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Targeting the *rpoB* Gene Using Nested PCR-Restriction Fragment Length Polymorphism for Identification of Nontuberculous Mycobacteria in Hospital Tap Water

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Nontuberculous mycobacteria (NTM) are ubiquitous in the environment and can cause nosocomial infections in immunocompromised patients. Recently the presence of NTM in public drinking water and hospital water distribution systems has been reported. Their ability to form biofilms and their resistance to chlorine both contribute to their survival and colonization in water distribution systems. Here we analyzed thirty-two hospital tap water samples that were collected from different locations in three hospitals so as to evaluate the prevalence of NTM species. The water samples were concentrated by membrane filtration and then eluted with sterilized water following sonication. Two-step direct PCR targeting the *rpoB* gene, restriction fragment length polymorphism (RFLP) using the *Msp*I restriction enzyme, and sequence analysis were performed for identification of NTM to the species level. The sequences of each PCR product were analyzed using BLASTN. Seven samples (7/32, 21.9%) were positive for NTM as determined by nested-PCR. The PCR-RFLP results indicated five different patterns among the seven positive PCR samples. The water-born NTM were identified, including *M. peregrinum*, *M. chelonae* (2 cases), *M. abscessus*, *M. gordonae* (2 cases), and *Mycobacterium* sp. JLS. The direct two-step PCR-RFLP method targeting the *rpoB* gene was effective for the detection and the differentiation of NTM species from hospital tap water.

***Keywords*:** nontuberculous mycobacteria, hospital tap water, *rpoB* gene, PCR-RFLP

Nontuberculous mycobacteria (NTM; also know as environmental mycobacteria, atypical mycobacteria, or mycobacteria other than tuberculosis) are considered a major cause of opportunistic infections in those persons who are immunocompromised, such as patients with the human immunodeficiency virus (HIV) (Covert *et al*., 1999; Katoch, 2004). NTM lung disease is also encountered with increasing frequency in the non-HIV-infected population (Brown-Elliott and Wallace, 2002; Henry *et al*., 2004). NTM are widely distributed in dust, soil, food, and water. Although the environmental reservoirs of NTM that cause infections in humans are not well understood, water has been considered a potential source of infectious NTM (Tsitko *et al*., 2006). The ability to form biofilms (Hall-Stoodley and Lappin-Scott, 1998), growth at low nutrient concentrations (Vaerewijck *et al*., 2005), an amoeba-associated lifestyle (Greub and Raoult, 2004), and resistance to chlorine (Le Dantec *et al*., 2002) contribute to their survival, persistence, growth, and colonization in water distribution systems. Following the first isolation of NTM from public drinking-water distribution systems in the early 1900s (Collins *et al*., 1984), several researchers have reported the presence of NTM in public drinking water (Vaerewijck *et al*., 2005), hospital tap water (Anaissie *et al*., 2002), swimming pools (Iivanainen *et al*., 1999), and air from hospital warm-water therapy pools (Angenent *et al*., 2005). NTM nosocomial infections that were acquired from drinking water, bathing, showering, or contact with medical equipment rinsed with tap water were recently reviewed (Anaissie *et al.*, 2002). *M. avium* complex (*M. avium* and *M. intracellulare*) causes the most common disseminated opportunistic infections in AIDS patients (Armstrong *et al*., 1985). Aronson *et al*. (1999) showed that large restriction fragment patterns of *M. avium* isolates from hospital water samples are similar to those isolates from patient with AIDS. In addition, *M. genavense* in hospital tap water has been reported to cause nontuberculous disseminated mycobacteriosis in patients with HIV (Hillebrand-Haverkort *et al*., 1999).

