Detection of Waterborne Pathogens by Polymerase Chain Reaction-Reverse Blot Hybridization Assay

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Dedicated to my family and my friends, who have encouraged me.

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ABBREVIATIONS

BHI	:	brain heart infusion
CFU	:	colony forming unit
CPC	:	cetylpyridinium chloride
CTAB	:	cethyltrimethyl ammonium bromide
DEPC	:	diethylpyrocarbonate
EDTA	:	ethylenediaminetetraacetic acid
NTM	:	nontuberculous mycobacteria
PCR	:	polymerase chain reaction
REBA	:	reverse blot hybridization assay
SDS	:	sodium dodecyl sulfate
SSPE	:	saline-sodium phosphate-EDTA
TBE	:	tris-borate EDTA
TNTC	:	too numerous to count
VBNC	:	viable but nonculturable

ABSTRACT

Detection of Waterborne Pathogens by Polymerase Chain Reaction-Reverse Blot Hybridization Assay

Traditionally, detection and enumeration of waterborne pathogens have focused on the use of selective culture and standard biochemical tests. This classical microbiological methodology relies on the cultivation of indicator bacteria, for example plate counts of total coliforms or *Escherichia coli.* Such methods have a variety of serious drawbacks, like no correlation to many waterborne pathogens and no valid identification of the pathogen. For example, opportunistic pathogen which normally occur at low levels in the environment tend to incur large errors in sampling and enumeration. Also, some waterborne pathogens (e.g., *Shigella* spp. and *Mycobacterium* spp.) are documented to remain viable for extended periods of time, but are unrecoverable by otherwise successful culture protocols. However, recent epidemiological evidence has shown that there is no correlation between *E. coli*/coliform count and pathological incidences.

Hence, conventional water testing methods are clearly outdated and do not adequately safeguard public health. Culture-based methods are time consuming, high cost, and labor intensive. Due to these limitations, examination of water samples for these opportunistic pathogen is usually not performed in routine assessment of water quality. In this regard, there exist absolute needs for developing more comprehensive system for assessing the safety of water. A sensitive, specific, and affordable test for rapid identification of waterborne pathogens is necessary to determine the impact of bacterial pathogens in water samples. The present study developed comprehensive system for assessing the safety of water using polymerase chain reaction-reverse blot hybridization assay (PCR-REBA). The aim of this study was to analyze the 16S rRNA gene diversity of Bacteria, as a supplementary tool to standard practices of microbiological quality in the whole water sample. This study described the development of waterborne pathogens monitoring system PCR-REBA. The PCR-REBA is capable of simultaneously detecting nineteen different types of waterborne pathogens in complex bacterial mixtures that are normally found in waters. PCR-REBA was developed to detect nineteen waterborne pathogens, using

two primer pairs for nested PCR and species-specific probes for each. The method was validated using a reference strain and established both sensitivity and specificity. To assess the ability of PCR-REBA to detect waterborne pathogens, diverse water samples were collected from eight wastewater treatment plants from November 2006 to July 2008. For detection to low population of target bacteria, membrane filtration method was conducted and amplified using the nested PCR for increase of sensitivity. Most of samples collected were positive for indicator bacteria and opportunistic pathogen. This study identified *Pseudomonas* spp. and *Yersinia* spp. which are the gram negative bacteria known to cause enteritis, and *Enterococcus* spp., *Listeria* spp. and *Staphylococcus* spp., the gram positive bacteria, in addition to the *E. coli* that are used as the indicator bacteria in the discharged water quality standard. In addition, as a result of PCR-REBA and sequence analysis after using cetylpyridinium chloride (CPC) and membrane filtration method, contamination of *Mycobacterium* spp. were confirmed in water samples. Isolated six strains of *Mycobacterium* spp. were potentially pathogenic mycobacterial species. Since the identified bacteria were continuously detected at all the sampling sites

regardless of the seasonal effect and the difference in the disinfection methods, these bacteria suggest to the indicator bacteria for water quality standard. This study shows that even though many innovative bacterial detection methods have been developed, PCR-REBA has the potential for becoming a standardized method for the detection of opportunistic pathogen in diverse water samples. Features of PCR-REBA include high overall rapidity, sensitivity and specificity for identifying waterborne pathogens.

Key Words: Waterborne pathogen, Polymerase chain reaction-reverse blot hybridization assay (PCR-REBA), Nested PCR, Cetylpyridinium chloride (CPC)

I. INTRODUCTION

Waterborne pathogens, disease causing germs in water, can be classified as bacteria, viruses, protozoa, or algae[1] (See Table 1 for a list of waterborne pathogens). Most of these infectious agents are spread by the fecal–oral route, in which water may play an intermediate role. This means that waterborne disease depends on factors such as environment, water, food, and poor hygiene (stemming from poverty, nutritional and immunity factors)[2]. However, waterborne pathogens not causing enteric illnesses (e.g., *Legionella* spp., *Mycobacterium* spp., and *Leptospira* spp.) have been also detected in water[3].

Globally, governments have taken measures to prevent outbreak of waterborne diseases. Protection of source water, advances in water treatment, and real-time monitoring of water-quality parameters represent some of the preventative measures that may one day relegate specific pathogen monitoring in areas with a high likelihood of a waterborne-disease outbreak[4].

Table 1. Examples of waterborne pathogens

Names of microorganisms	Major diseases	Sources
Salmonella Typhi	Typhoid fever	Human feces
Other *Salmonella*	Salmonellosis	Human feces
Shigella spp.	Cholera	Human feces
Enteropathogenic *E. coli*	Gastroenteritis	Human feces
Vibrio cholerae	Cholera	Human feces
Yersinia enterocolitica	Gastroenteritis	Human feces and animal feces
Various mycobacteria	Pulmonary illness	Soil and Water
Opportunistic bacteria	Variable	Natural waters

Modified from Lemarchand K *et al*., 2004[5].

Despite advances in preventing waterborne disease, outbreaks still occur, even in developed nations like the United States (*Cryptosporidium* spp.), Canada (*Escherichia coli* O157:H7), the United Kingdom, and many countries in Europe (several outbreaks of *Cryptosporidium* spp.). Thus, specific detection methods are still required to trace the origin of etiological agents and identify lapses in water treatment and new quality-control processes and procedures[3,4].

Pathogens are commonly found in low concentrations in water and thus cannot be easily detected and enumerated. As a result, it is extremely difficult to test water samples for each specific pathogen to assure water quality. Indicator microorganisms are used to indirectly suggest the presence of pathogens in water[5]. Total coliforms, fecal coliforms and fecal enterococci are the most commonly used indicator microorganisms for identifying of contamination in water.

However, some indicator organisms have disadvantageous survival characteristics such as *Yersinia* spp., *Staphylococcus aureus*, *Cryptosporidium* spp., *Giardia* spp. and *Mycobacterium* spp. In addition, one fecal indicator cannot represent the overall picture of the presence of enteric bacterial, viral and protozoan

contaminants in water[1,3,6] . Therefore, a combined application of alternative and conventional indicators should lead to a more comprehensive picture of fecal contamination, its source, and its association with bacteria pathogens.

Routine microbiological monitoring of waterborne pathogens is needed to prevent the spread of waterborne disease and to safeguard public health[7]. However, monitoring microbial parameters have a number of drawbacks. The current monitoring microbial parameters in water employs microbiological analysis which includes a process of water sampling and filtration followed by cultivation of the chosen microorganism using enrichment, selective cultures, and biochemistry tests[8]. The whole process usually takes 24 to 72 hours and still may not pick up all microorganisms if appropriate selective media and growth conditions are not provided[9].

Moreover, many organisms such as enterotoxigenic *E. coli*, *Vibrio cholerae*, *Enterococcus* spp. and *Mycobacterium* spp. may be present in water samples, but are unculturable[10]. Several problems arise with viable culture methods used for routine monitoring, including lack of growth of viable but nonculturable (VBNC) bacteria such as those stressed by chemicals in the water, time required for detection and confirmation of enteric bacteria (days),

and lack of specificity for detection of true fecal coliforms (*E. coli*)[11,12].

Therefore, extensive efforts have been spent to develop a more comprehensive method for monitoring all microbial water contaminants. Recently, researchers have applied molecular methods to the rapid detection of pathogens in food, soil, and water. Some of these methods allow detection of specific culturable and/or non-culturable bacteria within hours instead of the days required with complicated cultivation and additional identification process[13,14].

Molecular methods such as polymerase chain reaction (PCR)[15], real-time PCR[16], oligonucleotide arrays[17], reverse blot hybridization assay (REBA)[18] have been focused on the detection of waterborne pathogens. Many molecular methods are based on the molecular analysis of complex samples for detecting bacteria with respect to their rRNA genes.

These detection techniques can be classified into two major categories: PCR amplification and hybridization-based approaches using specific probes. Within these categories, the number of alternative methodologies increases continuously, and a vast number of variations have been proposed, each with its

inherent advantages and limitations but aimed at specific purposes[19]. These systems are usually used to confirm the diagnosis of specific clinical syndromes or specific bacterial strains[20]. Previous reports suggest that DNA-based methods targeting rRNA gene are more sensitive and rapid than the conventional bacteriological methods[21].

Most of molecular methods developed so far are based on the molecular analysis of bacteria with respect to their 16S rRNA or its own genes. These approaches take advantage of usefulness of 16S rRNA, a stable taxonomic marker for microorganisms, given that genetic variations in 16S rRNA are intergenus and interspecies. Thus, the variation in 16S rRNA can be utilized for the designing of species- and genus-specific genetic markers[22,23]. Currently, a number of studies have reported successful PCR primers and 16S rRNA-based oligonucleotide probes specific for bacterial pathogens such as *E. coli*[24], *Listeria monocytogenes*[25], *Salmonella* spp.[25], *Staphylococcus* spp.[26], *Enterococcus* spp.[27], and *Bacillus* spp.[28] in PCR amplification and DNA hybridization.

The major advantage of 16S rRNA-hybridization permits the analysis of unanalyzed bacteria, an impossible task with quantitative PCR-based methods. Oligonucleotide probes used in

16S rRNA-hybridization are phylogenetically targeted and specifically detect bacterial species or groups[29]. Probes can be designed for species-specific detection of a single or a few species or even a large group of related species. Probes are available for species present in intestinal samples, and researchers have estimated they can detect approximately 90% of fecal bacteria with a set of a few probes[29].

DNA-hybridization techniques have been reported to have successfully detected and identified waterborne pathogens[21,30]. DNA microarray is especially powerful since it can simultaneously detect multiple targets by one assay[31,32]. However, while an attractive model, DNA microarray is quite costly for many practical settings.

On the other hand, PCR-REBA employing multiple target probes is affordable in many practical settings including small clinical laboratories since it is relatively simple, cheap, and still as informative as DNA microarray. Several studies have reported the development of PCR-REBA, but those studies targeted no more than six waterborne bacterial species[33].

Therefore, the present study was set out to develop PCR-REBA which can be more comprehensive for the detection of waterborne pathogens. The development of a new PCR-REBA targeted to

one strain (*E. coli* O157:H7), ten species (*E. coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Y. pseudotuberculosis*, *Mycobacterium avium* complex and *M. marinum*, *Enterococcus faecalis*, and *Staphylococcus aureus*) and eight genera (*Shigella* spp., *Salmonella* spp., *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Yersinia* spp., *Mycobacterium* spp., and *Listeria* spp.) of waterborne pathogens simultaneously.

The PCR-REBA for detection and identification of waterborne pathogen was evaluated using water samples obtained from municipal wastewater treatment plants over a period of three years in Korea. Then, the results of PCR-REBA were compared with those of conventional microbiological methods.

II. MATERIALS AND METHODS

1. Development of PCR-REBA targeting waterborne pathogens

Bacterial reference strains and cultivation

Nineteen bacterial strains were obtained from the Clinical Microbiology Laboratory in the Department of Biomedical Laboratory Science, Yonsei University (Table 2). All of the strains were grown overnight at 37°C in brain heart infusion (BHI) broth and agar plates (Becton Dickinson, Franklin Lake, NJ, U.S.A.) excepted for genus *Mycobacterium* spp., *M. avium* and *M. marinum* were cultured at 37°C, 5% CO_2 in Middlebrook 7H9 broth and 7H11 agar plate (Becton Dickinson, Franklin Lake, NJ, U.S.A.).

