGCCTGACC	521–525
	WT5	CTGACCCACAAGCGCCGG	524–529
	WT6	CTGTCGGCGCTGGGCC	530–534
	MT1	AAGGAATTCTTCAGCACCAG	507AGC
	MT2	CAGCCAGCTGTCGGTGTTC	513GTG
	MT3	GTCGCAGTTCATGTATCAGA	516TAT
	MT4	CCGGCTGATGGCGCTG	531ATG
	MT5	CCGGCTGTTCGCGC	531TTC
	Universal	TCCGGTGGTCGCCGCT	

tide solutions (145 µl) were added to the slots and left for 60 min before removal. The membrane was removed from the miniblotter, inactivated in 100 mM NaOH for 9 min, and then washed in 2× SSPE/0.1% SDS for 5 min at 60°C. Membranes were then wrapped and stored at 4°C.

PCR for amplification of rpoB regions

All of the available partial and full *rpoB* genes of *M. leprae* from GenBank accession No. Z14314 were aligned. The two pairs of primers, 1220F and 1464R and 1240F and 1424R, where 1464R and 1424R are biotinylated, were prepared (Bioneer) and the amplification products of these primers were anticipated to be 220 bp and 185 bp in size (Table 1). For control purposes, plasmids carrying cloned *M. leprae* with resistance to rifampicin as wild-type and 5 rifampicin-resistant isolates of *M. leprae* were used in PCR reactions. The PCR reaction mix consisted of 2× premix (Genetbio), which contains 1 U *Taq* DNA polymerase, 250 µm dNTPs, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, and 1.5 mM $MgCl_2$ plus 10 pmol of each primer, DNA, and water to reach a final volume of 20 µl. Amplification was performed in a PTC-200 thermocycler (MJ Research). The reaction conditions for two-tube nested PCR using the outer primers (1220F and 1464R) were as follows: pre-denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec; and a final extension at 72°C for 7 min. The reaction conditions for two-tube nested PCR using the inner primers (1240F and 1424R) were as follows: pre-denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec; and a final extension at 72°C for 10 min. The nucleotide sequences of both strands of the relevant part of the *rpoB* gene were determined by direct sequencing of the PCR product using an ABI 3730 automated DNA sequencer (Cosmo GENTECH).

Reverse blot hybridization assay (REBA)

Hybridization was performed using the MN45 miniblotter (Immunetics). For this purpose, 20 µl of biotinylated PCR products were denatured at 25°C for 5 min in denaturation solution and diluted in 120 µl of hybridization solution (2× SSPE/0.1% SDS). The single-stranded PCR products were applied to a membrane in the miniblotter and hybridized at 50°C for 30 min. The membrane (or strips) was then washed twice in a water-bath (Gemini) in 100 ml (or 1 ml) 2× SSPE/0.5% SDS for 10 min at 55°C, incubated at 25°C with 1:2000 diluted streptavidin-alkaline phosphatase conjugate (Roche Diagnostics) in 2× SSPE/0.5% SDS for 45–60 min, washed twice with 100 ml 2× SSPE/0.5% SDS at 42°C for 10 min, rinsed once with 2× SSPE at room temperature for 5 min, and subjected to luminescent detection of hybrids with the ECL detection system (CDP-Star, Amersham Biosciences), followed by exposure to ECL Hyperfilm (Amersham Biosciences). After development of the ECL films, the autoradiographs were visually assessed.

For colorimetric detection, 1 ml of diluted NBT/BCIP stock solution (Roche Diagnostics) in TBS (pH 9.5, 20 µl/1 ml) was added, and the strips were incubated for 15–20 min. The coloring positive control probe was always used to check that hybridization or REBA process had been set up correctly. If the hybridization process is wrong, coloring positive control probe will not be detected.

Results

Suitability of the designed probes for detection of *M. leprae*

Six partially overlapping probes (WT1 through WT6 of 16 to 18 bases) that hybridize exclusively to wild-type *rpoB* se-