The accurate and rapid identification of NTM at the species level is very important for patient treatment. This is because the method of antimicrobial susceptibility testing or the treatment regimen prescribed is very different depending on the NTM species (Griffith *et al.*, 2007). However, it is not easy to identify closely related NTM species. NTM can be categorized by growth rates and pigment production for preliminary broad classification (Runyon, 1959). The conventional biochemical analysis is time consuming and has limited identification ability (Springer *et al*., 1996). A commercial diagnosis kit (AccuProbe: GenProbe, USA) utilizing nucleic acid probes is also available, but it covers a limited range of NTM species (Griffith *et al.*, 2007). Recently, many investigators have assessed the usefulness of molecular diagnosis methods (Lee *et al*., 2000; Adékambi and Drancourt, 2004),

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Table 1. Properties of the hospital water samples

Hospital	Cold water (Mean±SD[a])					Hot water (Mean±SD)				
	No.[b]	Temp. (°C)	pH	Free Cl (ppm)	Total Cl (ppm)	No.	Temp. (°C)	pH	Free Cl (ppm)	Total Cl (ppm)
A	5	27.9±3.54	7.2±0.08	0.1±0.04	0.2±0.06	5	45.8±3.25	7.2±0.08	ND[c]	ND
B	5	24.3±1.03	7.4±0.05	0.1±0.03	0.1±0.01	5	45.3±12.49	7.4±0.06	ND	ND
C	6	24.1±0.37	7.2±0.05	0.2±0.07	0.2±0.05	6	43.9±8.29	7.2±0.04	ND	ND

[a] All data were expressed as the Mean±Standard Deviation (SD).
[b] Number of samples obtained at different locations
[c] ND, not detected

such as PCR-restriction fragment length polymorphism analysis or sequence analysis, in efforts to improve the accuracy of NTM identification.

Although species level identification of NTM is becoming increasingly important, especially as it relates to nosocomial infections, only a few studies have reported the prevalence of different NTM species from hospital tap water in Korea. The purpose of this study is to evaluate the diversity and distribution of NTM in hospital tap water and to evaluate the feasibility of *rpoB* targeted PCR-RFLP for the identification of NTM to the species level.

Materials and Methods

Collection and preparation of water samples

A total of thirty-two tap water samples were collected in sterile sample bottles from three general hospitals in Seoul on July 2007. The properties of the water samples were summarized in Table 1. Each water sample (1 L volume) was examined immediately by measuring pH, temperature, and chlorine levels using DPD (*N*, *N*-diethyl-*ρ*-phenylenediamine) chlorine reagents according to the manufacturer's instructions (Hach, USA). The water samples were filtered through 0.45 μm pore size membrane filters (Millipore Corp., USA) by vacuum filtration. After filtration, the membrane filters were aseptically transferred to 50 ml tubes and 20 ml of filtered, sterilized water was added. Sonication was applied in an ultrasonic water bath for 5 min to elute particles on the membrane, using a Branson ultrasonics 5510 (Branson, USA). After sonication, each 1.5 ml water sample was centrifuged at 13,000 rpm for 10 min. The pellet was resuspended in 200 μl of distilled water and then used in the subsequent experiments. All water samples were analyzed for NTM by direct two-step PCR and sequencing. Each 20 μl water sample was inoculated onto both LJ (Lowenstein-Jensen) medium (BD, USA) and Middlebrook 7H10 agar enriched with 10% OADC (oleic acid, BSA, dextrose, and catalase) (BD, USA), then cultured for 4 weeks at 37°C.

Table 2. Bacterial strains used in this study

Bacteria	Strains
Mycobacterium species	
M. abscessus	ATCC[a] 19977
M. chelonae	ATCC 35752
M. gordonae	ATCC 14470
M. peregrinum	ATCC 14467
M. tuberculosis H37Rv	ATCC 27294
Gram-negative bacilli	
Escherichia coli	ATCC 25922
Legionella pneumophila	ATCC 33152
Pseudomonas aeruginosa	ATCC 27853
Gram-positive bacilli	
Staphylococcus aureus	ATCC 25923

