

Isolation of Environmental Mycobacteria from Diverse Water Samples Using Cetylpyridinium Chloride

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Despite of the increasing importance of environmental mycobacteria, detection and identification of mycobacteria from environmental sources including water have been fraught with technical difficulties. Although, several protocols to optimize isolation of mycobacteria from water sources have been reported, standard method has not yet been established. In this study, usefulness of cetylpyridinium chloride (CPC), a cationic quaternary ammonium compound, for the isolation of environmental mycobacteria from diverse water samples was evaluated. For this, water samples from diverse water sources such as effluent water, lake water, and underground water were collected, treated with diverse concentrations of CPC, and plated on the solid agar plates. Subsequently individual colonies grown on the plates were sequence analyzed for identification of each colony. In brief, the results from this study showed that the growth of mycobacteria was enhanced by use of CPC as a pre-treatment reagent to water samples by inhibiting growth of other non-mycobacteria in water. In fact, the effect of CPC to decontaminate non-mycobacteria for isolation of mycobacteria was better than 1~4% of NaOH, which is a routinely used decontaminating reagent widely employed for culturing mycobacteria from sputum specimens. Therefore, the results from this study seems to support that the CPC pre-treatment may be useful for isolation of mycobacteria from diverse sources including clinical specimens which are often contaminated with other bacteria.

Key Words: Environmental mycobacteria; Decontamination reagents; Cetylpyridinium chloride; Environmental water samples

INTRODUCTION

Recently, there have been increasing evidences showing that nontuberculous mycobacteria from environmental sources are implicated in a variety of human diseases. mycobacteria are common saprophytes found in all natural ecosystems including water, soil, food, dust, and aerosols (Dailloux et al., 1999). Mycobacteria have been also demonstrated to be present in drinking water (Goslee et al.,

1976; Kubalek et al., 1995; Kubalek et al., 1996; Covert et al., 1999; Falkinham et al., 2001; Le Dantec et al., 2002; Vaerewijck et al., 2005), drinking water distribution systems (Chang et al., 2002; Le Dantec et al., 2002a; Le Dantec et al., 2002b; Vaerewijck et al., 2005), hot water systems (Du Moulin et al., 1988), spas (Embil et al., 1997), and pools (Leoni et al., 1999; Livanainen et al., 1999). Mycobacteria are not killed by common disinfectants and can tolerate wide ranges of pHs and temperatures, these organisms can persist in water for long periods (Le Dantec et al., 2002).

Despite of the increasing importance of mycobacteria in clinical aspects, detection and identification of mycobacteria from environmental sources have been extremely hindered (Kazda, 1983; Du Moulin, 1986; Falkinham, 1996). This difficulty is often due to various growth rates and specific growth requirements of mycobacteria, contamination by

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other bacteria that outgrow mycobacteria, and the nature of diverse sources of environmental samples. For this reason, choice of pretreatment methods to limit other bacteria than mycobacteria and fungal overgrowth and enhance growth of mycobacteria (Thomson et al., 2008) is very important.

In past, substances which can kill other bacteria and pathogenic fungi have shown to increase the mycobacterial culture positivity and reduce the contamination rate. Mineral acids (HCl) and bases (eg, NaOH), organic acids (eg, oxalic acid), and detergents have been used as decontaminating agents (Songer JG, 1981). However, the pretreatment method can also prevents the detection of certain species of mycobacteria and reduce the rate of positive samples and the number of colonies seen. Several authors have described the use of cetylpyridinium chloride (CPC) for decontamination of diverse water samples from environmental sources (Du Moulin, 1978; Schulze-Robbeke et al., 1991; Le Dantec et al., 2002b; Falkinham, 2004; Thomson et al., 2008). CPC is an antiseptic that kills bacteria and other microorganisms, and used in some types of mouthwashes, toothpastes, anti-sore throat sprays.

The present study was set to evaluate the usefulness of CPC as a method for pretreatment of water samples for isolation of mycobacteria.

MATERIALS AND METHODS

Collection of water samples

A total of 10 water samples were collected from 3 different types of water sources; effluent water, lake water, and underground water. A total of 8 water samples from waste water treatment plants, 1 surface water from the lake Maeji, and 1 from underground water at Wonju-si, Gangwon province, Korea were taken according to the standard methods (American Public Health Association, 1995; Korea ministry of Environment. 2002). Samples were transported to the laboratory and were kept at 4°C until analysis. All samples were analyzed within 24 h of collection.