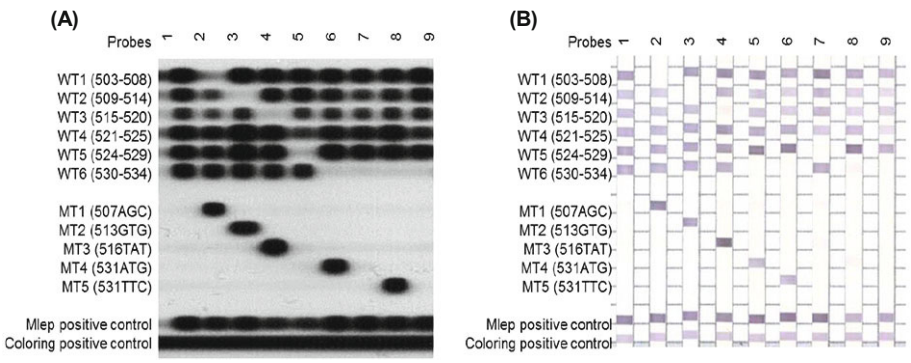

Fig. 2. Detection of rifampicin resistance by luminescent (A) and colorimetric (B) detection using the PCR-REBA. The results were obtained using the protocol described in Materials and methods with biotinylated primers generated by PCR using the *M. leprae* clone DNA from the following sources: Lanes: 1, wild-type *M. leprae*; 2, positive control *rpoB* 507AGC; 3, positive control *rpoB* 513GTG; 4, positive control *rpoB* 516TAT; 5, positive control *rpoB* 526GAC; 6, positive control *rpoB* 531ATG; 7, positive control *rpoB* 531TGG; 8, positive control *rpoB* 531TTC; 9, positive control *rpoB* 533GTG.

Table 2. Specificity of the REBA Mlep-Rifa assay for 64 bacterial reference strains

Genus	Species	Reference strains	Results of REBA Mlep-Rifa data interpretation
Mycobacterial strains			
Mycobacterium	*M. leprae*	clinical isolates	*M. leprae*
	M. tuberculosis H37Rv	ATCC 27294	ND
	M. avium	ATCC 25291	ND
	M. intracellulare	ATCC 13950	ND
	M. abscessus	ATCC 19977	ND
	M. massiliense	clinical isolates	ND
	M. bolleti	clinical isolates	ND
	M. chelonae	ATCC 35749	ND
	M. fortuitum	ATCC 49403	ND
	M. marinum	ATCC 927	ND
	M. mageritense	ATCC 700351	ND
	M. peregrinum	ATCC 14467	ND
	M. kansasii	ATCC 12478	ND
	M. gastri	ATCC 15754	ND
	M. celattum	ATCC 51130	ND
	M. celattum	ATCC 51131	ND
	M. terrae	ATCC 15755	ND
	M. nonchromogenicum	ATCC 19530	ND
	M. gordonae	ATCC 14470	ND
	M. szulgai	ATCC 35799	ND
	M. mucogenicum	ATCC 49650	ND
	M. phlei	ATCC 11758	ND
	M. malmoense	ATCC 29571	ND
	M. triviale	ATCC 23292	ND
	M. aurum	ATCC 23366	ND
	M. farcinogen	ATCC 35753	ND
	M. gilvum	ATCC 43909	ND
	M. neoaurum	ATCC 25795	ND
	M. parafortuitum	ATCC 19686	ND
	M. xenopi	ATCC 19250	ND
	M. genavense	ATCC51233	ND
Non-mycobacterial strains			
Norcadia	*N. abscessus*	BAA-279	ND
	N. asiatica	CIP 108374	ND
	N. asteroides	ATCC 19247	ND
	N. brevicatena	ATCC 15333	ND
	N. carnea	ATCC 6847	ND
	N. cyriacigeorgica	CIP 48295	ND
	N. elegans	CIP 108553	ND
	N. farcinica	ATCC 3318	ND
	N. flavorosea	CIP 104511	ND
	N. nova	ATCC 33726	ND
	N. otitidiscaviarum	ATCC 14629	ND
	N. salmonicida	ATCC 27463	ND
	N. seriolae	ATCC 43993	ND
	N. uniformis	CIP 104824	ND
	N. vaccinii	ATCC 11092	ND
	N. pseudosporangifera	CIP 104825	ND
	N. violaceofusca	CIP 104780	ND
Escherichia	*E. coli*	ATCC 25922	ND
	E. coli	ATCC 35218	ND
Klebsiella	*K. pneumoniae*	ATCC 13883	ND
Pseudomonas	*P. aeruginosa*	ATCC 27853	ND
Enterobacter	*E. aerogenes*	ATCC 1304	ND
Citrobacter	*C. freundii*	ATCC 6750	ND
Shigella	*S. boydii*	DML 399*	ND
	S. dysenteriae	DML 400*	ND
	S. flexneri	ATCC 9199	ND
Salmonella	*S. typhi*	ATCC 19430	ND
	S. typhimurium	ATCC 13311	ND
Staphylococcus	*S. aureus*	ATCC 29213	ND
	S. aureus	ATCC 25923	ND
Enterococcus	*E. faecium*	ATCC 19434	ND
Streptococcus	*S. pneumoniae*	ATCC 49619	ND
	S. agalactiae	ATCC 13813	ND

Abbreviations: ATCC, American type culture collection; CIP, Collection Institut Pasteur; DML, Diagnostic Microbiology Laboratory, Biomedical Laboratory Science, Yonsei University; ND, Not detected.