[a] ATCC, American Type Culture Collection

PCR amplification of the *rpoB* gene

For direct PCR, DNA was extracted from the water samples by a simple boiling method, as described in Afghani and Stutman (1996) with some modifications. After centrifugation at 13,000 rpm for 10 min, the supernatants were used as a template for genus specific *rpoB* targeted PCR. DNA from the reference bacterial strains was extracted as described above. The strains used in this study are listed in Table 2. The first round of PCR was performed with a primer set designed by Lee *et al.* (2000). The forward primer rpoB-F; 5'-TCAAGGAGAAGCGCTACGA-3' was used in combination with the reverse primer rpoB-R; 5'-GGATGTT GATCAGGGTCTGC-3'. For the nested PCR, the forward primer N-rpoB-F; 5'-GACCTGGCCCGCGTCGGTCG-3' was used in combination with the reverse primer N-rpoB-R; 5'-C TGCGGTGTGATCGCCTCCAC-3'. The PCR reactions were performed in a 50 μl reaction mixture containing 22.1 μl of H_2O, 10 μl of 5× PCR buffer, 2.5 μl of 25 mM $MgCl_2$, 5 μl of dNTP mix (2 mM of each dNTP), 25 pmol of forward primer, 25 pmol of reverse primer, 2 U of Go*Taq* DNA polymerase (Promega, USA), and 5 μl of a single boiled water sample or, for the nested PCR, 10 μl of the first PCR amplification product was used as a DNA template. Two microliters of the first PCR product was used in the nested PCR reaction for the control references strains. For the first PCR, the PCR mixture was first incubated at 94°C for 2 min 30 sec, then conducted by running 29 cycles of amplification (94°C for 30 sec, 53°C for 30 sec, 72°C 30 sec), with a final extension at 72°C for 7 min. The same conditions were used for the nested PCR with the exception of the annealing temperature (62°C). The PCR products were separated on a 1.2% agarose gel and purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) for use subsequent experiments.

Restriction fragment length polymorphism (RFLP) analysis

For the nested PCR-RFLP analysis, the 327 bp nested PCR products were digested with the *Msp*I restriction enzyme

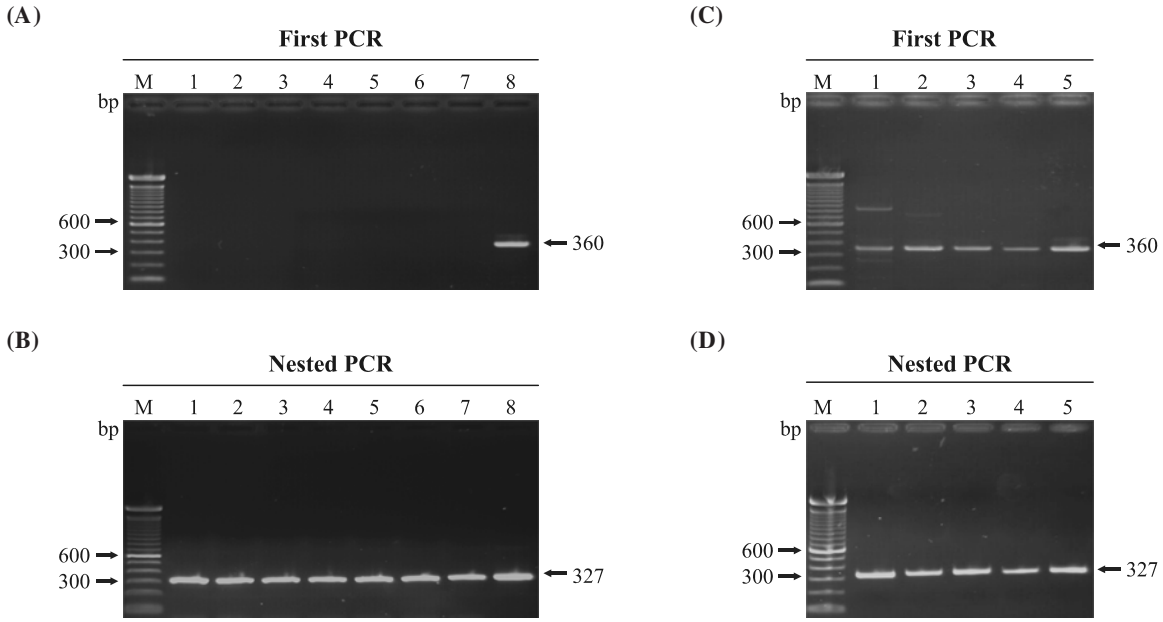

Fig. 1. Gel electrophoresis analysis of the PCR products. (A) and (B) Products were obtained from the hospital tap-water samples (lanes 1~7) using direct two-step PCR. *M. tuberculosis* H37Rv (lane 8) was used as a positive control. (C) and (D) Products were obtained from the reference mycobacterial strains using direct two-step PCR. Lane 1, *M. peregrinum* ATCC 14467; 2, *M. chelonae* ATCC 35752; 3, *M. abscessus* ATCC 19977; 4, *M. gordonae* ATCC 14470; and 5, *M. tuberculosis* H37Rv. Lane M is a 100 bp DNA size marker.