Table 2. Bacterial strains used in this study

Genus	Species	Source
Escherichia	*coli* O157:H7	ATCC[a] 43894, STEC
	coli O157:H7	ATCC 35150
Salmonella	*coli*	ATCC 25922
	Typhi	ATCC 19430
Shigella	*dysenteriae*	DML[b] 400
	flexneri	ATCC 9199
	sonnei	ATCC 25931
	boydii	DML 399
Klebsiella	*pneumoniae*	ATCC 35657
Citrobacter	*freundii*	ATCC 6750
Enterobacter	*aerogenes*	ATCC 13048
Pseudomonas	*aeruginosa*	ATCC 27853
Yersinia	*pseudotuberculosis*	ATCC 29833
	enterocolitica	ATCC 9610
Enterococcus	*faecalis*	ATCC 19433
Listeria	*monocytogenes*	ATCC 15313
Staphylococcus	*aureus*	ATCC 25923
Mycobacterium	*avium*	ATCC 25291
	marinum	ATCC 927

[a]American Type Culture Collection.
[b]Diagnostic Microbiology Laboratory.

Genomic DNA extraction from reference strains

The bacterial genomic DNA was prepared using the cethyltrimethyl ammonium bromide (CTAB) method as previously described[17].

The concentration of genomic DNA was determined by optical density readings at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). The extracted DNA was determined by electrophoresis of each DNA through a 1% (w/v) tris-borate EDTA (TBE) agarose gel. The DNA was stored at 4°C until required.

Preparation of PCR primers for 16S rRNA gene

To design specific primer molecules, 16S rRNA gene sequences of diverse waterborne pathogenic strains were compiled from the GenBank database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), and the sequence data for the target bacteria were aligned using the clustral method (http://www.-cmbi.kun.nl/bioinf/tools/clustalws.html). The present study used partial 16S rRNA gene sequences flanking variable regions (V), nucleotide position from 40 to 500 relative to the published *E. coli* 16S rRNA gene sequence. The primer sequence was chosen from conserved regions in aligned 16S rRNA gene sequences. All primers were provided by Bioneer (Daejeon, Korea)(Table 3).

Table 3. Oligonucleotide sequences used to amplify 16S rRNA gene

Primer		Oligonucleotide sequence (5' to 3')	Nucleotide position of *E. coli*
1st PCR	16S-SF2*	CR†K†GCY†TAAY†ACATGCAAGTCGA	42 - 65
	16S-RSH-A5**	TGGCACGD†AGTTR†GCCGK†K†GCTT	498 - 520
2nd PCR	16S-SF	AAY†ACATGCAAGTCGAR†CK	50 - 68
	16S-R5H-A	ACR†D†AK†TTRGCCGKK†GCTT	498 - 516

* 16S-SF_2 : 42-65 of *E. coli* 16S rRNA numbering.

** 16S-R5H-A_5 : 498-520 of *E. coli* 16S rRNA numbering.

†Y=C:T, M=C:A, R=G:A, D=G:A:T, K=T:G.

PCR amplification for waterborne pathogens and other opportunistic pathogen

In order to directly amplify 16S rRNA genes from environmental samples, the DNA fragment was amplified by using the nested PCR using the two primer sets targeted to conserved regions. For the first round of PCR amplification, 16S-SF_2 and 16S-RSH-A_5 were used, and the 16S-SF and 16S-R5H-A were used for the second round of PCR amplification. The 5' end of reverse primers was labeled with biotin to generate hybridization signaling in REBA. The amplification reactions were performed in a thermal cycler (GeneAmp PCR® System 2700, Applied Biosystems, Foster city, CA, U.S.A.).

PCR amplification was carried out in a 25 ㎕ reaction volume containing *Taq* buffer (10 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl), 200 μM dNTPs, 20 pmole of each primers, 1 unit of *Taq* DNA polymerase (Cosmo Co., Seoul, Korea), 10 ng of genomic DNA extracted from bacterial culture. Sterile distilled water added to give a 25 ㎕ final volume.

For the nested PCR, the first 15 cycles of PCR were performed as follows: initial denaturation at 95°C for 30 seconds, annealing at

62°C for 30 seconds, and extension at 72°C for 1 minute. Subsequently, the 35 cycles of the second PCR were performed by a program consisting of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 1 minute. After the final cycle, samples were maintained at 72°C for 10 minutes to complete the synthesis of all strands. In every PCR assay, sterile distilled water in place of bacterial genomic DNA was added to PCR mixture to act as a PCR negative control.

Following the amplification, 5 ㎕ of each PCR products were electrophoresed on a 1.8% TBE agarose gel to confirm successful amplification. The gel was stained with 0.05 ㎎/㎖ ethidium bromide solution, visualized with an Agaro-power^{TM} system (Bioneer Co., Daejeon, Korea), and photographed with the Gel Doc EQ system (Bio-Rad, Hercules, CA, U.S.A.).

Analysis of sensitivity for nested PCR

To determine the sensitivity of PCR, the genomic DNA from *E. coli* was serially ten-fold diluted to establish a concentration range from 100 ng to 100 fg. PCR amplification was performed in the presence of primer pair with 16S-SF and 16S-R5H-A, 16S-SF_2 and 16S-R5H-A_5.

Preparation of oligonucleotide probes for REBA

While the conserved regions were adopted as universal PCR primers for all the bacteria, variable regions of the 16S rRNA genes were utilized for the probe molecules to gram negative, gram positive and to each species or genus of bacteria targeted[24]. Table 4 lists all probes used in this study with their sequences and modification. Target bacterial species selected in this study included the strains of common waterborne pathogens that may cause diarrhea or vomiting such as *E. coli, E. coli* O157:H7 and *Shigella* spp.. And major disease-causing mycobacteria in contaminated water are extremely difficult to culture such as *M. avium* complex, and *M. marinum* these were also targeted.

The genus-specific oligonucleotide probes were designed based on the 16S rRNA gene sequences of the waterborne pathogenic bacteria obtained from the NCBI database (Bethesda, MD). Sequence data of the strains were aligned by the clustal W method (http: //www.cmbi.kun.nl/ bioinf/tools/clustalw.shtml). The selection of oligonucleotide probes was designed to avoid self complementarity more than three nucleotides of probe. BLAST search (National Center for Biotechnology Information of the National Library of Medicine website) was utilized to

retrieve the sequence homology for each probes.

Oligonucleotide probes were labeled at the 5’ end with the amine group for covalently binding with membrane. An optimal concentration of each probe was determined by binding varied amounts of the probe in such a way that all the probes resulted in equally intense signals relative to the catchall at a concentration ranging from 10 to 400 pmole.

Table 4. List of oligonucleotide probes for identification of waterborne pathogens

Probe name	Oligonucleotide sequence (5' to 3')	Modification
Universal probe for 16S rRNA gene	CCAGACTCCTACGGGAGGCAGCAGT	5'-amine
Gram-negative bacteria	GCTGGTCTGAGAGGATGAYCA	5'-amine
Gram-positive bacteria	AAA ACGGGTGAGTAACACGTGG	5'-amine
E. coli O157:H7	GTAACAGGAAGAAGCTTGCTTCTT	5'-amine
Shigella / *E. coli* spp.	GGGAGTAAAGTTAATACCTTTGCTC	5'-amine
Citrobacter freundii	TAGCACAGAGGAGCTTGCTCCTTGGG	5'-amine
Salmonella / *Citrobacter* spp.	TGTTGTGGTTAATAACCGCAGCAA	5'-amine
Klebsiella pneumoniae	GTTAATAACCTCATCGATTGACGT	5'-amine
Enterobacter / *Klebsiella* spp.	AACGTTAAGGTTAATAACCTTGG	5'-amine
Pseudomonas aeruginosa	GCAGTAAGTTAATACCTTGCTGTT	5'-amine
Yersinia enterocolitica	CCAATAACTTAATACGTTGTTGG	5'-amine
Yersinia pseudotuberculosis	GGTTGAGTTTAATACGCTCAATC	5'-amine
Yersinia spp.	CGGGAAGTAGTTTACTACTTTGCCGG	5'-amine
Mycobacterium avium	TCAAGACGCATGTCTTCTGGTG	5'-amine
Mycobacterium marinum	CCACGGGATTCATGTCCTGT	5'-amine
Mycobacterium spp.	CTTTTGCGGTGTGGGATGGGC	5'-amine
Enterococcus faecalis	ACAGTTTATGCCGCATGGCATAAGAGT	5'-amine
Listeria spp.	AAGAGCTTGCTCTTCCAAAGTTAGTGGC	5'-amine
Listeria spp. / *Staphylococcus aureus*	CATATGTGTAAGTAACTGTGCACAT	5'-amine

Covalent coupling of probe to the membrane

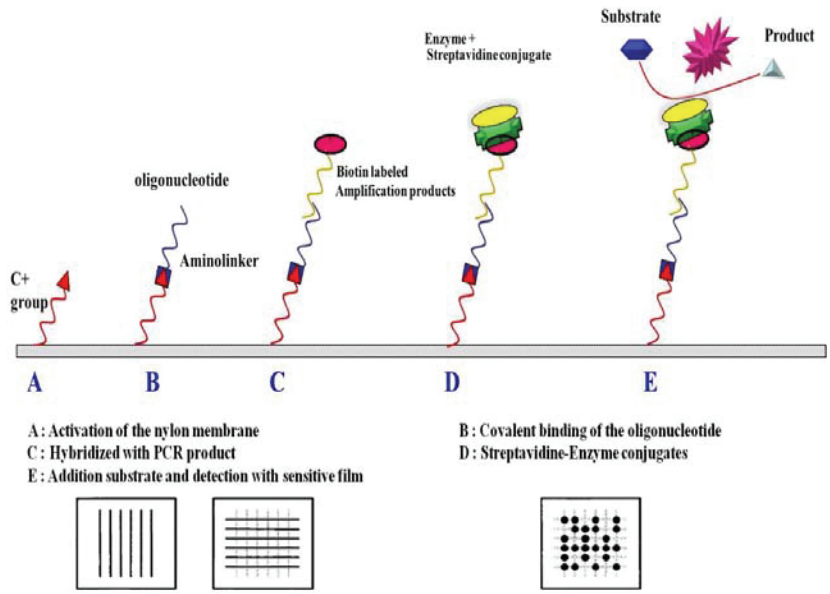
Preparation of the membrane containing probe molecules was performed using a system described elsewhere[34,35]. The amine groups were used to bind probes with the Biodyne C membrane (Pall Corporation, Port Washington, NY, U.S.A.) which contains the carboxyl groups. The Biodyne C membrane was activated by incubation for 15 minutes in 10 ㎖ of freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC, Sigma-Aldrich, St. Louis, MO, U.S.A.) with sterile distilled water at room temperature. All amino-linked oligonucleotides probes were diluted to the optimal concentration in total volume of 150 ㎕ with pH 8.4, 0.5 M sodium bicarbonate ($NaHCO_3$, Duksan, Ansan, Korea).

Following a brief wash with sterile distilled water, 150 ㎕ of each diluted oligonucleotide was applied to MN45 miniblotter system (MN45; Immunetics, Boston, MA, U.S.A.). The first two and the last slot were used to mark the lane with 1:100 diluted stamp ink.

After incubation, the residual solutions were removed from the slots by aspiration. The membrane was detached from the miniblotter and inactivated by incubation with 0.1 M sodium

hydroxide (NaOH, Duksan, Ansan, Korea) for 9 minutes at room temperature, followed by a brief wash with 5 minutes incubation in 2 × saline-sodium phosphate-EDTA (SSPE)/0.1% SDS at 60°C. The membrane was sealed in a plastic bag and stored at 4°C until use (Fig. 1).

Figure 1. The principle of the Reverse blot hybridization assay.



Modified from Bunschoten A *et al*., 2000[35].

REBA

To perform REBA, the membrane was incubated in 2 × SSPE/0.1% SDS for 5 minutes at room temperature and then inserted into the miniblotter apparatus. The lines of the previously applied oligonucleotides were perpendicular to the sample lanes. Residual liquid was removed by vacuum aspiration. The biotin-labeled PCR amplicons of 16S rRNA gene were hybridized to the oligonucleotides membrane using the following protocol.

Twenty-five micrometer of each PCR product was mixed with an equal volume of denaturated solution (0.2 N NaOH, 0.2 mM EDTA) at room temperature for 10 minutes and then diluted with 100 $\mu\ell$ of 2 × SSPE/0.1% SDS. One hundred fifty micrometer of diluted PCR-amplified biotinylated product was added to one slot of the miniblotter. The entire apparatus was then incubated at 50°C for 90 minutes. Following hybridization, the samples were removed using an aspirator. The membrane was removed from the miniblotter and washed twice with 2 × SSPE/0.5% SDS at 62°C, for 10 minutes.