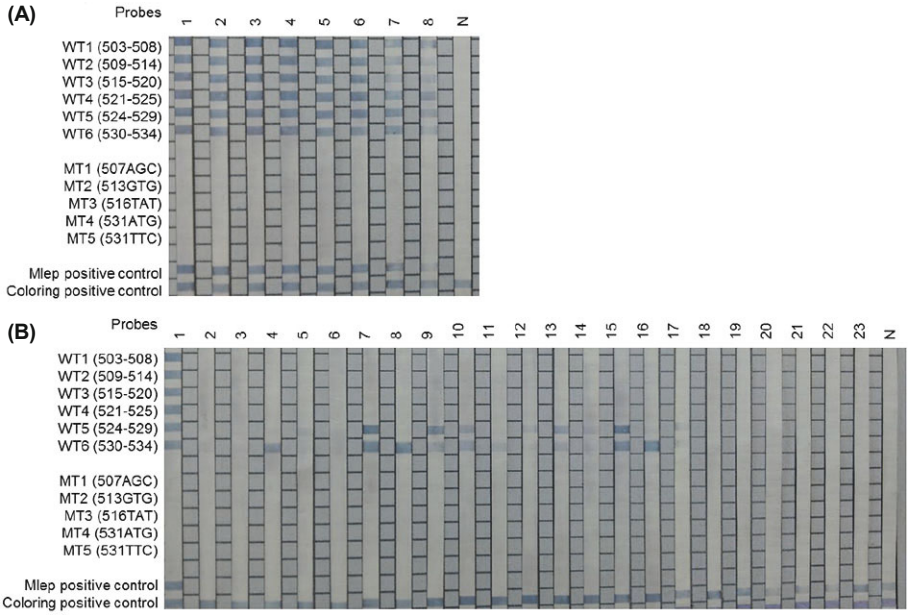

Fig. 3. (A) Sensitivity of the PCR-REBA. Serially diluted *M. leprae* strain 4264 DNA from 10 ng (lane 1), 1 ng (lane 2), 100 pg (lane 3), 10 pg (lane 4), 1 pg (lane 5), 100 fg (lane 6), 10 fg (lane 7), and 1 fg (lane 8) were used to determine the detection limit of the PCR-REBA. N, negative control. (B) Specificity of the PCR-REBA with DNAs from 23 different *Mycobacterium* species. Lanes: 1, *M. leprae*; 2, MTB H37Rv; 3, *M. avium*; 4, *M. intracellulare*; 5, *M. abscessus*; 6, *M. chelonae*; 7, *M. fortuitum*; 8, *M. marinum*; 9, *M. kansasii*; 10, *M. gastri*; 11, *M. celatum*; 12, *M. terrae*; 13, *M. nonchromogenicum*; 14, *M. gordonae*; 15, *M. szulgai*; 16, *M. scrofulaceum*; 17, *M. mucogenicum*; 18, *M. malmoense*; 19, *M. phlei*; 20, *M. triviale*; 21, *M. genavense*; 22, *M. mageritense*; 23, *M. xenopi*; and N, negative control.

quence in the rifampicin resistance-determining region (RRDR) were tested (Fig. 1). The reactivity of an amplified fragment with one or more of the 6 wild-type probes (WT1 to WT6) is prevented if a mutation is present in one or more of the probe regions. Five additional probes [MT1 (507AGC), MT2 (513GTG), MT3 (516TAT), MT4 (531ATG), and MT5 (531TTC)] were expected to hybridize to mutant sequences of the five most commonly observed mutations. When all of the wild-type probes produced a positive signal and none of the MT probes reacted, the *M. leprae* isolate was considered to be susceptible to rifampicin (Fig. 2; Lane 1). When at least one negative signal was obtained with the WT probes and none of the MT probes reacted, the isolate was considered rifampicin-resistant (Fig. 2; Lanes 5, 7, 9). When a positive reaction was obtained with one of the five MT probes and was accompanied by a negative signal with the corresponding WT probes, the isolate was considered to be resistant to rifampicin (Fig. 2; Lanes 2, 3, 4, 6, and 8). The DNA strip tests were validated with regard to the *M. leprae* identification band, which was positive with an intensity equal to or greater than that obtained with the universal positive control, demonstrating the presence of *M. leprae* DNA (Fig. 2B).