Decontamination and filtration of water samples

In order to evaluate the effect of CPC as a pretreatment method for water samples for detecting environmental

mycobacteria, CPC and NaOH were compared using lake and ground water samples. For this, water samples were analyzed by the method reported by previous researchers (Neumann et al., 1997; Covert, 1999; Falkinham et al., 2001). In brief, 200 ml of water samples were exposed to from 0.01% to 0.1% of CPC (Sigma Chemical Co., St. Louis, U.S.A) and to 1% to 4% of NaOH for 30 min at RT. All water samples were filtered through 0.45 µm nitrocellulose membrane filter (Millipore Corp., Bedford, Mass.) by vacuum filtration after exposure to CPC and NaOH. Water samples were decontaminated by CPC and NaOH.

In the second set of experiments, evaluate the effect of CPC for the isolation of mycobacteria from 10 water samples. Water samples were exposed to from 0.01% to 0.1% of CPC (Sigma Chemical Co., St. Louis, U.S.A.) at RT. And then, water samples were filtered through 45 µm nitrocellulose membrane filter (Millipore Corp., Bedford, Mass.) by vacuum filtration after exposure to CPC.

Processing of cultural detection of nontuberculous mycobacteria

After filtration, the membranes were rinsed with PBS and transferred to Middlebrook 7H10 agar plates (Difco Laboratories, Detroit, Mich.) containing 500 mg of Amphotericin B (Sigma Chemical Co. St. Louis, U.S.A.) per liter. The plates were then sealed and incubated at 37°C with 10% CO₂ for 8 weeks. All plates were examined at weekly intervals for 8 weeks. After 8 weeks of incubation, all the colonies grown on the plate were enumerated, acid fast stained and individual colonies which looked distinct each other were then restreaked onto the new Middlebrook 7H10 agar plate (Fig. 1).

DNA extraction for PCR amplification

Subsequently, DNA was extracted from a single isolated colony by CTAB method as previously described (Lemarchand et al., 2005), and subjected for PCR amplification of 16S rRNA gene. The quality and the quantity of the extracted DNA were determined by electrophoresis using a 1% agarose gel.