The membrane was transferred to a roller bottle and incubated in the presence of 1:2000 of streptavidin-conjugated alkaline phosphatase (Roche, Penzberg, Upper Bavaria,

Germany) diluted with 2 × SSPE/0.5% SDS for 1 hour at 42°C. The membrane was washed twice with 2 × SSPE/0.5% SDS for 10 minutes at 42°C and then twice with 2 × SSPE for 5 minutes at room temperature.

For luminescent detection, the membrane was incubated with CDP-star detection solution (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 4 minutes and sandwiched between two sheets of transparency film. The Hyper™film (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was exposed for 30 minutes and 1 hour and then developed. For repeated use (up to 12 times), the membrane was stripped twice by 30 minutes incubation in 1% SDS at 80°C. The membrane was incubated in 20 mM EDTA (Duksan, Ansan, Korea) for 15 minutes at room temperature and stored at 4°C.

Sensitivity comparison of PCR and REBA

For analyzing sensitivity of PCR, the bacterial number of *E. coli* O157:H7 ATCC 43894 was measured by colony-forming units (CFU) counts. The bacteria were grown for 18 hours at 37°C and the resultant bacterial colonies were counted to express bacteria concentrations as CFU per ㎖ of culture[36].

Physiological saline (0.85% NaCl) was used for ten-fold

diluted bacterial cells to achieve the final bacterial number ranging from 1.2×10^8 to 1.2×10^1 CFU/㎖. The bacteria solution was then used to measure the number of bacteria by CFU count and to extract total RNA.

Cultured cells were lysed in 1 ㎖ TRIZOL reagent (Invitrogen, Carlsbad, CA, U.S.A.). Total bacterial RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure[37]. The extracted RNA was resuspended in 25 ㎕ of diethylpyrocarbonate-treated water (DEPC, Biosewoom Inc., Seoul, Korea). Concentration of total RNA was determined by optical density readings at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). The extracted RNA was treat with RNase-free DNase I (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions.

Complementary DNA (cDNA) was generated using RT-Premix (Bioneer Co., Daejeon, Korea) for 60 minutes at 42℃, and the reaction was terminated by heating at 94℃ for 5 minutes. Nested PCR was carried out with the cDNA template as previously described for determining sensitivity of REBA. The results were compared with those of CFU counts obtained from same bacterial culture.

2. Application of REBA for detection of waterborne pathogens in diverse water samples

Site description and water sample collection

Water samples were provided by the Division of Biotechnology, Catholic University, Seoul. Water samples were collected from geographically dispersed sites in Figure 2. In this study six sites of wastewater treatment plants were monitored for over two months of period between 2006 and 2008. The discharging effluents and reusable water from seven municipal wastewater treatment plants have been surveyed nine months period between November 2006 and July 2007, eight municipal wastewater treatment plants surveyed eleven months period September 2007 and July 2008. The sites include examples of eight specific discharge types.

At each sampling site, water temperature, biochemical oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (T-N), total phosphorus (T-P), and suspended solid (SS) were measured in turbid water samples, defined by the Division of Biotechnology, Catholic University in accordance with the standard methods[38].

Water samples were collected aseptically in 2,000 ㎖ sterile disposable plastic containers and stored in the dark inside a cool box during transport to the laboratory. The samples were handled according to guidelines suggested by the Ministry of Environment of Korea and analyzed within 6 hours from sampling.

The samples were then transported to an laboratory for additional analysis. Various approaches for culturing the target bacteria following filtration were generally accessed. The target bacteria were cultured either by using pre-enrichment using broth media or by placing a filter paper on the differential and selective media[38].

Figure 2. The locations of the six water sampling sites in Korea. A: Incheon city, B: Bucheon city, C: Jungnang-gu, Seoul city, D: Yeoju-gun, E: Munmak, wonju city, F: Wonju city.

Microbiological characteristics of water samples

Laboratory analyses included measurements of water quality, indicator bacteria, and waterborne pathogens. Filtration processes for bacteriological analyses were done within 6 hours. Total bacteria, total coliforms, *E. coli*, *S. aureus,* and Enterobacteriaceae were enumerated using membrane filtration techniques and identified using the spread plate method[38].

The presence of bacterial colony of total coliform was measured by m-Endo agar plates (Becton Dickinson, Franklin Lake, NJ, U.S.A.) incubated at 35°C for 24 hours. *E. coli* enumeration was done using EC plates incubated for 24 hours at 37°C. Total bacteria, Enterobacteriaceae and *S. aureus* were measured by standard plate count agar, MacConkey agar and mannitol salt agar, respectively.

Decontamination and filtration of water samples for cultural detection of nontuberculous mycobateria (NTM)

Experiments were carried out on the efficacy of chemical agents suitable for using with the membrane filtration assay to affect a substantial reduction in background organism populations and to

allow the optimal recovery and quantitation of NTM in water samples.

In order to detect environmental NTM, water samples pretreated with cetylpyridinium chloride (CPC) and NaOH were compared with drinking water, lake water, and groundwater samples. The water samples were analyzed by a method previously reported method[39,40]. In brief, 200 ㎖ of water samples were exposed to 0.01 ~ 0.1% CPC (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 1 ~ 4% NaOH (Duksan, Ansan, Korea) for 30 minutes at room temperature. After decontamination of water samples by exposure to CPC and NaOH, all water samples were filtered through 0.45 ㎛ pore size nitrocellulose membrane filters (Millipore Corp., Billerica, MA, U.S.A.) by vacuum filtration.

Bacterial culture of mycobacteria in water samples

After filtration, the membranes were rinsed with 1 × phosphate buffered saline (PBS) and transferred to Middlebrook 7H11 (Becton Dickinson, Franklin Lake, NJ, U.S.A.) agar plates. The plates were sealed and incubated at 37°C with 10% CO_2 for 8 weeks[41].

All plates were examined at weekly intervals for 8 weeks. After

8 weeks of incubation, all the colonies grown on the plates were enumerated. Individual colonies that look distinct from each other were then subcultured onto a new Middlebrook 7H11 agar plate. Growth of NTM was confirmed by staining for acid-fast bacilli.

Identification of mycobacteria by sequence analysis of 16S rRNA gene

Subsequently, DNA was extracted from a single isolated colony by the CTAB method, as stated above, and was subjected to PCR amplification of the 16S rRNA gene. The quality and the quantity of the extracted DNA were determined by electrophoresis on 1% TBE agarose gel.

All mycobacteria isolates cultured on the filter membranes were subjected to sequence analysis of 16S rRNA gene. The PCR primers, 16S–SF and 16S–R5H-A, were used in PCR amplification as previously described. The amplification reactions were performed in a thermal cycler (GeneAmp PCR® System 2700, Applied Biosystems, Foster city, CA, U.S.A.). The PCR conditions used were denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds and the total 35 cycles

of PCR were processed. Successful amplification was confirmed by agarose gel electrophoresis.

Sequence analysis of the PCR products was requested to Genotech (Daejeon, Korea). The results of sequence analysis were analyzed by comparing them with GenBank database in National Center for Biotechnology Information (web site: http://www.ncbi.nlm.nih.gov). Species identification was done on the basis of sequences homology to corresponding sequences in the GenBank database.

Enumerating process for waterborne pathogens

After decontamination procedure, membrane filtration was carried out in two steps. As shown in Figure 3**,** within 6 hours after sampling, microorganisms were harvested by filtrating 200 ㎖ of water sample through 1.2 ㎛ pore size membrane filters (Millipore Corp., Billerica, MA, U.S.A.) to remove floating matters. Then, the water sample were filtered through 0.45 ㎛ pore size membrane filters. After filtration, the membranes containing the bacteria were placed on a selective and differential media and incubated at 37°C for periods ranging from 1 day to 8 weeks.

As shown in the Figure 4, each pathogen were isolated from

enriched media to identify each species, subcultured on selective media, and then tested for species characteristics. The membrane filters were subsequently transferred onto MacConkey agar (Becton Dickinson, Franklin Lake, NJ, U.S.A.) for gram negative bacteria, onto MacConkey Sorbitol agarfor *Escherichia coli* O157:H7, onto xylose lysine desoxycholate (XLD) agar for *Shigella* spp., onto Salmonella Shigella (SS) agar for *Salmonella* spp. and *Shigella* spp., onto Pseudomonas F agar for *Pseudomonas aeruginosa*, to Cefsulodin Irgasan Novobiocin (CIN) agar for *Yersinia* spp., onto PALCAM medium for *Listeria* spp., onto bile esculin azide agar for *Enterococcus faecalis*, onto mannitol salt for *Staphylococcus aureus*, and onto Middlebrook 7H9 broth and Middlebrook 7H11 agar for *Mycobacterium* spp.

Bacterial colonies with typical *E. coli* O157:H7 and *Shigella* morphology were tested by slide agglutination with *E. coli* O15:H7*, Shigella* antiserum (Becton Dickinson, Franklin Lake, NJ, U.S.A.). Most bacteria colonies became evident after incubation at 37°C for 24 hours, but *Mycobacterium* spp. took 2 weeks to 8 weeks to grow[42]. The organism species were identified by the appropriate API® strips (BioMerieux, rue des Aqueducs, Craponne, France).

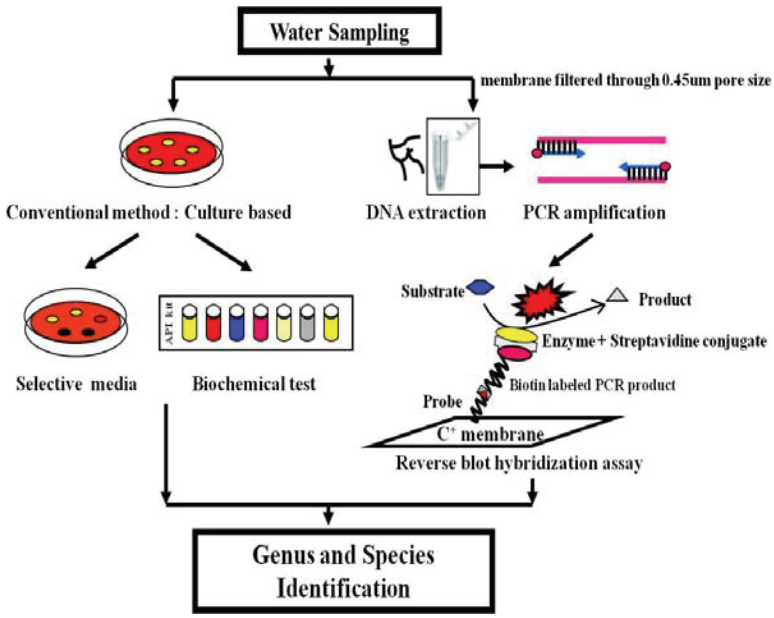


Figure 3. Schematic illustration of bacteria species identification.

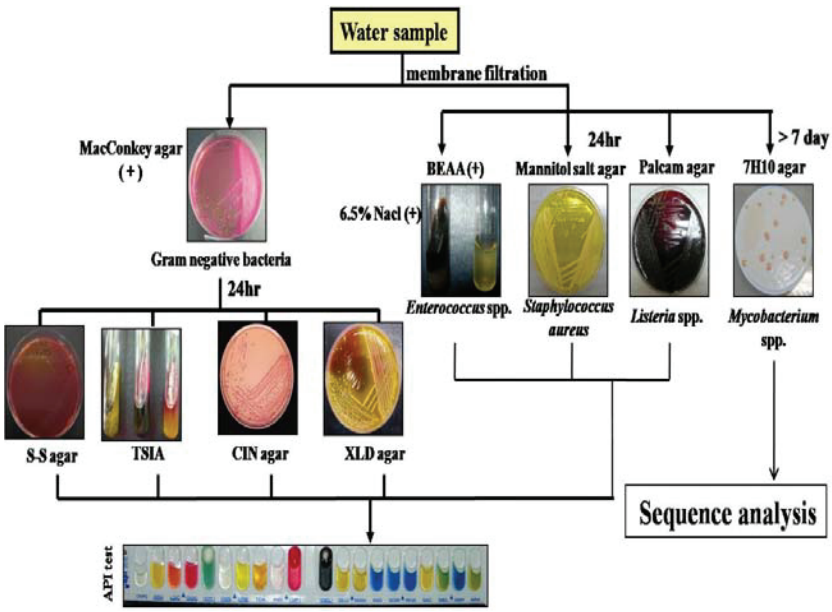


Figure 4. Identification of microorganisms in diverse water samples used in this study using conventional tests, selective media, and API® strips.