Specificity and sensitivity of PCR-REBA

Mycobacterium tuberculosis H37Rv (MTB H37Rv), 29 Nontuberculous mycobacteria (NTM) strains, and 33 non-mycobacterial strains were used to determine the specificity of the PCR-REBA (Table 2). Although all these strains except *M. leprae* showed no signal in the *M. leprae* specific positive control probe band, some of the WT5 and WT6 probe bands were observed in PCR-REBA (Fig. 3). The analytical sensitivity of the assay was determined through the use of a 10-fold dilution (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg) of standard DNA isolated from the *M. leprae* 4264 strain. The detection range of the PCR-REBA assay was 10 fg to 1 fg (Fig. 3).

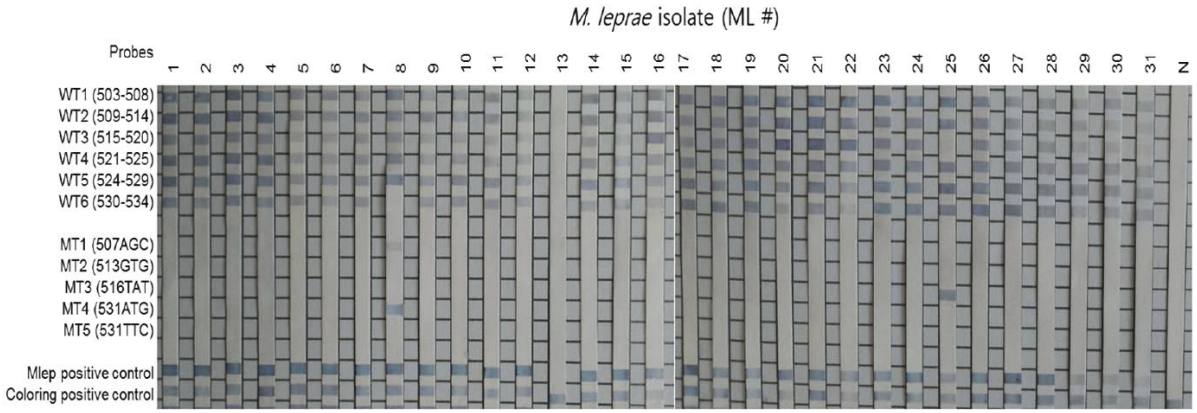

Fig. 4. Detection of rifampicin resistance in 31 clinical specimens using PCR-REBA. ML clinical isolates No. 1-7, 9-12, 14-24, and 26-31 show rifampicin susceptibility. ML 8 shows mixed 507AGC and 531ATG. ML 13 is not detected, and No. 25 shows 516TAT and ΔWT6 (deletion of 530-534).

Detection of rifampicin resistance in *M. leprae* clinical isolates using PCR-REBA

Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, have been reported to result in resistance to rifampicin in several mycobacterial species including *M. leprae*. The resistance pattern was previously determined by phenotypic drug susceptibility testing. Of 31 clinical isolates of *M. leprae* provided by the Korean Leprosy Control Association, 28 isolates are predicted to be fully susceptible to rifampicin and two showed mutations in the *rpoB* gene and one isolate was not detected. Of the results, one of the isolates (ML 8) showed a mixed infection with MT1 (mutation of ACC to AGC in codon position 507) and MT4 (mutation of TCG to mutation ATG in codon position 531), one isolate (ML 25) showed a positive signal MT3 (mutation of GAT to TAT in codon position 516) and ΔWT6 (deletion of 530-534) (Fig. 4).

To confirm the results of false-negatives and the PCR-REBA assay of those 31 isolates, we confirmed by direct *rpoB* gene sequence analysis. We observed a 100% concordance between the RRDR sequence in *rpoB* and the DNA strip test results. Especially, the specific mutations detected by PCR-REBA were confirmed 531ATG and 507AGC in isolates ML8 and 516TAT and 533 GTG in isolates ML25 by *rpoB* gene sequence analysis, respectively (Fig. 5).