(New England Biolabs Inc., USA). After the reaction, the restriction patterns were analyzed on a 4% Metaphore agarose gel (Lonza, USA). The fragment sizes were estimated using a 50 bp DNA ladder (Invitrogen, USA) as a standard size marker. The restriction profiles of the PCR products from the water samples were compared with those of the reference *Mycobacterium* species.

Sequencing analysis

The purified nested PCR products (327 bp) from the seven water samples and reference mycobacterial strains were directly sequenced using the N-rpoB-F and N-rpoB-R primers. The E-values used to identify NTM to the species level were obtained from the BLAST program at the National Center for Biotechnology Information website (http://www.

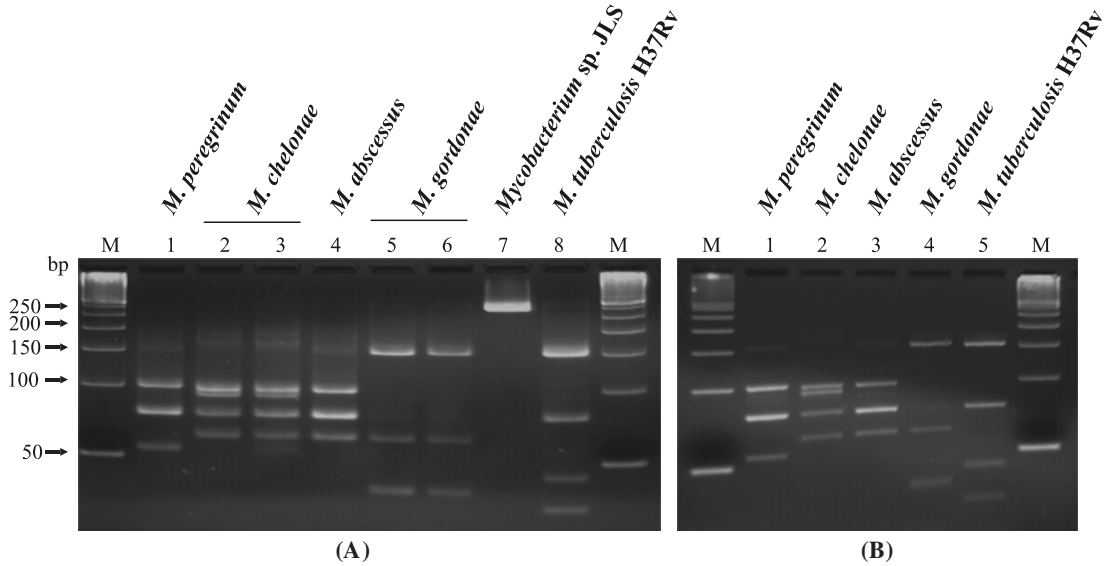

Fig. 2. The profiles of the *rpoB* nested PCR-RFLP following *Msp*I digestion and separation on a 4% Metaphore agarose gel. (A) PCR-RFLP profiles from the water samples. *M. tuberculosis* H37Rv (lane 8) was used as a positive control. (B) PCR-RFLP profiles from the reference mycobacterial strains. Lane M is a 50 bp DNA size marker.

ncbi.nlm.nih.gov/BLAST). The sites recognized by the *Msp*I restriction enzyme were confirmed using the BioEdit Sequence Alignment Editor (Version 7.0).

Validation of the nested PCR-RFLP analysis for detection of NTM in a mixed bacteria solution

Our current nested PCR-RFLP analysis was validated using bacteria associated with nosocomial infections. Tests were conducted using *Escherichia coli*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Staphylococus aureus*, and *Mycobacterium abscessus*. Bacteria were grown on Luria-Bertani (LB) agar plates for *E. coli*, *P. aeruginosa*, and *S. aureus* for 1 day at 37°C; on buffered charcoal yeast extract (BCYE) agar plates for *L. pneumophila* for 3 days at 35°C in 2.5% CO_2; or on Middlebrook 7H10 agar plates for *M. abscessus* for 7 days at 37°C. An inoculum consistent with a 0.5 McFarland turbidity standard ($\sim 1\times10^8$ CFU/ml) for each bacteria was prepared in saline (0.85% NaCl). Fifty microliters of each test bacterial suspension was added to 5 ml of saline, mixed thoroughly, and then ten-fold serially diluted in saline to 10^{-5}. *M. tuberculosis* H37Rv was used as a positive control for the PCR. DNA extraction and the nested PCR-RFLP were done as described above.