DNA purification from waterborne pathogens and other opportunistic pathogens

Wastewater samples were filtered through 0.45 μm pore size membrane filters. The filtered membranes containing the bacteria were pretreated with lysozyme (Roche, Penzberg, Upper Bavaria, Germany) at 37°C overnight to impair bacterial cell walls. Then, DNA was extracted from the collected bacteria using the CTAB method.

Application of PCR and REBA for detection of waterborne pathogens

Nested PCR amplification for amplifying the 16S rRNA gene was performed using primers listed in Table 3 under the same conditions described above. The amplified PCR products were then subjected to REBA, as previously stated.

III. **RESULTS**

1. Development of REBA targeting waterborne pathogens

Efficiency of 16S rRNA gene PCR primer for detection of waterborne pathogens

The 16S rRNA genes have been most frequently used for detection and identification of bacteria, primarily as a result of the inherent signal amplification afforded by the large number (10^3~10^5) of rRNA copies available for hybridization or further amplification.

Comparison of the anatomy in 16S rRNA genes have shown the presence of relatively conserved areas and variable regions (V region) between different bacterial species[43]. Species-specific DNA probes have been designed based on variable 16S rRNA regions and used for detection of specific target cells[44].

To construct the probe for REBA to detect waterborne pathogens, four consensus primers, 16S-SF, 16S-SF_2, 16S-R5H-A, and 16S-A_5 were designed from the conserved regions flanking the variable region of the bacterial 16S rRNA gene.

Molecular weights of these PCR products were approximately 500 bp (Fig. 5).

The PCR primer pairs of 16S-SF and 16S-R5H-A, 16S-SF_2 and 16S-A_5 were able to successfully amplify the 16S ribosomal RNA gene of all nineteen bacterial strains.

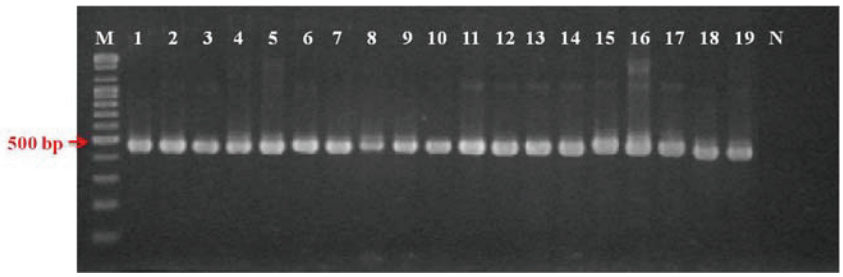


Figure 5. PCR amplification of waterborne pathogen DNA using 16S rRNA gene primers. Lane M; 100 bp DNA ladder (Bioneer Co., Daejeon, Korea), lane 1; *E. coli* O157:H7 ATCC 43894, lane 2; *E. coli* O157:H7 ATCC 35150, lane 3; *E. coli* ATCC 25922, lane 4; *S. flexneri* ATCC 9199, lane 5; *S. sonnei* ATCC 25931, lane 6; *S. dysenteriae* DML 400, lane 7; *S. boydii* DML 399, lane 8; *S.* Typhi ATCC 19430, lane 9; *C. freundii* ATCC 6750, lane 10; *K. pneumoniae* ATCC 35657, lane 11; *E. aerogenes* ATCC 13048, lane 12; *Y. enterocolitica* ATCC 9610, lane 13; *Y. pseudotuberculosis* ATCC 29833, lane 14; *P. aeruginosa* ATCC 27853, lane 15; *E. faecalis* ATCC 19433, lane 16; *L. monocytogenes* ATCC 15313, lane 17; *S. aureus* ATCC 25923, lane 18; *M. avium* ATCC 25291, lane 19; *M. marinum* ATCC 927, N; Negative control.

Sensitivity of PCR amplification from reference strains

Serially diluted *E. coli* O157:H7 ATCC 43894 genomic DNA amounts ranging from 100 ng to 10 fg were used to determine the sensitivity of the PCR assay (Fig. 6).

Under the experimental conditions, PCR products amplified between 100 ng and 100 fg could be on 1.8% TBE agarose gel. This results, indicates that the 16S rRNA gene target was amplified in all template dilutions down to 100 fg of genomic DNA. According to an estimated DNA concentration per *E. coli* O157:H7 cell of 5.1 fg/cell, this detection limit correspond to approximately 19 cells[45,46].

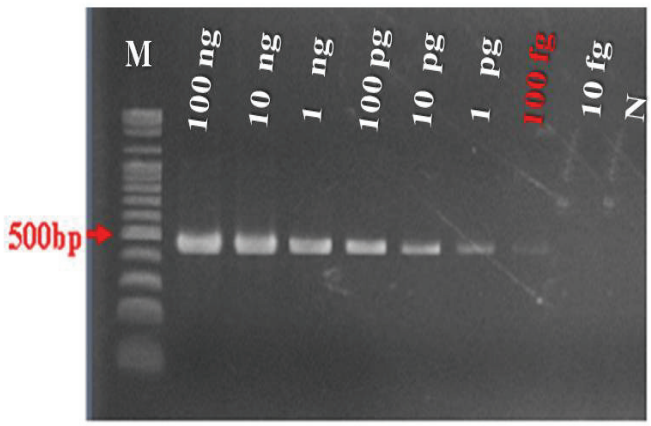


Figure 6. Sensitivity of the PCR using 16S rRNA gene primers and genomic DNA of *E. coli* O157:H7 ATCC 43894. Lane M; 100 bp DNA ladder (Bioneer Co., Daejeon, Korea), lane 1; 100 ng, lane 2; 10 ng, lane 3; 1 ng, 4: 100 pg, lane 5; 10 pg, lane 6; 1 pg, lane 7; 100 fg, lane 8; 10 fg, lane N; negative control.

Development of REBA for the detection of waterborne pathogens

Target bacterial species selected in this study included strains of common waterborne pathogens that may cause gastroenteritis such as *E. coli*, *E. coli* O157:H7, *Shigella* spp., *Salmonella* spp., *C. freundii*, *K. pneumoniae*, *E. aerogenes*, *P. aeruginosa*, *Yersinia* spp., *Y. enterocolitica*, *Y. pseudotuberculosis*, *E. faecalis*, *Listeria* spp., *S. aureus*, *M. avium* complex and *M. marinum* that are major mycobacteria causing diseases by contaminated water and extremely difficult to culture were also targeted.

PCR amplicons of the bacteria 16S rRNA genes using consensus primers were hybridized to the immobilized probes on the membrane. Designed probes in this study are listed in Table 4.

The specificities of the probes for detection of each target bacteria are shown in Figure 7. As shown in Figure 7, hybridization results precisely agreed with those predicted from the probe sequences. The nine species-specific probes (*C. freundii*, *K. pneumoniae*, *P. aeruginosa*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *M. avium* complex, *M. marinum*, *E. faecalis*, and *S. aureus*), one strain-specific probe (*E. coli* O157:H7), and three genus-specific probes (*Yersinia* spp., *Listeria* spp., and *Mycobacterium* spp.) were hybridized to their targets and did not show any cross reactivity.

On the other hand, some probes were not specific to the relevant genus. For example, *Shigella* spp. and *E. coli*, *Salmonella* spp. and *Citrobacter* spp., *Klebsiella* spp. and *Enterobacter* spp., *Listeria* spp. and *Staphylococcus aureus* hybridized to the same probe, since they have the same 16S rRNA gene sequences. Therefore, all probes did not show cross-reaction with other DNA sequences.

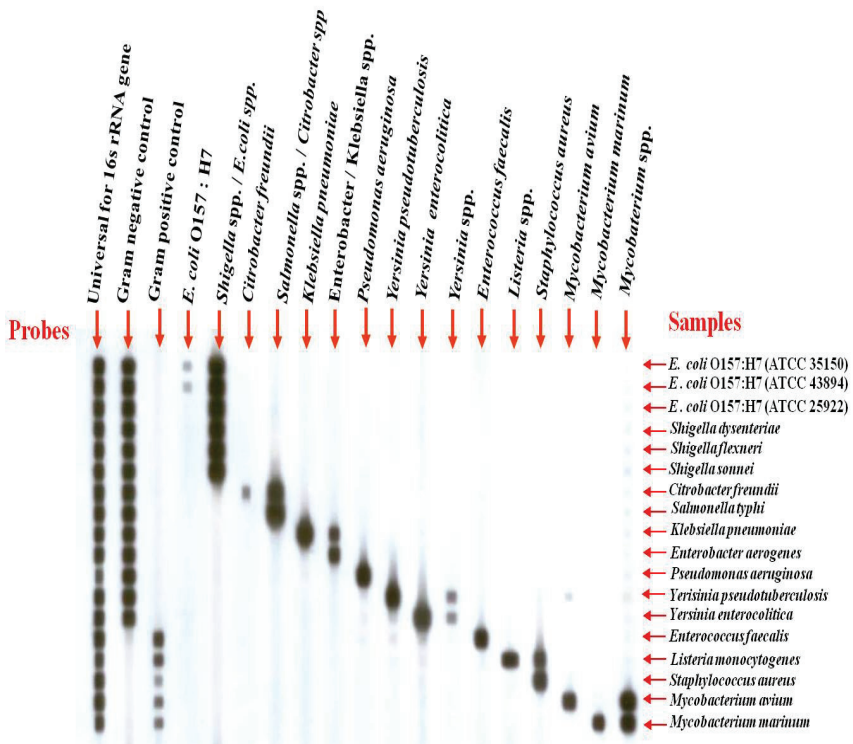


Figure 7. Specificity of the REBA from reference strains of waterborne pathogens. PCR amplicons obtained from the reference strains of bacterial DNA were hybridized to the probes bound to the membrane.

Sensitivity analysis of REBA

Alignment of waterborne pathogens 16S rRNA gene sequences revealed conserved regions where the DNA sequences were identical among all species and divergent regions where the sequences differ between each individual species or groups of species. A conserved region was used to design the universal probe, while divergent regions were used to design the gram negative, gram positive and species-specific probes.

Different parameters for array hybridization, such as temperature, incubation time, salt composition, and shaking speed were optimized for both the specificity and sensitivity of the array system.

The detection limit of REBA was determined using a serially diluted genomic DNA of reference strains. A series of 10-fold genomic DNA dilutions from 10 ng to 1 fg were hybridized to their probes in nylon membrane. As shown in Figure 8, the minimum detection limit of the REBA ranged from 1 pg to 1 fg for different species. The nineteen probes did not show any cross-reaction with other DNA sequences. Therefore, all bacteria species could be differentiated by their specificity with probes and target genes.

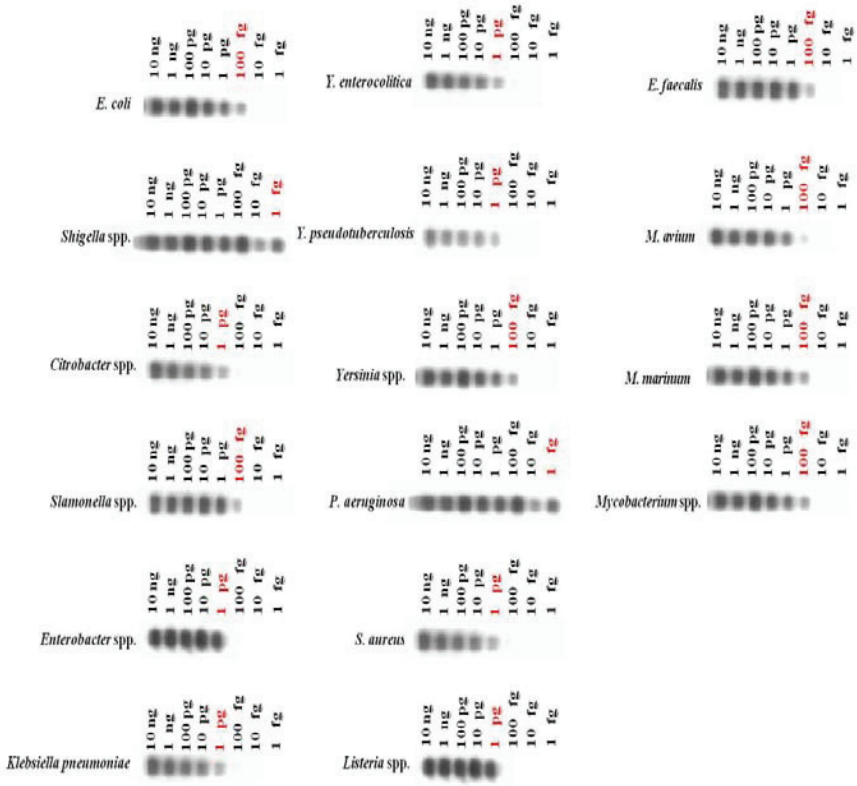


Figure 8. Determination of REBA sensitivity using reference strains of waterborne pathogens. PCR amplicons from the reference DNA of bacterial strains were hybridized to their respective probe.