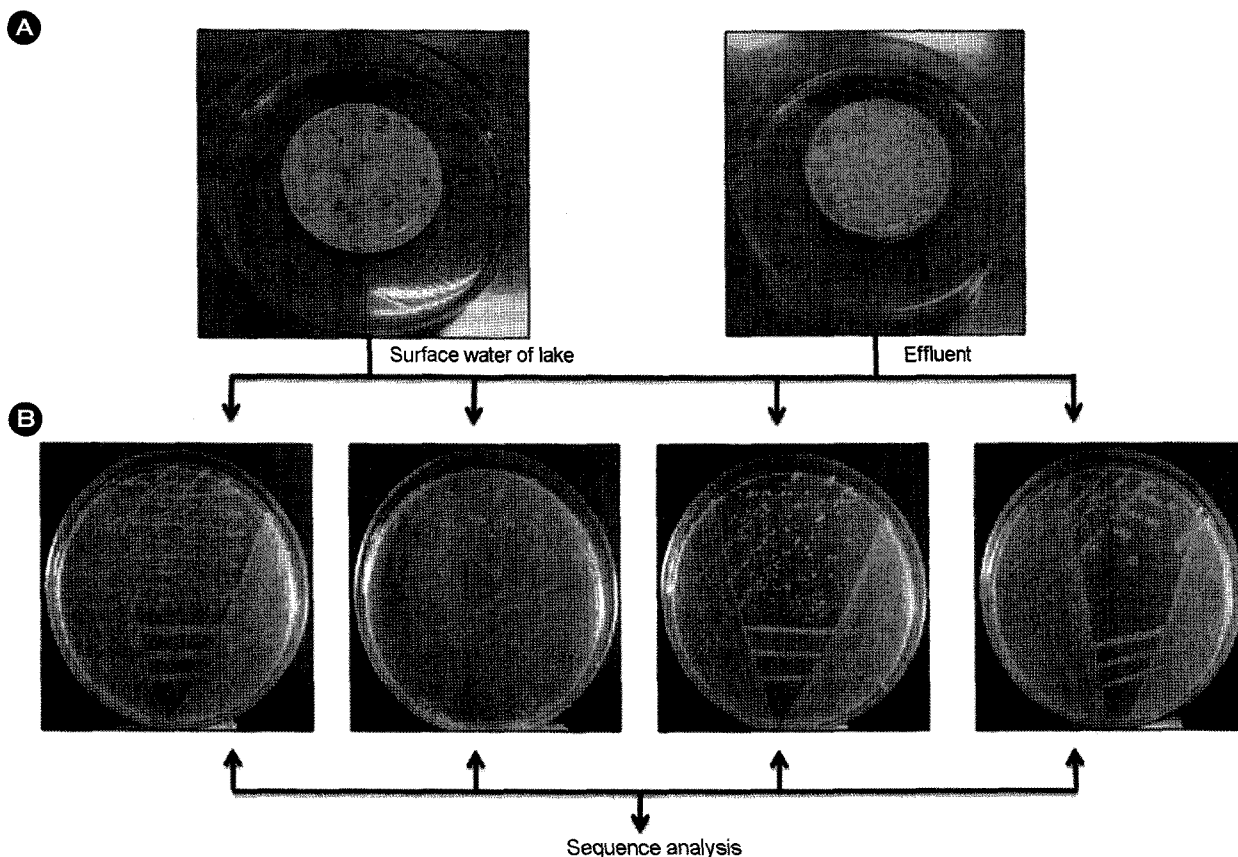


Fig. 1. Isolation of non mycobacterial and mycobacterial colonies from effluent and surface water of lake by treated 0.05% CPC. (A) Microorganisms grown on the filtered membrane were incubated for 8 weeks at Middlebrook 7H10 agar plate, and then (B) single isolated individual colonies obtained from plate (A) were restreaked at new Middlebrook 7H10 agar plate for further identification of isolates. The filter membranes in (A) had been used for passing water samples treated with CPC. Subsequently DNAs were extracted from individual colonies and then their 16S rRNA gene were PCR-sequence analyzed for identification of species.

Identification of nontuberculous mycobacteria by 16S rRNA gene sequencing

All *Mycobacterium* isolates that were cultured on the filter membranes were subjected to 16S rRNA gene sequencing. To design primer molecules, data base of 16S rRNA sequences of mycobacteria were compiled from GenBank database of National Center for Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov>), and the sequence data for mycobacteria were aligned by using the Clustral method (<http://www.cmbi.kun.nl/bioinf/tools/clustalws.html>). The PCR primers were as following: 16S - SF: 5'-AAYACATGCAAGTCGARCK-3' and 16S - R5H-A: 5'-ACRDAKTTRGCCGKKGCTT-3' (479 base pairs in length). The amplification reactions were performed with a Thermal cycler (GeneAmp PCR[®] System 2700). The PCR condition used was denaturation at 95°C for 30 s, annealing

at 52°C for 30 s, and extension at 72°C for 30 s and a total of 35 cycles were used. Successful amplification was confirmed by using agarose gel electrophoresis. Sequencing of the PCR products was carried out at genotech (Daejeon, Korea). The sequences were analyzed with databases GenBank. Species identification was made on the basis of the sequence homology to the respective sequences in the GenBank database of National Center for Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov>).

RESULTS AND DISCUSSION

First of all, in order to compare the effect of CPC and NaOH treatment on mycobacterial isolation, water samples were taken from the lake and underground water. Water samples were then exposed to from 0.01% to 0.1% of CPC and from 1% to 4% of NaOH for 30 min at room

Table 1. Effects of different concentrations of CPC and NaOH on microbial growth isolated from 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

Table 2. 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	11	12	9	5	1
0.05%	18	9	8	8	0	1
0.08%	5	2	0	2	0	0
0.1%	0	1	0	0	0	0

*TNTC: Too Numerous To Count

temperature, and subsequently plated on solid agar media. As shown in the Table 1, when 1% to 4% of NaOH were used, too many microbial organisms grew on the plate (to numerous to count; TNTC), making impossible to identify any colonies. On the other hand, at the concentration of 0.04% or more of the CPC, countable numbers of individual colonies were grown on the plate, making possible to identify colony isolates. Next, for identification of individual colonies, each colonies grown on the plates were subjected for sequence analysis, and numbers of non-mycobacterial colonies and mycobacterial colonies were counted (Table 1).