Discussion

Leprosy remains difficult to diagnose and treat in low- and mid-level developing countries, especially in rural areas. Active leprosy prevalence decreased during the two last decades, whereas the incidence rate did not decrease. Therefore, leprosy is still an actively transmitted disease (Rodrigues and Lockwood, 2011). Leprosy can be cured if the standard regimen of multidrug therapy (MDT) is properly implemented following World Health Organization (WHO) recommendations, which include a 6-month regimen for paucibacillary cases and a 12-month regimen (formerly 24 months) for multibacillary (MB) cases, both combining rifampicin (RIF) and dapsone (DDS), plus clofazimine (CLO) (Jing *et al.*, 2009). Because of its great potency, RIF is the key component

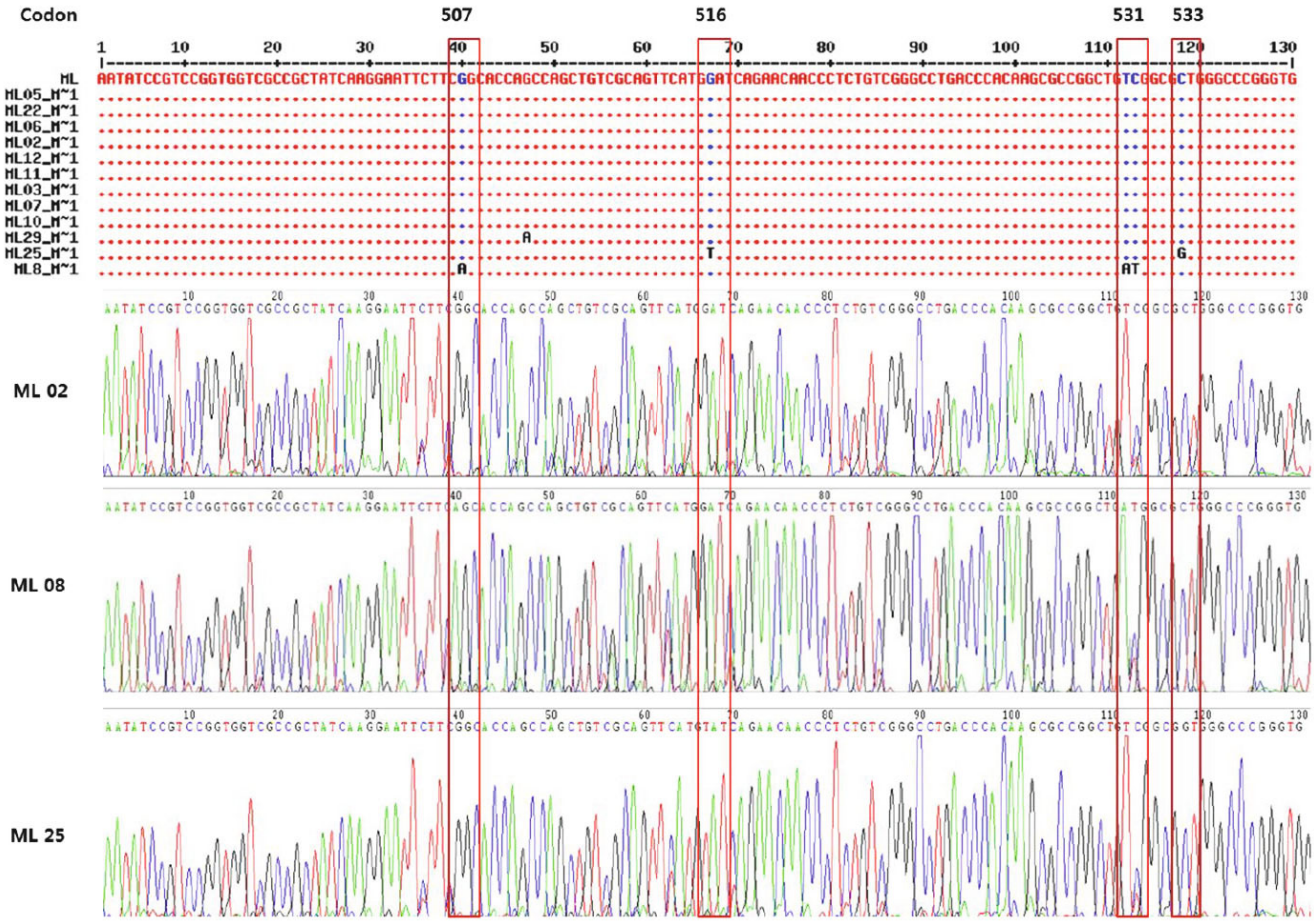

Fig. 5. The results of confirmed clinical samples of *M. leprae* by direct DNA sequence analysis of the PCR-amplified region containing the 81-bp RRDR of the *rpoB* gene. Wild-type clinical isolate numbers included ML2, ML3, ML5, ML6, ML7, ML10, ML 11, ML 22, and ML29. ML 8 contained nucleotide substitutions of TCG to ATG at codon position 531 and GGC to AGC at codon position 507. ML25 contained nucleotide substitution of GAT to TAT at codon position 516 and CTG to GTG at codon position 533. Color options are presented for consensus values, red color (90–100% consensus value); blue color (50–89% consensus value); black color (0–49% consensus value).