Results

PCR targeting the *rpoB* gene

To develop a method for rapid detection and differentiation of NTM to the species level, we applied an in-house PCR method to assay water samples obtained from hospital taps. Here we used the *rpoB* gene for detection of the mycobacteria genus. Thirty-two water samples concentrated by filtration were tested. None of the PCR products was detectable in the first round PCR from the water samples (Fig. 1A). As anticipated, a 360 bp PCR product was amplified in the positive control (Fig. 1A, lane 8; *M. tuberculosis* H37Rv). The inner primers were newly designed for the nested PCR in this study. Of the thirty-two water samples, seven samples (7/32, 21.9%) produced 327 bp PCR products in the nested reaction (Fig. 1B, lanes 1~7), whereas the reference mycobacterial strains produced detectable PCR products in both the first and the nested reactions (Fig. 1C and D).

RFLP analysis

After purification of the PCR products, the *Msp*I restriction enzyme was reacted with each sample for the discrimination of NTM to the species level. As shown in Fig. 2A, five different RFLP profiles were generated from the nested PCR products obtained from the water samples. These restriction fragment profiles were then compared with those of the reference mycobacterial strains (Fig. 2A vs B). The RFLP profiles of the PCR products from the water samples correlated with those of the reference strains. Based on the similarity of the RFLP profiles between Fig. 2A and B, we identified the NTM species to be *M. peregrinum* (1 case; hospital A, cold water; Fig. 2A, lane 1), *M. chelonae* (2 cases; hospital B, hot water; Fig. 2A, lanes 2 and 3), *M. abscessus* (1 case; hospital C, hot water; Fig. 2A, lane 4), and *M. gordonae* (2 cases; hospital A, hot water; Fig. 2A, lanes 5 and 6). One sample (Fig. 2A, lane 7) could not be assigned to the species level because the PCR product was not digested by the *Msp*I restriction enzyme. For further investigation and confirmation of the NTM identities, all the *rpoB* nested PCR products were sequenced and analyzed.

Sequence analysis

We analyzed the PCR product sequences using the BLASTN public database and the BioEdit sequence alignment editor.