Comparison of sensitivities of PCR and REBA

Already known numbers of *E. coli* O157:H7 was serially diluted from 1.2×10^8 CFU/㎖ to 1.2×10^1 CFU/㎖. These serially diluted *E. coli* O157:H7 samples were used to confirm CFU and total RNA extraction for determining sensitivity of PCR and REBA.

The sensitivity of PCR was estimated by RNA obtained from serially diluted *E. coli* O157:H7 cultured cells. The sensitivities of REBA using single PCR, one set of primers (16S-SF, 16S-R5H-A) and nested PCR using two sets of PCR primers (16S-SF, 16S-R5H-A and 16S-SF_2, 16S-R5H-A_5) were compared to each other (Figs. 9A and 9B). The results showed that the sensitivities of both PCRs were between 1.2×10^5 CFU/㎖ and 1.2×10^4 CFU/㎖, respectively.

Subsequently, the sensitivity of the REBA using each PCR amplicons were also compared (Figs. 9C and 9D). The sensitivity of hybridization ranges were from 1.2×10^4 CFU/㎖ to 1.2×10^3 CFU/㎖ (Figs. 9C and 9D), which showed more than at least 10 times higher sensitivity in REBA than that of in PCR.

In some cases, the PCR product was not detected on the

ethidium bromide-stained agarose gel. However, after hybridization of the PCR products to the REBA, hybridized signals were clearly visualized. The PCR method followed by hybridization of the products would appeared to improve detection sensitivity.

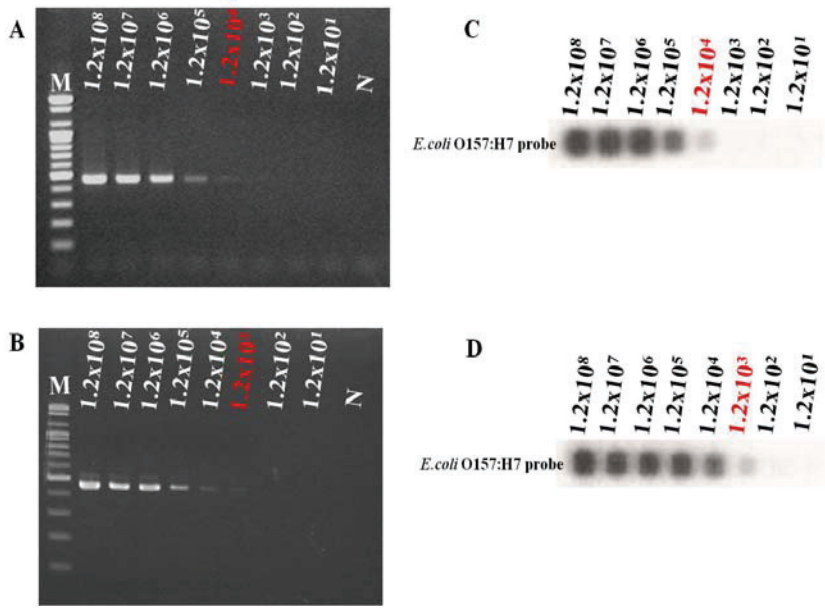


Figure 9. Comparison of sensitivities of PCR and REBA using RNA of *E. coli* O157:H7 ATCC 43894. The sensitivity of (A) single PCR using one set of primers (16S-SF, 16S-R5H-A) and (B) nested PCR using two sets of PCR primers (16S-SF, 16S-R5H-A and 16S-SF_2, 16S-R5H-A_5). The sensitivity of the REBA using PCR amplicons from single (C) and nested PCR (D) was compared. To compare the sensitivity of the PCR with that of REBA, PCR amplicons obtained from (A) and (B) were subsequently hybridized to the REBA membrane containing probe for *E. coli* O157:H7, M; 100 bp DNA ladder (Bioneer Co., Daejeon, Korea). (C) shows the REBA result with (A), and (D) shows the REBA results with (B).

2. Applications of REBA for detection of waterborne pathogens in diverse water samples

Microbiological data for water quality parameters

The microbiological analyses of water samples were performed and reported in Figure 10 and Supplemental data 1-6. Microbial indicators included total coliform, *E. coli*, *S. aureus*, Enterobacteriaceae. Figure 10 summarized microbial indicator concentration of eight water samples. Nearly all the collected samples were positive for opportunistic pathogen (*S. aureus*) and indicator bacteria (total microorganism, total coliform, *E. coli*, Enterobacteriaceae). In general, no apparent relationship between concentrations of microbiological parameters and disinfectant treatment was observed.

In the total coliform count, *E. coli* is one of the indicator bacteria, exhibited its largest populations in September 2010. An increase in the number of microbes in summer is implies that temperature greatly influences the proliferation of indicator bacteria and other microorganisms.

Members of the family Enterobacteriaceae are gram negative and non-spore former. For example, fecal bacteria such as *E. coli*

and *Salmonella* spp., are widely distributed in soil and water[45]. Their presence was observed in season changes. Therefore, the presence of Enterobacteriaceae is more significant than that of the specified indicator organisms.

Unlike other bacteria, *S. aureus* is most prevalent in winter which is a widespread gram positive cocci, and causes food poisoning and dermatitis. *E. coli* and total coliform are not well correlated with *S. aureus*.

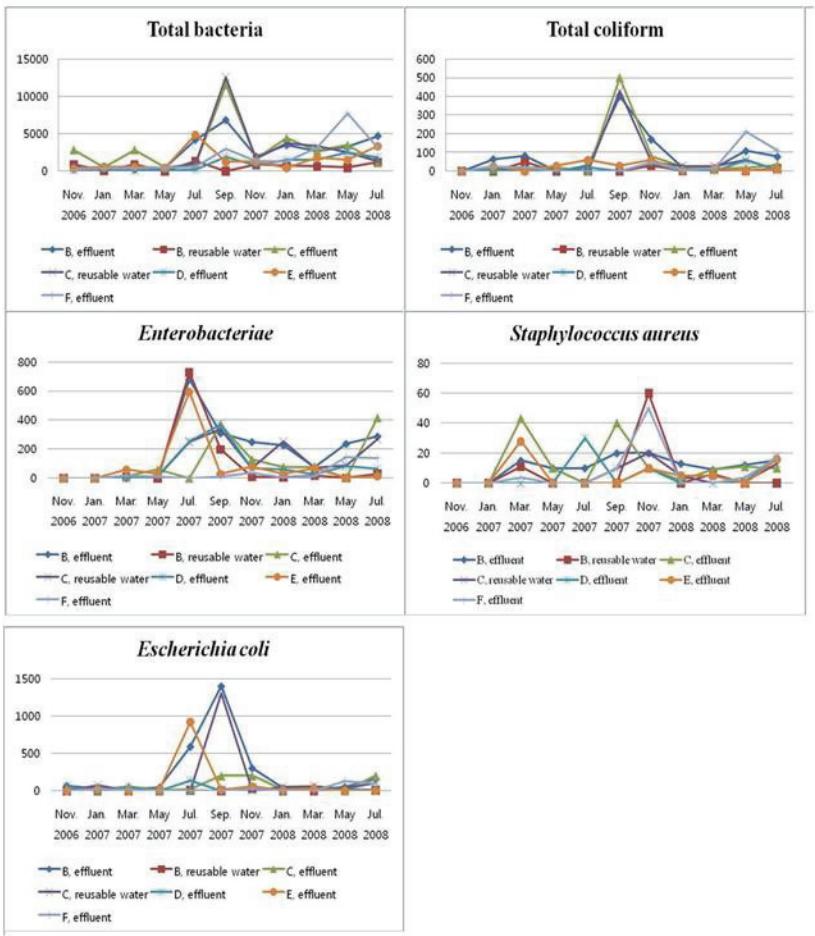


Figure 10. Microbiological data for water quality parameters in treated wastewater.

Comparison of decontamination efficiencies in isolation of mycobacteria from water samples

Mycobacteria has been identified in a broad range of environmental sources, including water, soil, food, plants, dust, and aerosols[45,47].

In order to compare the effect of CPC and NaOH treatment on mycobacterial isolation, water samples were taken from the lake and underground water. Those samples were exposed to 0.01% to 0.1% CPC and 1% to 4% NaOH for 30 minutes at room temperature and were subsequently plated on solid agar media.

The effects of the different chemical decontamination methods were significantly different from each other. As shown in Table 5, when treated with 1% to 4% NaOH, innumerable microbial organisms grew on the plate (too numerous to count or TNTC), making it impossible to identify any colonies. On the other hand, treated at more than 0.04% of cetylpyridinium chloride (CPC), countable numbers of individual colonies grew on the agar plate and colony isolating was possible. This study demonstrated that use of CPC for decontamination is effective for isolating mycobacteria from water samples.

Table 5. Effects of different concentrations of CPC and NaOH on microbial growth isolated from the surface water of lake and underground water

		Surface water of lake		Underground water	
		Non mycobacterial colony	Mycobacterial colony	Non mycobacterial colony	Mycobacterial colony
Cetylpyridinium chloride (CPC)	0.01%	TNTC*	TNTC	TNTC	TNTC
	0.02%	TNTC	TNTC	TNTC	TNTC
	0.04%	10	3	7	3
	0.05%	6	4	3	2
	0.08%	4	1	0	0
	0.10%	0	0	0	0
Sodium hydroxide (NaOH)	1%	TNTC	TNTC	TNTC	TNTC
	2%	TNTC	TNTC	TNTC	TNTC
	3%	TNTC	TNTC	TNTC	TNTC
	4%	TNTC	TNTC	5	3

*TNTC : too numerous to count

Optimization of CPC concentration for isolation of mycobacteria from water samples

In order to evaluate the usefulness of CPC for more diverse water samples, ten water samples from various water sources: eight waste water sample from water treatment plants, the surface water from one lake, and underground water. The water samples were treated with different concentrations of CPC and were cultured on the plates. The experimental results indicated that all mycobacterial species detected in this study are not equally resistant to the all decontamination procedures.

As shown in Table 6, the counts of mycobacterial colonies from the water samples ranged from 1 CFU/100 ㎖ to TNTC. The highest level of countable mycobacterial growth was obtained from diverse water samples treated with 0.04% CPC. At the same concentration, the non-mycobacterial growth became controllable and mycobacterial colonies were also countable.

The optimal CPC concentration to isolate mycobacteria appeared to be 0.04%. Identified each colony of mycobacteria or non-mycobacteria was the based on sequence analysis of each individual colony in the previous experiment. Overall, 0.04% CPC appeared to be the most effective in

reducing non-mycobacterial contaminants, allowing good recovery of mycobacteria from water samples.

Treatment with 0.04% CPC worked the best, as it succeeded in totally eliminating contamination; both slow and rapid growers could be isolated. Thus, this study used decontamination treated with 0.04% CPC concentration for the efficient recovery of mycobacteria from effluent.

Table 6. Effects of different concentrations of CPC on microbial growth isolated from diverse water samples

	Effluent (n=8)		Surface water of lake (n=1)		Underground water (n=1)	
	Non mycobacterial colony	Mycobacterial colony	Non mycobacterial colony	Mycobacterial colony	Non mycobacterial colony	Mycobacterial colony
0.01%	TNTC*	TNTC	TNTC	TNTC	TNTC	TNTC
0.02%	TNTC	TNTC	TNTC	TNTC	12	4
0.04%	26	14	10	20	5	3
0.05%	18	11	10	15	0	1
0.08%	5	2	0	2	0	0
0.1%	0	1	0	0	0	0

*TNTC : too numerous to count

Identification of mycobacteria using the culture methods and 16S rRNA gene sequence analysis

All isolated colonies were confirmed by 16S rRNA gene sequence analysis developed in this study. Among one hundred fifty nine individual colonies, eighty six colonies (54%) were determined to contain eight non-mycobacterial species (Table 7) and seventy three colonies (45%) were determined to contain six mycobacterial species (Table 8).