Subsequently, in order to evaluate the usefulness of CPC with more diverse water samples, 10 water samples were taken from various water sources: 8 waste water treatment plants, 1 surface of the lake, and 1 underground water. Then, the water samples were treated with different concentration of CPC and cultured on the plates. As shown in the Table 2,

the counts of mycobacterial colonies isolated from water samples ranged from 1 CFU/200 ml to too numerous to count. The highest level of mycobacterial growth was obtained with diverse water samples by using 0.04% CPC treatment. From the concentration of 0.04% CPC, the number of non-mycobacterial growth started to be controllable, and mycobacterial colonies were countable. In fact, the optimized concentration of the CPC for isolation of Mycobacteria seems to reside between 0.04~0.05%. The identification of each colony to be mycobacteria or non-mycobacteria was made by sequence analysis of each individual colonies as previous experiment. Overall, 0.04% CPC appeared to be the most effective both in reducing non-mycobacterial contaminants and in allowing good recovery of mycobacteria from water samples.

As a result of sequence analysis of a total of 134 individual colonies, 86 colonies (64%) were determined to

Table 3. Identification of non mycobacterial isolates based on 16S rRNA-gene sequence analysis

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

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

Table 4. Identification of mycobacterial isolates based on 16S rRNA-gene sequence analysis

Sources	Species isolated	Highest similarity, %*	No. of colonies sequenced (Total = 48)
Effluent	<i>Mycobacterium gilvum</i>	99%	3
	<i>Mycobacterium lentiflavum</i>	98%	2
	<i>Mycobacterium mageritense</i>	100%	8
	<i>Mycobacterium peregrinum</i>	98%	5
	<i>Mycobacterium wolinskyi</i>	98%	5
Surface water of lake	<i>Mycobacterium mageritense</i>	100%	20
Underground water	<i>Mycobacterium mageritense</i>	98%	5

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

be 8 non-mycobacterial species (Table 3), whereas 48 colonies (36%) to be 5 mycobacterial species (Table 4). Despite measures to control contamination with CPC, *Herbaspirillum* spp. and *Bacillus* spp. overgrowth were found to be a major problem with water samples. On the other hand, *Mycobacterium mageritense* was the most frequently isolated mycobacterial species being isolated from all 3 kinds of water samples tested. Besides, *M. gilvum*, *M. lentiflavum*, *M. peregrinum*, and *M. wolinskyi* which are considered to be human opportunistic were also isolated.

There have been a number of studies using different methods to isolate mycobacteria from water samples. In this study, with the use of CPC at the optimized concentration between 0.04% and 0.05%, mycobacteria were successfully isolated from diverse water samples. This study confirms the findings by previous authors: that is, CPC inhibits growth of non-mycobacteria, while enhances growth of mycobacteria. In addition to previous findings, however, this study further suggests the optimum concentration of the

CPC (0.04% and 0.05%) for the isolation of mycobacteria from diverse water samples. In summary, the present study showed that treatment with CPC is an effective procedure for retrieving pure cultures of mycobacteria from cultures initially positive with contamination. The correlation between the quality of water and the contamination level of water with mycobacteria seemed to need further investigation. In short, we believe that the experience from our study can be benefit to the future studies which aim to investigate presence of mycobacteria in diverse environmental sources including water samples.