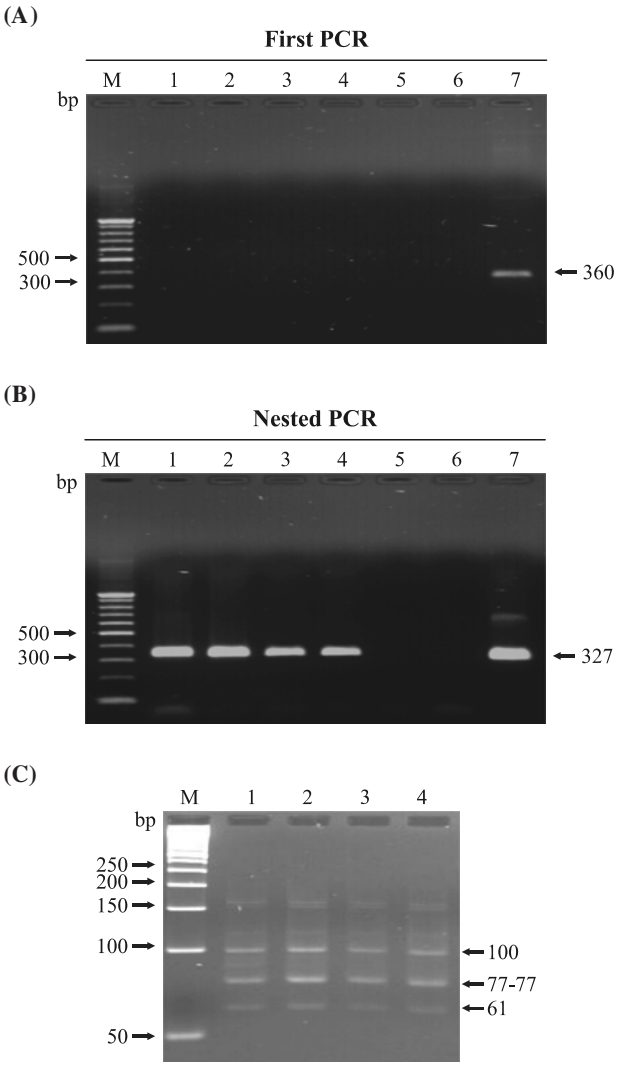

Fig. 3. Validation of the *rpoB* nested PCR-RFLP for the detection of NTM in a mixed bacterial solution. (A) Fifty microliters of the each bacterial (*E. coli*, *L. pneumophila*, *P. aeruginosa*, *S. aureus*, and *M. abscessus*) solution with a 0.5 McFarland turbidity was added to 5 ml of saline solution, and then the mixed solution was tenfold serially diluted (10^0 to 10^{-5}). None of the PCR products were amplified in the first PCR. *M. tuberculosis* H37Rv (lane 7) was used as a positive control and produced a 360 bp PCR amplicon. (B) The 327 bp nested PCR products were amplified in a 10^3 dilution of the mixed bacterial solution (lanes 1~4). Lane 7 is a positive control (*M. tuberculosis* H37Rv). M is a 100 bp DNA ladder (A and B). (C) PCR-RFLP profiles following *Msp*I digestion of the nested PCR amplicons in Fig. 3B, lanes 1~4, after separation on a 4% Metapore agarose gel.

Table 3. Calculated fragment sizes of the *rpoB* gene amplicons following *Msp*I digestion from different species of mycobacteria

Lane no.	*Mycobacterium* species	GenBank accession no.	Restriction fragments size (bp)
1	*M. peregrinum* strain CIP 105382T	AY147166	102-77-77-53
2∼3	*M. chelonae* strain CIP 104535T	AY147163	100-94-78-61
4	*M. abscessus* strain CIP 104536T	AY262741	100-77-77-61
5∼6	*M. gordonae* strain 126/1/03	AM885890	156-64-39-38
7	*Mycobacterium* sp. JLS	CP000580	No digestion
8	*M. tuberculosis* H37Rv[a]	U12205	154-77-44-33

[a] Reference strain

To correlate the sample sequences and the database sequences, we considered ≥99% sequence identities for the *rpoB* gene to correspond to the species level. The results from sequence identification exactly matched those of the nested PCR-RFLP analysis.

For lane 1 in Fig. 2A, the sequences represented the *M. peregrinum* strain CIP 105382T with 99.3% identity (290/292, E-value; 5e-145). The sequences were very closely matched between lanes 2 and 3 (Fig. 2A), and they were identified as the *M. chelonae* strain CIP 104535T with 99% identity (295/298, E-value; 7e-149) and 99.7% identity (301/302, E-value; 3e-152), respectively. Lane 4 (Fig. 2A) was identified as the *M. abscessus* strain CIP 104536T with 99% identity (296/299, E-value; 2e-149). Lanes 5 and 6 (Fig. 2A) were identified as the *M. gordonae* strain 126/1/03, both with 99.3% identity (286/288, E-value; 5e-145). The PCR product in lane 7 (Fig. 2A) was not identified in the nested PCR-RFLP analysis because it did not digest with the *Msp*I restriction enzyme. In the sequences analysis, the *rpoB* gene sequence was 99.3% (296/298, E-value; 1e-146) identical to the database sequence for *Mycobacterium* sp. JLS (1 case; hospital B, cold water). *M. tuberculosis* H37Rv was used as a control strain (Fig. 2A, lane 8).

The exact sizes of the *Msp*I fragments were calculated based on the sequences results, and the species-specific banding patterns are summarized in Table 3. Multiple fragments of the same size resolved as bands with an increased intensity in the gel. Here we obtained unambiguous results for the identification of *Mycobacterium* species using culture-independent molecular methods.