The analysis of 16S rRNA genes were subjected to GenBank database in National Center for Biotechnology Information (web site: http://www.ncbi.nlm.nih.gov/) data. According to the sequence analysis, mycobacterial species were found to belong to *Mycobacterium gilvum, M. peregrinum*, *M. lentiflavum*, *M. mageritense, M. mucogenicum* and *M. wolinskyi*. These strains had more than 98 % homologies with the respective reference strains. *M. mageritense* and *M. mucogenicum* were the most commonly existing mycobacterial species that were isolated from all three kinds of water samples. *M. gilvum*, *M. lentiflavum*, *M. peregrinum*, and *M. wolinskyi*, considered to be human opportunistic pathogens, were also isolated. Both rapid- and

slow-growing mycobacteria including human potentially pathogenic species were isolated[44].

There have been many studies using different methods to isolate mycobacteria from water samples. Mycobacteria were successfully isolated from diverse water samples treated with optimized concentration (0.04%) of CPC. This study confirmed the findings of previous reports that CPC inhibits the growth of non-mycobacteria and facilitates the growth of mycobacteria[47].

Table 7. Identification of non-mycobacterial isolates based on 16S rRNA gene sequence analysis

Sources	Species isolated	Highest similarity, %*	No. of colonies sequenced (Total = 86)
Effluent	*Herbaspirillum rubrisubalbicans*	99%	18
	Burkholderia spp.	98%	11
	Bacillus cereus	98%	8
	Serratia marcescens	99%	5
	Acinetobacter spp.	99%	5
	Micrococcus luteus	99%	2
Surface water of lake	*Aeromonas hydrophila*	99%	15
	Arthrobacter davidanieli	98%	5
Underground water	*Serratia marcescens*	98%	12
	Acinetobacter spp.	98%	5

*** Identified by comparison against NCBI Blast (http://www.ncbi.nlm.nih.gov/blast/)**

Table 8. Identification of mycobacterial isolates based on 16S rRNA gene sequence analysis

Sources	Species isolated	Highest similarity, %*	No. of colonies sequenced (Total = 73)
Effluent	*Mycobacterium gilvum*	99%	3
	Mycobacterium lentiflavum	98%	2
	Mycobacterium mageritense	100%	8
	Mycobacterium mucogenicum	98%	5
	Mycobacterium peregrinum	98%	5
	Mycobacterium wolinskyi	98%	5
Surface water of lake	*Mycobacterium mageritense*	100%	20
	Mycobacterium mucogenicum	98%	8
	Mycobacterium peregrinum	98%	7
Underground water	*Mycobacterium mucogenicum*	98%	5
	Mycobacterium mageritense	98%	5

Detection of waterborne pathogens and other opportunistic pathogens using nested PCR

After filtering of the water samples without culture methods, genomic DNA were extracted from filter membranes and PCR amplification repeatedly failed PCR processing. Determining the concentration of bacteria from water samples for PCR and REBA is generally done by the following two steps. Firstly, the water samples were passed through membrane filters using a 1.2 ㎛ pore size filter to remove the organic matrix. Then, bacteria were involves enumerated by passing through a 0.45 ㎛ pore size membrane filter[48].

Water samples containing individual waterborne pathogens were amplified with a pathogen specific primer and placed on membrane for REBA. The ability of REBA was assessed to specifically detect PCR amplified products.

Applications of REBA and comparison to culture methods for identification of waterborne pathogens

To assess the ability of REBA to detect waterborne pathogens, water samples collected from the wastewater treatment plants were analyzed

between November 2006 and July 2008. The REBA results were compared with those identified by the culture methods (Table 9 and Supplemental data 7-16, Fig. 11).

The presence of seventeen bacteria groups, including *E. coli* O157:H7, *E. coli*, *Shigella* spp., *Salmonella* spp., *C. freudii*, *Enterobacter* spp., *Klebsiella* spp., *P. aeruginosa*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *Yersinia* spp., *Listeria* spp., *S. aureus*, *E. faecalis*, *M. avium*, *M. marinum,* and *Mycobacterium* spp. was analyzed using REBA. Those bacteria were too difficult to culture the strains of common waterborne pathogens causing diarrhea. But the REBA was able to detect non-pathogenic bacteria in water samples using gram positive bacteria and gram negative bacteria probes.

When membrane filtration and decontamination are applied to as the concentrate bacteria, enumeration of mycobacteria can be done by means of REBA. Features of REBA include high overall sensitivity and specificity for identifying waterborne pathogens. The difficult culture methods can be replaced by REBA assay without culturing process.

Table 9. Detection of waterborne pathogens using REBA in treated water samples

Samples	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *M. avium* *S. aureus* *Y. pseudotuberculosis* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *M. avium* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp. *Yersinia* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *E. faecalis* *M. avium* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp. *Yersinia* spp.	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *K. pneumoniae* *M. marinium* *S. aureus* *Shigella* spp. *Salmonella* spp. *Citrobacter* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *S. aureus* *M. marinum* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp. *Yersinia* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp.

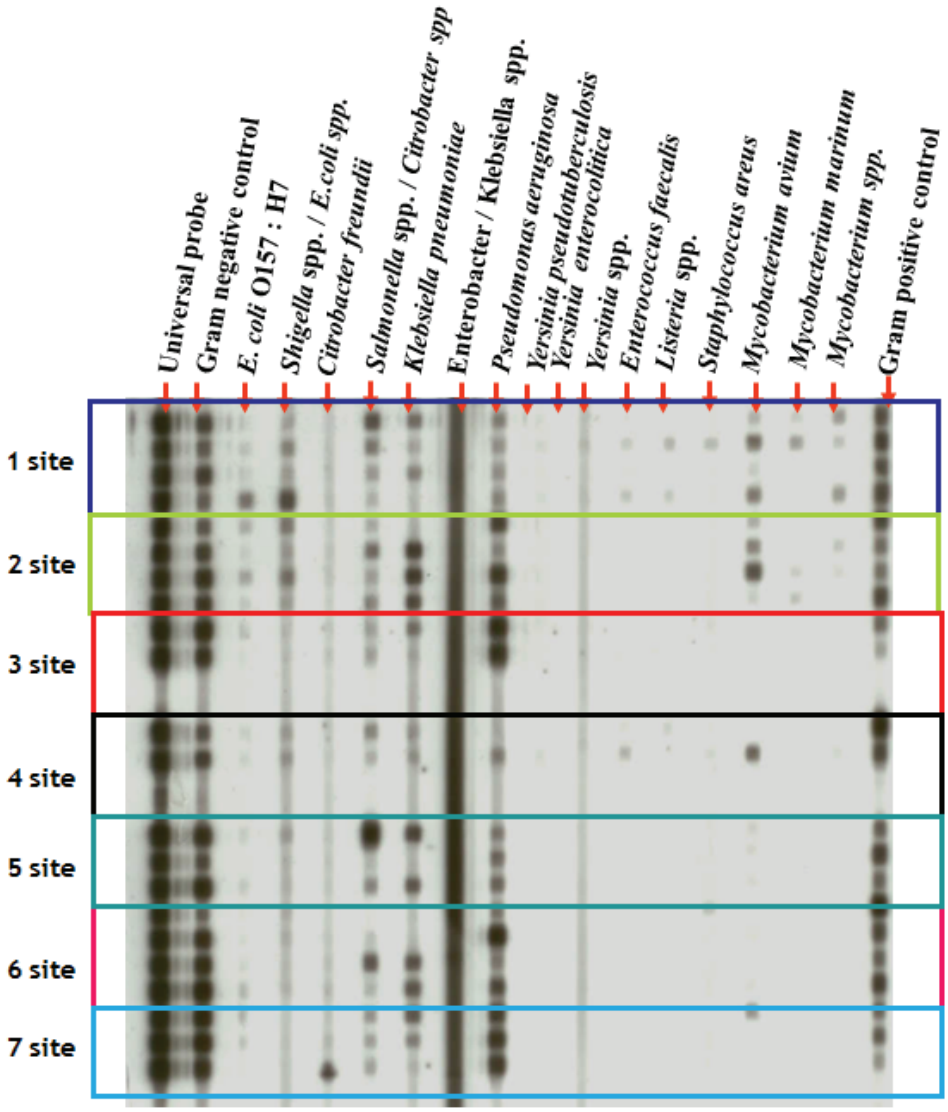


Figure 11. An application example in REBA for detection of waterborne pathogens.

IV. DISCUSSION

Clean safe water is an essential constituent to support our life. Microbial pathogens in water resources can cause bacterial enteric disease as the etiologic agent of outbreaks. In this regard, a comprehensive system to monitor of bacterial contamination in water are required to assess the safety of water source[4,49]. Furthermore, routine microbiological monitoring method for opportunistic pathogen in water is needed to prevent the spread of waterborne diseases and to protect public health. However, the lack of accurate, rapid, and cost-effective diagnostic tests poses a major challenge in prevention and control of infections and outbreaks produced by waterborne pathogens[7].

REBA has been shown to be useful for identification and typing of several bacterial species, but has been used infrequently for direct detection of pathogens in diverse samples[18,46,50]. The checkerboard REBA technique outlined in this study offers a number of advantages for detection and identification of multiple species of bacteria in large numbers of samples containing complex mixtures of microorganisms[51]. The REBA certainly satisfied requirements for simultaneous detection system of microbial

contaminants in water than other molecular methods. It is more convenient, less expensive, and easier to perform because it uses commonly available reagents, less expensive equipment, and can analyze 45 samples within a single run. The REBA format uses a non precipitating ECL substrate. Hence, it has the advantage in that nylon membrane can be stripped and successfully reused at least 5 times[15].

The aim of this study was to set up and develop a rapid, simple and specific technique for monitoring waterborne pathogens that may be present in water. The monitoring system was evaluated using effluents from eight municipal wastewater treatment plants between November 2006 and July 2008.

Nineteen target bacterial species selected in this study include species of opportunistic pathogens found in most bacterial waterborne outbreak (Table 2). As shown in Figure 7, REBA results precisely agreed with those predicted from the probe sequences. None of the nineteen specific probes cross-reacted with any non-target species among the reference strains or isolates. Detection limits of the REBA varied among the reference strains, from 1 pg to 1 fg of genomic DNA (Fig. 8). The sensitivity of the assay was 1.2×10^3 CFU/㎖ for *E. coli* O157:H7 16S rRNA gene (Fig. 9).

Effluents, one kind of water source, are reused for irrigation purposes in many countries around the world, all of the populated continents[52]. Therefore, mandated jurisdictions generally monitor and enforce effluent standards for wastewater treatment plants and drinking-water quality using bacterial indicators of fecal pollution and the presence of pathogens. Nowadays, guidelines and specific regulations are based on determining the levels of indicator organisms (coliform bacteria—either fecal coliform or *E. coli*) and the physical and chemical parameters of interest, including pH, temperature, dissolved oxygen, organic matter, particulate matter, and specific conductance[53,54].

The removal of coliform bacteria by disinfection procedures used in wastewater treatment plants or by those used to treat drinking water does not necessarily mean that pathogenic viruses and protozoa have also been removed[55-59]. Treatments such as UV irradiation and chemical disinfection are clearly more effective in eliminating bacteria than bacteriophages and viruses[59,60].

Therefore, indicators may be more suitable than coliform bacteria to assess the removal of pathogens during treatment and their subsequent presence in reclaimed water and fresh water. However, there are many other

species of bacteria that should not be present in the water as contaminants. There have been extensive efforts to develop a more comprehensive method for monitoring all microbial contaminants in water.

In this study, microbiological analyses were conducted according to guidelines suggested by the Ministry of Environment of Korea. The microbiological analyses of water samples from the eight wastewater treatment plants performed are presented in Supplemental data 1~6 and Figure 10. Most of the samples collected were positive for indicator bacteria and diverse opportunistic pathogens. It is known that indicator bacteria such as total coliform or *E. coli* are not suitable to evaluate various pollutions. Meanwhile, gram positive bacteria such as *S. aureus* are known to be more useful as the indicator bacteria than total coliform in evaluating contamination in water.

As shown in Figure 10, this study showed the distribution of opportunistic pathogen that exist in discharged water, causing various diseases in humans and domestic animals through environmental pollution pathway. The experimental results showed that the total coliform or *E. coli*, the indicator bacteria in the water from the waste water treatment plants was detected much more than 1.5 to 32 times during the summer season (July to

September). The increase in the number of bacteria in summer was also found in the cases of other indicator bacteria, which means that the growth of indicator bacteria was affected by temperature.

However, the *S. aureus* population was not affected by season changes. Rather, the total number of *S. aureus* was relatively high detection rate during the winter. Thus, it was assumed that *S. aureus* may be designated as a distinctive indicator bacterium, different from the indicator bacteria of *E. coli* that are easily affected by the season changes. Moreover, it was found that there was not a significant correlation between the discharged water disinfection method and survival of bacteria. The number of bacterial cells was smaller in the discharged water from small villages than in urban discharged water.