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REFERENCES

- American Public Health Association. Standard methods for the examination of water and wastewater. 1995. p.120-150 19th ed. American Public Health Association, Washington, D.C., USA.
- Chang CT, Wang LY, Liao CY, Huang SP. Identification of nontuberculous mycobacteria existing in tap water by PCR-restriction fragment length polymorphism. *Appl Environ Microbiol.* 2002. 68: 3159-3161.
- Covert TC, Rodgers MR, Reyes AL, Jr Stelma GN. Occurrence of nontuberculous mycobacteria in environmental samples. *Appl Environ Microbiol.* 1999. 65: 2492-2496.
- Daillox MC, Weber LM, Hartemann P. Water and nontuberculous mycobacteria. *Water Res.* 1999. 33: 2219-2228.
- Du Moulin GC, Stottmeier KD. Use of cetylpyridinium chloride in the decontamination of water for culture of mycobacteria. *Appl Environ Microbiol.* 1978. 36: 771-773.
- Du Moulin GC, Stottmeier KD. Waterborne mycobacteria: an increasing threat to health. *ASM News.* 1986. 52: 525-529.
- Du Moulin GC, Stottmeier KD, Pelletier PA, Tsang AY, Hedley-Whyte J. Concentration of *Mycobacterium avium* by hospital hot water systems. *JAMA.* 1988. 260: 1599-1601.
- Embil J, Warren P, Yakrus M, Stark R, Corne S, Forrest D, Hershfield E. Pulmonary illness associated with exposure to *Mycobacterium avium* complex in hot tub water. Hypersensitivity pneumonitis or infection? *Chest.* 1997. 111: 813-816.
- Falkinham JO 3rd. Epidemiology of infection by nontuberculous mycobacteria. *Clin Microbiol Rev.* 1996. 9: 177-215.
- Falkinham JO 3rd, Norton CD, LeChevallier MW. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other Mycobacteria in drinking water distribution systems. *Appl Environ Microbiol.* 2001. 67: 1225-1231.
- Falkinham JO 3rd, nontuberculous mycobacteria in the environment. *Clin Chest Med.* 2002. 23: 529-551.
- Falkinham J. Environmental sources of *Mycobacterium avium* linked to routes of exposure. Pathogenic mycobacteria in water: a guide to public health consequences, monitoring management. 2004. IWA Publishing, London, United Kindom.
- Goslee S, Wolinsky E. Water as a source of potentially pathogenic mycobacteria. *Am Rev Respir Dis.* 1976. 113: 287-292.
- Kazda JF. The principles of the ecology of mycobacteria, The biology of mycobacteria, 1983. vol. 2. cademic Press, London, United Kingdom.
- Korea ministry of Environment. Bacterial contamination guidelines for drinking water in Korea. 2002.
- Kubalek I, Komenda S. Seasonal variations in the occurrence of environmental mycobacteria in potable water. *APMIS.* 1995. 103: 327-330.
- Kubalek I, Mysak J. The prevalence of environmental mycobacteria in drinking water supply systems in a demarcated region in Czech Republic, in the period 1984~1989. *Eur J Epidemiol.* 1996. 12: 471-474.
- Le Dantec C, Duguet JP, Montiel A, Dumoutier N, Dubrou S, Vincent V. Chlorine disinfection of atypical mycobacteria isolated from a water distribution system. *Appl Environ Microbiol.* 2002. 68: 1025-1032.
- Le Dantec C, Duguet JP, Montiel A, Dumoutier N, Dubrou S, Vincent V. Occurrence of mycobacteria in water treatment lines and in water distribution systems. *Appl Environ Microbiol.* 2002. 68: 5318-5325.
- Leoni E, Legnani P, Mucci MT, Pirani R. Prevalence of mycobacteria in a swimming pool environment. *J Appl Microbiol.* 1999. 87: 683-688.
- Lemarchand K, Berthiaume F, Maynard C, Harel J, Payment P, Bayardelle P, Masson L, Brousseau R. Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. *J Microbiol Methods.* 2005. 63: 115-126.
- Livanainen E, Northrup J, Arbeit RD, Ristola M, Katila ML, Von Reyn CF. Isolation of mycobacteria from indoor swimming pools in Finland. *APMIS.* 1999. 107: 193-200.
- Schulze-Robbecke R, Weber A, Fischeder R. Comparison of decontamination methods for the isolation of mycobacteria from drinking water samples. *J Microbiol Methods.* 1991. 14: 177-183.
- Songer JG. Methods for selective isolation of mycobacteria from the environment. *Can J Microbiol.* 1981. 27: 1-7.
- Vaerewijck MJ, Huys G, Palomino JC, Swings J, Portaels F. Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol Rev.* 2005. 29: 911-934.