Validation of the nested PCR-RFLP analysis

The current nested PCR-RFLP analysis used for the detection of NTM species was validated using a mixed bacterial solution. The bacteria cells (*E. coli*, *L. pneumophila*, *P. aeruginosa*, *S. aureus*, and *M. abscessus*) were mixed in saline solution and then PCR was conducted. No PCR products were detected from the 10^0 to 10^{-5} sample dilutions following the first PCR (Fig. 3A, lanes 1∼6). As anticipated, the positive control strain (*M. tuberculosis* H37Rv) amplified a 360 bp PCR product (Fig. 3A, lane 7). However, nested PCR products of 327 bp were observed in the 10^0 to 10^{-3} sample dilutions (Fig. 3B, lanes 1∼4) and the positive control (lane 7). After purification, each of the nested-PCR products from the mixed bacterial solution was cut with *Msp*I. The PCR- RFLP analysis showed exactly the same pattern (100-77-77-61) as that of the *M. abscessus* (Fig. 3C, lanes 1∼4 vs Fig. 2A, lane 4 and Fig. 2B, lane 3).

Discussion

NTM are considered normal inhabitants of natural reservoirs, such as natural water sources, soil, aerosols, animals, and humans (Primm *et al*., 2004). Among a wide variety of environmental reservoirs, water is known to be a primary source of mycobacterial disease, especially in immunocompromised patients (Chang *et al*., 2002; Prime *et al*., 2004; Vaerewijck *et al*., 2005). NTM have an extremely hydrophobic outer membrane that is composed of lipids and mycolic acids, and this hydrophobicity plays a major role in water survival via biofilm formation on hydrophobic surfaces (Hall-Stoodley and Lappin-Scott, 1998; Recht *et al*., 2000). The hydrophobicity of the mycobacterial membrane also contributes to aerosolization, which is an important characteristic as it relates to transmission (Reddy, 1998). Several authors have demonstrated the presence of mycobacteria in hospital water distribution systems. According to Chang *et al*. (2002), 20.4% (10/49) of hospital water samples tested positive for NTM, including *M. szuigai*, *M. simiae*, *M. scrofulaceum*, *M. gastri*/*M. kansasii*, *M. gordonae*, and *M. fortuitum,* via culture methods. However, none of the water samples tested positive by direct PCR-RFLP that targeted the heat shock protein 65. Shin *et al*. (2007) reported that half of the samples from the hospital tap water system (50/100) were positive for NTM, and these isolates were identified as *M. gordonae* (type I, III-1, and IV), *M. mucogenicum*, *M. kansasii* III, and *M. chelonae*.

In the present study, the hospital tap water samples included both slowly growing NTM (SGM; *M. gordonae*) and rapidly growing NTM (RGM; *M. abscessus*, *M. chelonae*, *M. peregrinum*, and *Mycobacterium* sp. JLS) species. According to the recently released guidelines for NTM diagnosis by the American Thoracic Society (Griffith *et al*., 2007), identification of RGM species, such as *M. chelonae*, *M. abscessus*, and *M. fortuitum*, is strongly recommended. 16S rRNA sequencing is commonly used for NTM species identification; however, closely related species, such as *M. chelonae* and *M. abscessus*, are known to be difficult to distinguish via this method (Hall *et al*., 2003; Yam *et al*., 2006). In the present study, we were able to discriminate between *M. chelonae* and *M. abscessus* from tap-water samples on a 4% Metapore agarose gel (Fig. 2A, lanes 2 and 3 vs lane 4; Fig. 2B, lane 2 vs 3). *M. peregrinum* belongs to the *M. fortuitum* group (Brown-Elliott and Wallace, 2002), which was also clearly

distinguished from the *M. chelonae-M. abscessus* group (Fig 2A, lane 1 vs lanes 2∼4; Fig. 2B, lane 1 vs lanes 2 and 3).