Additionally, according to the results of REBA (Table 9), it was difficult to monitor gram positive opportunistic pathogen which have different physiological properties from those of total coliform, *Mycobacteirium* spp. and *Listeria* spp.. Different from the conventional indicator organisms, they will survive in water environment since they are more resistant to chlorination, the conventional water disinfection method.

This study also included the classical microbiological identification using API® strip kit to examine the treated effluent wastewater samples. The results of the current study showed that, microbiological data identified the gram negative bacteria known to cause bacterial enteritis including *E. coli*, *Pseudomonas* spp. and *Yersinia* spp. and the gram positive bacteria including *Enterococcus* spp., *Listeria* spp. and *Staphylococcus* spp. Since the indentified bacteria were continuously detected in all the sampling sites regardless of the seasonal changes and the varied disinfection methods, it may be appropriate to add these type of bacteria to the current indicator bacteria for water quality monitoring.

Recently, there has been increasing evidence that mycobacteria from environmental sources are implicated in a variety of human diseases. mycobacteria are common saprophytes found in natural water, soil, food, dust, and aerosols[61]. Mycobacteria were also found even in drinking water[62], drinking water distribution systems[63,64], hot water systems[65], surface water, and diverse water samples[66,67]. Moreover, mycobacteria are not killed by common disinfectants and can tolerate wide ranges of pHs and temperatures, and those can persist in water for a long period of time[68].

Despite of the increasing clinical importance of mycobacteria, detection and

identification of mycobacteria from environmental sources have been greatly hindered due to their varied growth rates and specific growth requirements of mycobacteria and contamination by outgrowing other bacteria[69]. Therefore, proper limiting pretreatment methods is very important for growth enhancing the mycobacteria and growth suppressing the non-mycobacteria and fungi[70].

In the previous reports, substances that can kill other bacteria and fungi were able to increase the growth of mycobacterial culture with low contamination. Mineral acids (e.g., HCl) and bases (e.g., NaOH), organic acids (e.g., oxalic acid), and detergents have been used as decontaminating agents[71]. However, the pretreatment methods may interfere with the detection of certain species of mycobacteria resulting in reduction of both the rate of positive samples and the number of visible colonies seen.

According to the results of REBA, contamination with *Mycobacterium* spp. was observed in water samples. However, *Mycobacterium* spp. were not identified in water sample because of difficulties in isolation : growth rates and specific growth requirements. Water pretreatment method is necessary to limit non-mycobacterial and fungal overgrowth, resulting in enhanced detection of slow grower *Mycobacterim* spp. A number of different protocols have been described, but a standard protocol has not been established yet.

Several authors have described the use of cetylpyridinium chloride (CPC) for decontamination of diverse water samples from environmental sources[72-75]. CPC is an antiseptic which killing bacteria and other microorganisms. It has been used in some types of mouthwashes, toothpastes, and anti-sore throat sprays. This study demonstrated that filtration and decontamination agent using CPC is a more effective disinfecting method for isolating mycobacteria from water samples. The mycobacteria were grown according to the culture method, followed by filtration (Table 9 and Supplemental data 7-16).

In this study, the filtration procedure was a more effective method than centrifugation for isolation of potential waterborne pathogens from water samples. Unfortunately, there have been no direct comparative studies, but it has been able to isolate mycobacteria from CPC treated water samples using centrifugation[71]. Previous reports have demonstrated that the filtration method is the more effective than the centrifugation method in isolating mycobacteria[71].

Middlebrook 7H11 agar plate was selected as a selective medium for detection of *Mycobacterium* spp. However, other microorganisms were observed after 3 day of incubation on Middlebrook 7H11 agar plate containing four anti microbial agents (polymyxin B, carbenicillin, amphotericin B and trimethoprim

lactate). For this reason, this study evaluated the usefulness of CPC as a method for the pretreatment of water samples and for the isolation of mycobacteria .

In this study, more than six of environmental isolates including potentially pathogenic mycobacterial species such as *M. mageritense* and *M. mucogenicum, M. gilvum*, *M. lentiflavum*, *M. peregrinum*, *M. wolinskyi*, *M. avium* and *M. marinum* were detected in water samples. Considering medical significance, identification of mycobacteria from their reservoir is very important[69].

In the present study, the efficiency of REBA as a waterborne pathogen identification protocol was systemically evaluated. Diverse opportunistic pathogen isolated from the eight wastewater treatment plants were quantified using both the standard culture-based enumeration method and the newly developed REBA.

At present, the conventional method of environment sample screening is less sensitive and cannot identification of bacteria to a species level. The culture procedure requires 3~7 days to have enough number of cell for analysis. Meanwhile, the REBA method was found to be more sensitive in species identification. This method will particularly useful in identifying waterborne pathogens. It overcomes many of the limitations of cultural

microbiology method including loss of organism viability during sample process, a problem of enumerating difficult to cultivate (or even uncultivable) species.

The experimental results in this study suggested that the REBA method is a sensitive and specific diagnostic procedure for the simultaneous detection and identification of waterborne pathogens. This method can be adopted as an useful modality for detection and identification of waterborne pathogens.

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SUPPLEMENTAL DATA

1. Water quality parameters in treated wastewater (November 2006 and January 2007)

	Site description		Treatment	Disinfection	Bacterial water quality (CFU/㎖)				
					Total	*E. coli*	*S. aureus*	Total Coliform	Enterobacteriaceae
Nov. 2006	B	effluent	A_2O	Chlorine	213	65	21	75	1760
		Reusable water	A_2O	UV	863	1	ND	1	208
	C	effluent	Conventional Activated Sludge	Chlorine	2850	45	8	126	888
		Reusable water	Activated Sludge	Ozone	137	ND	ND	ND	1480
	D	effluent	SBR	Chlorine	550	63	2	7	410
	E	effluent	Activated Sludge	UV	343	2	12	100	1180
	F	effluent	Contact Oxidation	Chlorine	200	1	3	10	1500
Jan. 2007	B	effluent	A_2O	Chlorine	630	29	3	65	320
		Reusable water	A_2O	UV	100	1	ND	ND	ND
	C	effluent	Conventional Activated Sludge	Chlorine	513	16	1	10	160
		Reusable water	Activated Sludge	Ozone	255	80	2	20	ND
	D	effluent	SBR	Chlorine	376	1	1	1	ND
	E	effluent	Activated Sludge	UV	550	29	2	28	ND
	F	effluent	Contact Oxidation	Chlorine	343	19	12	26	ND

ND : Non Detection

2. Water quality parameters in treated wastewater (March 2007 and May 2007)

		Site description	Treatment	Disinfection	Bacterial water quality (CFU/mℓ)				
					Total	*E. coli*	*S. aureus*	Total Coliform	Enterobacteriaceae
Mar. 2007	B	effluent	A_2O	Chlorine	213	18	15	83	ND
		Reusable water	A_2O	UV	863	ND	10	49	11
	C	effluent	Conventional Activated Sludge	Chlorine	285	62	43	17	14
		Reusable water	Activated Sludge	Ozone	147	ND	ND	ND	ND
	D	effluent	SBR	Chlorine	137	50	ND	10	ND
	E	effluent	Activated Sludge	UV	550	4	27	2	60
	F	effluent	Contact Oxidation	Chlorine	343	15	4	21	20
May 2007	B	effluent	A_2O	Chlorine	450	40	10	ND	ND
		Reusable water	A_2O	UV	ND	ND	ND	ND	ND
	C	effluent	Conventional Activated Sludge	Chlorine	500	10	10	20	60
		Reusable water	Activated Sludge	Ozone	300	ND	ND	ND	10
	D	effluent	SBR	Chlorine	100	ND	ND	ND	17
	E	effluent	Sludge	UV	400	20	ND	30	30
	F	effluent	Contact Oxidation	Chlorine	400	ND	ND	ND	10

ND : Non Detection

3. Water quality parameters in treated wastewater (July 2007 and September 2007)

	Site description		Treatment	Disinfection	Bacterial water quality (CFU/㎖)				
					Total	*E. coli*	*S. aureus*	Total Coliform	Enterobacteriaceae
Jul. 2007	B	effluent	A_2O	Chlorine	4200	590	10	30	680
		Reusable water	A_2O	UV	1340	10	ND	ND	730
	C	effluent	Conventional Activated Sludge	Chlorine	900	20	ND	ND	ND
		Reusable water	Activated Sludge	Ozone	1030	ND	ND	ND	250
	D	effluent	SBR	Chlorine	215	140	30	20	255
	E	effluent	Activated Sludge	UV	4870	920	ND	60	595
	F	effluent	Contact Oxidation	Chlorine	570	ND	ND	ND	ND
Sep. 2007	A	effluent	A-zenit-P	UV	1180	900	ND	210	170
	B	effluent	A_2O	Chlorine	6920	1400	20	400	310
		Reusable water	A_2O	UV	ND	ND	ND	ND	200
	C	effluent	Conventional Activated Sludge	Chlorine	11600	200	40	500	370
		Reusable water	Activated Sludge	Ozone	12600	1300	10	430	340
	D	effluent	SBR	Chlorine	1910	ND	ND	ND	370
	E	effluent	Sludge	UV	1230	20	ND	30	30
	F	effluent	Contact Oxidation	Chlorine	2950	ND	ND	ND	10

ND : Non Detection

4. Water quality parameters in treated wastewater (November 2007 and January 2008)

		Site description	Treatment	Disinfection	Bacterial water quality (CFU/㎖)				
					Total	*E. coli*	*S. aureus*	Total Coliform	Enterobacteriaceae
Nov. 2007	A	effluent	A-zenit-P	UV	740	70	20	40	80
	B	effluent	A_2O	Chlorine	1950	300	60	170	250
		Reusable water	A_2O	UV	870	30	10	30	10
	C	effluent	Conventional Activated Sludge	Chlorine	1690	200	20	80	130
		Reusable water	Activated Sludge	Ozone	1660	10	10	50	70
	D	effluent	SBR	Chlorine	740	50	10	40	70
	E	effluent	Activated Sludge	UV	1300	60	10	60	80
	F	effluent	Contact Oxidation	Chlorine	1330	30	50	50	40
Jan. 2008	A	effluent	A-zenit-P	UV	4100	10	60	18	273
	B	effluent	A_2O	Chlorine	3500	32	13	19	228
		Reusable water	A_2O	UV	750	2	ND	2	6
	C	effluent	Conventional Activated Sludge	Chlorine	4400	18	2	24	78
		Reusable water	Activated Sludge	Ozone	3750	50	5	29	255
	D	effluent	SBR	Chlorine	1500	6	ND	ND	63
	E	effluent	Sludge	UV	450	11	5	6	29
	F	effluent	Contact Oxidation	Chlorine	1250	ND	ND	6	3

ND : Non Detection

5. Water quality parameters in treated wastewater (March 2008 and May 2008)

		Site description	Treatment	Disinfection	Bacterial water quality (CFU/㎖)				
					Total	*E. coli*	*S. aureus*	Total Coliform	Enterobacteriaceae
Mar. 2008	A	effluent	A-zenit-P	UV	3850	ND	11	20	163
	B	effluent	A_2O	Chlorine	2700	12	9	18	78
		Reusable water	A_2O	UV	670	1	6	11	21
	C	effluent	Conventional Activated Sludge	Chlorine	2750	57	09	16	78
		Reusable water	Activated Sludge	Ozone	3350	64	ND	28	72
	D	effluent	SBR	Chlorine	1500	20	ND	3	28
	E	effluent	Activated Sludge	UV	1800	31	5	17	70
	F	effluent	Contact Oxidation	Chlorine	3150	3	ND	15	17
May 2008	A	effluent	A-zenit-P	UV	6300	144	15	211	262
	B	effluent	A_2O	Chlorine	3250	50	12	109	238
		Reusable water	A_2O	UV	490	ND	ND	2	3.5
	C	effluent	Conventional Activated Sludge	Chlorine	3500	ND	11	18	2
		Reusable water	Activated Sludge	Ozone	2500	30	2	60	88
	D	effluent	SBR	Chlorine	2500	23	2	56	84
	E	effluent	Sludge	UV	1500	1	ND	2	5
	F	effluent	Contact Oxidation	Chlorine	7750	129	4	213	146