of the regimen; its monthly administration permits supervision of each dose, significantly minimizing the problem of patient compliance. The major objective of combining RIF with DDS and CLO is to ensure the elimination of spontaneously occurring RIF-resistant mutants before the conclusion of chemotherapy (de Carsalade *et al*., 1997). However, even in the best leprosy control program, it is difficult to persuade patients to adhere to the self-administered daily therapy (de Carsalade *et al*., 1997), suggesting that RIF resistance may still develop if the DDS-CLO component is not taken regularly. RIF-resistance may be reduced if all of the components are fully administered under supervision, which could be achieved if a monthly-administered MDT regimen can be developed. Such a regimen would facilitate integration of antileprosy chemotherapy within the general health services. Because RIF is the drug with the most bactericidal activity by far against *M. leprae* (Ji and Grosset, 2000), the fully supervised, monthly-administered regimens should always contain RIF, except in those instances in which the strain of *M. leprae* is resistant to RIF. Since the regimens should be effective for all MB patients, including those who have relapsed from previous treatment, and because patients who have suffered relapses should not be treated with combinations consisting of only RIF plus a single new antimicrobial drug, the regimens should include two antimicrobial agents in addition to RIF (Ji and Grosset, 2000).

In recent years, many important advances have been made in molecular diagnostics for detecting rifampicin resistance based on the *rpoB* genotype, in identifying highly effective drugs and designing multidrug regimens for treatment, and in determining the genomic structure and functions of leprosy bacillus (de Carsalade *et al*., 1997; Ji and Grosset, 2000). Using the new information about specific sequences of *M. leprae*, several gene probes and gene amplification systems for confirming diagnosis and monitoring treatment have been developed. Among these, PCR-based methods have been useful in confirming the diagnosis in paucibacillary leprosy, where few bacilli are present (Kramme *et al*., 2004). RNA-targeting systems for monitoring the progress of treatment, hybridization techniques for analyzing specimens with nonspecific histological features, and molecular methods for direct detection of rifampicin resistance are other major technological advances with immense applied value (Katoch, 2002).

Codons 516, 526, 531, and 533 in *rpoB* gene of *M. leprae* are known to be responsible for rifampicin resistance. However, it remains unclear whether mutations that have not been reported previously can confer rifampicin resistance. Our results show that not all mutations in the *rpoB* gene detected in *M. leprae* clinical samples confer rifampin resistance. *M. leprae* is not cultivable. Therefore, it has been very difficult to analyze the mutation-susceptibility relationship. In the present study it has been convincingly demonstrated that detection of rifampicin resistance by molecular methods is a feasible and practical alternative to the use of clinical samples. The results of the test were 100% concordant with those of PCR sequencing. However, there are a few limitations to this study. First, some of the PCR-REBA results showed a positive band with some probes (524-529 and 530-534) for other Mycobacteria species though these positive bands are not seen with all 6 wild type-specific probes. It would seem that WT5 and 6 probe had high levels of identity (i.e., >94%) between the *rpoB* genes of the cross reacting NTMs from the identity of sequences which were aligned with the NCBI blast (de Zwaan *et al*., 2014). Second, the *rpoB* mutation was present in only 3 of 31 (9.7%) leprosy patients. Therefore, it is necessary to conduct more tests with a larger number of samples. Third, although several mutations were detected by the PCR-REBA, the number of sites within *rpoB* that can be monitored by positive controls (Fig. 2) is limited by the restricted set of base changes described. Identification of a multidrug-resistant strain of *M. leprae* from a patient who received mono- or multidrug therapy for leprosy in Korea was recently reported (You *et al*., 2005). The faint possibility that new mutations might be missed by the system cannot be excluded, and more extensive evaluation is required.

The PCR-REBA can be successfully applied in a clinical laboratory setting when rapid sensitive testing is required for the correct management of patients and in cases of resistant *M. leprae*.

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