Among the NTM identified by the nested PCR-RFLP analysis in the present study, *M. chelonae* and *M. gordonae* are known to be the most frequently reported NTM occurring in drinking water (Vaerewijck *et al*., 2005). The important clinical manifestation of *M. chelonae* (RGM) infection is skin, bone, and soft tissue disease (Wallace *et al*., 1992; Uslan *et al*., 2006). *M. gordonae* (SGM) is frequently considered a common laboratory contaminant. Although *M. gordonae* is known to be nonpathogenic, there are reported cases of *M. gordonae* infections, especially in patients with AIDS (Bonnet *et al*., 1996; Rusconi *et al*., 1997). A small number of sporadic infection cases due to *M. peregrinum* (RGM) have been reported, and the related diseases include chronic lung disease, sternal wound infections, and cutaneous disease (Brown-Elliott and Wallace, 2002). Cutaneous disease due to accidental trauma, acupuncture, or surgery in a variety of clinical settings was also reported in connection with *M. abscessus* (RGM) infection (Wallace *et al*., 1983; Song *et al*., 2006). *Mycobacterium* sp. JLS (RGM) detected in this study is a PAH (polycyclic aromatic hydrocarbon)-degrading mycobacteria, and it was first isolated from creosote-contaminated soil (Miller *et al*., 2004). *Mycobacterium* sp. JLS is nonpathogenic, but the clinical implication of its detection needs to be evaluated. The *Mycobacterium avium* complex, the most common opportunistic bacterial pathogens causing disseminated disease in patients infected with HIV, was not detected in this study. Although NTM are uncommonly encountered clinical pathogens, they can cause clinical diseases under some circumstances. Therefore, when NTM are isolated from specimens, the isolates should be considered with the clinical aspects. For the treatment of NTM diseases, identification of NTM to the species level is strongly recommended, as suggested by Griffith *et al*. (2007).

In the present study, none of the mycobacteria were isolated from the water samples under our culture conditions. The properties of the water samples from the three hospitals were not significantly different (Table 1). The concentration of free and total chlorine was almost undetectable in the hot water samples. The environmental conditions of the water distribution system, including pH, temperature, nutrition, etc., can influence the growth of mycobacteria (George and Falkinham, 1986; Kubálek *et al*., 1995). Although many authors have reported isolates of NTM from water distribution systems, a number of authors failed to isolate NTM (Vaerewijck *et al*., 2005). It may be assumed that very low numbers of (or dead) mycobacteria temporary existing in the water samples or unfavorable culture conditions might have influenced our results. The significance of our results is that we were able to clearly detect the existence of NTM species in the water samples using nested PCR-RFLP analysis. Therefore, our direct detection and discrimination method for NTM in water samples will be very useful to predict the prevalence of NTM species, especially fastidious NTM and slowly growing mycobacteria.

Lastly, we validated the current nested PCR-RFLP method for the detection of NTM using a mixed bacterial solution. *rpoB* is the gene encoding a highly conserved subunit of the bacterial RNA polymerase (Drancourt *et al*., 2004). Therefore, we assessed the primers used in this study to evaluate whether they amplified the *rpoB* gene regions from non-mycobacteria, such as *E. coli*, *L. pneumophila*, *P. aeruginosa*, and *S. aureus*. None of the above bacteria produced products in the first or the nested PCR (data not shown). In addition, we tested the feasibility of the nested PCR-RFLP using a saline solution that included the above non-mycobacteria and *M. abscessus*. In the nested PCR, a 327 bp PCR product was detected in the mixed bacterial solution (Fig. 3B) and the RFLP pattern coincided with those of *M. abscessus* (Fig. 3C, lanes 1∼4 vs Fig. 2A, lane 4 and Fig. 2B, lane 3). Although, we verified that the detected NTM from the hospital tap water samples as correct using only a limited number of non-mycobacteria, these non-mycobacteria are the most common nosocomial-bacteria (Carroll, 2002; Vonberg and Gastmeier, 2007). Therefore, we infer that our current nested PCR-RFLP is a very promising tool for the detection and discrimination of NTM species in water containing several different bacteria.

In conclusion, we have shown the existence of NTM species, including *M. peregrinum*, *M. chelonae* (2 cases), *M. abscessus*, *M. gordonae* (2 cases), and *Mycobacterium* sp. JLS, in hospital tap water distribution systems using culture-independent molecular methods. Although we could not isolate NTM from the water samples, our culture-independent method will be a useful tool as part of epidemiological investigations. In further research, the relationship between NTM isolates from water distribution systems and their clinical implications should be intensively evaluated in connection with public health.

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