ND : Non Detection

6. Water quality parameters in treated wastewater (July 2008)

		Site description	Treatment	Disinfection	Bacterial water quality (CFU/㎖)				
					Total	*E. coli*	*S. aureus*	Total Coliform	Enterobacteriaceae
Jul. 2008	A	effluent	A-zenit-P	UV	3650	28	7	108	7935
	B	effluent	A_2O	Chlorine	4700	155	15	78	288
		Reusable water	A_2O	UV	1200	7	ND	15	32
	C	effluent	Conventional Activated Sludge	Chlorine	1100	203	10	33.5	415
		Reusable water	Activated Sludge	Ozone	1300	108	13	5	270
	D	effluent	SBR	Chlorine	1850	18	18	5	66
	E	effluent	Sludge	UV	3350	7	16	12	17
	F	effluent	Contact Oxidation	Chlorine	3100	98	17	109	139

ND : Non Detection

7. Comparison of microorganisms detected in this study using the culture method and REBA (November 2006)

Nov. 2006 Samples	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* *C. freundii* *S. aureus*	*C. freundii* *P. aeruginosa* *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *S. aureus* *K. pnemoniae* *P. aeruginosa* *Shigella* spp. *Citrobacter* spp.	*E. coli* *P. aeruginosa* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp.	*E. coli* O157:H7 *E. coli* *S. aureus* *Listeria* spp.	*E. coli* *C. freundii* *S. aureus* *Klebsiella* spp. *Listeria* spp.	*E. coli* *C. freundii* *S. aureus* *Klebsiella* spp.
Culture method	*E. coli* *S. aureus*	*C. freundii* *P. aeruginosa* *Klebsiella* spp. *Enterobacter* spp.	*K. pnemoniae* *P. aeruginosa* *Shigella* spp. *Citrobacter* spp.	*E. coli* *P. aeruginosa* *Klebsiella* spp. *Listeria* spp.	*E. coli* *Listeria* spp.	*Citrobacter* spp. *Klebsiella* spp. *Listeria* spp.	-

8. Comparison of microorganisms detected in this study using the culture method and REBA (January 2007)

Jan. 2007 Samples	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157: H7 *E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *Klebsiella* spp. *Citrobacter* spp. *Enterobacter* spp.	*E. coli* *P. aeruginosa*	*E. coli* O157: H7 *E. coli* *E. faecalis* *S. aureus* *P. aeruginosa* *Citrobacter* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Yersinia* spp.	*E. coli* O157:H7 *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Yersinia* spp.	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterococcus* spp.	*E. coli* *P. aeruginosa* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Enterobacter* spp.
Culture method	*E. coli* *P. aeruginosa* *S. aureus* *Klebsiella* spp. *Enterobacter* spp.	*P. aeruginosa*	*E. coli* *E. faecalis* *S. aureus* *P. aeruginosa* *Citrobacter* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*S. aureus* *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *P. aeruginosa* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterococcus* spp.	*E. coli* *P. aeruginosa* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Enterobacter* spp.

9. Comparison of microorganisms detected in this study using the culture method and REBA (March 2007)

Mar. 2007 Samples	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *Citrobacter* spp. *Klebsiella* spp. *Mycobacterium* spp.	*E. coli* O157: H7 *E. coli* *C. freundii* *P. aeruginosa* *K. pnemoniae* *E. faecalis* *S. aureus* *Yersinia* spp. *Listeria* spp.	*E. coli* *S. aureus* *Klebsiella* spp.	*E. coli* O157:H7 *E. coli* *S. aureus* *Citrobacter* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *P. aeruginosa* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Klebsiella* spp. *Enterococcus* spp.	*E. coli* *C. freundii* *E. faecalis* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Mycobacterium* spp.	*E. coli* *E. faecalis* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Listeria* spp. *Mycobacterium* spp.
Culture method	*E. coli* *E. coli* *Citrobacter* spp. *Klebsiella* spp. *Mycobacterium* spp..	*E. coli* *E. coli* *C. freundii* *P. aeruginosa* *K. pnemoniae* *E. faecalis* *S. aureus*	*E. coli* *S. aureus* *Klebsiella* spp.	*E. coli* *S. aureus* *Citrobacter* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *P. aeruginosa* *S. aureus* *Shigella* spp. *Klebsiella* spp. *Enterococcus* spp.	*E. coli* *C. freundii* *E. faecalis* *S. aureus* *Salmonella* spp. *Klebsiella* spp. *Listeria* spp.	*E. coli* *S. aureus* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Listeria* spp. *Mycobacterium* spp.

10. Comparison of microorganisms detected in this study using the culture method and REBA (May 2007)

May 2007 Samples	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Y. pseudotuberculosis* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.	*K. pneumoniae* *P. aeruginosa*	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *Shigella* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *Shigella* spp. *Enterobacter* spp.
Culture method	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Y. pseudotuberculosis* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.	*K. pneumoniae* *P. aeruginosa*	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *S. aureus* *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *C. freundii* *K. pneumoniae* *Shigella* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *S. aureus* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *K. pneumoniae* *Shigella* spp. *Enterobacter* spp.

11. Comparison of microorganisms detected in this study using the culture method and REBA (July 2007)

Jul. 2007 Samples	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *K. pneumoniae* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.
Culture method	*E. coli* *P. aeruginosa* *K. pneumoniae* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *K. pneumoniae* *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *C. freundii* *K. pneumoniae* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *S. aureus* *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.

12. Comparison of microorganisms detected in this study using the culture method and REBA (September 2007)

Sep. 2007 Samples	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *C. freundii* *P. aeruginosa* *K. pneumoniae* *Salmonella* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *Shigella* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Salmonella* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *K. pneumoniae* *E. faecalis* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.
Culture method	*E. coli* *C. freundii* *P. aeruginosa* *K. pneumoniae* *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *Shigella* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Salmonella* spp. *Mycobacterium* spp.	*E. coli* *P. aeruginosa* *K. pneumoniae* *E. faecalis* *S. aureus* *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *S. aureus* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Mycobacterium* spp.

13. Comparison of microorganisms detected in this study using the culture method and REBA (November 2007)

Nov. 2007 Samples	A, effluent	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *E. faecalis* *M. avium* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Citrobacter* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *M. marinum* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Mycobacterium* spp.
Culture method	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *K. pneumoniae* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.	*E. coli* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Citrobacter* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp.

14. Comparison of microorganisms detected in this study using the culture method and REBA (January 2008)

Jan. 2008 Samples	A, effluent	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *K. pneumoniae* *M. avium* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *K. pneumoniae* *M. avium* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *C. freundii* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.
Culture method	*E. coli* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp.	*E. coli* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *S. aureus* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *K. pneumoniae* *P. aeruginosa* *Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *P. aeruginosa* *C. freundii* *K. pneumoniae* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.

15. Comparison of microorganisms detected in this study using the culture method and REBA (March 2008)

Mar. 2008 Samples	A, effluent	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *M. marinum* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *P. aeruginosa* *S. aureus* *M. avium* *Shigella* spp. *Salmonella* spp. *Yersinia* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *Yersinia* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *M. marinum* *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *M. avium* *Shigella* spp. *Salmonella* spp.	*E. coli* O157:H7 *E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *Shigella* spp. *Yersinia* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.
Culture method	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* *P. aeruginosa* *S. aureus* *Shigella* spp. *Salmonella* spp. *Yersinia* spp.	*E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *Yersinia* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *Shigella* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *S. aureus* *Shigella* spp. *Yersinia* spp. *Mycobacterium* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Enterobacter* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.

16. Comparison of microorganisms detected in this study using the culture method and REBA (May 2008)

May 2008 Samples	A, effluent	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Salmonella* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.
Culture method	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Salmonella* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.

17. Comparison of microorganisms detected in this study using the culture method and REBA (July 2008)

Jul. 2008 Samples	A, effluent	B, effluent	B, reusable water	C, effluent	C, reusable water	D, effluent	E, effluent	F, effluent
REBA	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Salmonella* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* O157:H7 *E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.
Culture method	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp	*E. coli* *P. aeruginosa* *Shigella* spp. *Salmonella* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Shigella* spp. *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp.	*E. coli* *C. freundii* *P. aeruginosa* *K. pneumoniae* *Shigella* spp. *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *C. freundii* *P. aeruginosa* *Staphylococcus* spp. *Salmonella* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.	*E. coli* *K. pneumoniae* *P. aeruginosa* *E. faecalis* *Citrobacter* spp. *Salmonella* spp. *Klebsiella* spp. *Listeria* spp. *Staphylococcus* spp. *Mycobacterium* spp.

ABSTRACT IN KOREAN

Polymerase Chain Reaction - Reverse Blot Hybridization Assay를 이용한 수인성 미생물의 검출

수인성 병원성 미생물 모니터링은 전통적인 미생물의 분리, 동정 방법을 토대로 수 시료 내에 유해세균의 유무를 확인하기 위해 지표미생물을 사용한다. 지표미생물은 개체수가 많고 실험하기 용이한 대장균 (*Escherichia coli*)이나 대장균군 (Total coliforms) 을 이용하여 간접적으로 세균의 오염도를 평가한다. 대부분의 수인성 병원성 미생물 오염도가 지표미생물의 오염도와 연관이 있음에도 불구하고 미생물학적 특성이 다르거나 배양 조건이 까다로운 미생물은 지표미생물만으로 그 오염현황을 제대로 반영하지 못한다는 문제점이 있다. 또한 *Shigella* spp.와 *Mycobacterium* spp.같은 수인성 병원성 미생물들은 배양 방법으로 검출되지 않는 특성을 가지고 장기간 수 환경에 존재하기도 한다. 기존의 전통적인 미생물 분리, 동정방법으로 유해세균을 분석하려면 각 유해세균에 적합한 조건을 맞춰야 하므로 많은 시간, 경비, 인력이

소요되어 현실적으로 모니터링을 하기 힘든 실정이다. 따라서 기존의 배양법에 비해 신속, 정확하며 여러 종류의 미생물을 동시에 검출할 수 있는 모니터링 시스템 개발이 필요하다. 본 연구에서는 수계 내의 오염도를 판단하는 지표미생물인 *E. coli* 를 포함한 총 19 종의 수인성 병원성 미생물을 검출하기 위하여 16S DNA 유전자를 이용한 각각의 대상세균에 특이적인 probe 를 고안하여 polymerase chain reaction-reverse blot hybridization assay (PCR-REBA) 기법을 확립하였고 모니터링을 위한 시스템으로 개발하였다. 개발된 REBA 기법의 유용성을 평가하기 위하여 2006 년 11 월부터 2008 년 7 월까지 8 곳의 하수종말처리장에서 다양한 소독공정방법으로 처리된 방류수를 대상으로 세균의 오염도를 분석하였다. 방류수 시료를 대상으로 지표미생물과 유해세균을 검출하기 위하여 고전적인 미생물학적 배양을 통한 분리, 동정방법과 시료를 배양하지 않고 PCR-REBA 를 병행하였다. 수중에 미량으로 존재하는 대상세균들을 배양하지 않고 직접 검출하기 위해 막여과과정을 실시한 후, 여과된 막에서 DNA 를 분리하였고 검출 과정의 민감도를 상승시키기 위해 nested PCR 을 사용하였다. 검출된 미생물들은 방류수 수질기준에서 지표미생물로 사용되고 있는 대장균종 이외에 그람 음성세균 중 장염을 유발할 수 있는 것으로 알려진 *Pseudomonas* spp., *Yersinia* spp.등과 그람 양성 세균인 *Enterococcus* spp., *Listeria* spp.,

Staphylococcus spp. 가 검출되었다. 또한, 전처리 과정을 추가하여 여과과정을 시행한 후 PCR-REBA 와 염기서열 분석 결과 기회감염균으로 알려진 6 종의 *Mycobacterium* spp. 도 검출되었다. 검출된 세균들은 계절의 변화나 소독 방법의 차이와 관계없이 모든 시료채취 장소에서 지속적으로 검출되어 이들 세균들을 수질기준 지표세균으로 추가하여 사용할 수 있을 것으로 사료된다. 수인성 병원성 미생물을 검출하기 위한 PCR-REBA 기법의 유용성을 평가한 결과, 미생물의 배양기간이 소요되는 미생물학적 검출 기법에 비해 PCR-REBA 기법이 높은 민감도와 특이도를 나타내는 것으로 확인되었다. 본 연구에서는 PCR-REBA 기법이 인력, 시간 및 비용이 많이 소요되는 기존의 배양법에 의한 미생물 검사법을 대체 가능한 수계 내 병원성 미생물 검출기법으로 사용될 수 있을 것으로 사료된다.

핵심되는 말 : 수인성 병원성 미생물, Polymerase chain reaction-reverse blot hybridization assay (PCR-REBA), Nested PCR, Cetylpyridinium chloride (CPC)