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NOTE

Distinct difference of *flaA* genotypes of *Legionella pneumophila* between isolates from bath water and cooling tower water

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ABSTRACT

To investigate the genetic difference of *Legionella pneumophila* in human-made environments, we collected isolates of *L. pneumophila* from bath water ($n = 167$) and cooling tower water ($n = 128$) primarily in the Kanto region in 2001 and 2005. The environmental isolates were serogrouped and sequenced for a target region of *flaA*. A total of 14 types of *flaA* genotypes were found: 10 from cooling tower water and nine from bath water. The *flaA* genotypes of isolates from cooling tower water were quite different from those of bath water.

Key words *flaA*, genotype, *Legionella pneumophila*.

In Japan, The National Epidemiological Surveillance of Infectious Diseases data indicate that hot springs and public baths are the major sources of *Legionella* rather than cooling towers (1). We previously analyzed 27 epidemiologically unrelated *Legionella pneumophila* serogroup 1 isolates (10 from cooling towers, 10 from public spas and/or hot spring baths, and seven from patients with bath-related infections) with SBT using the six loci (*flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA*) proposed by the European Working Group on *Legionella* Infections (http://www.ewgli.org/) (2, 3). The 27 isolates were divided into 14 types (2). Notably, all 10 isolates from cooling towers displayed the same type (*flaA*1, *pilE*4, *asd*3, *mip*1, *mompS*1, *proA*1). Only one *flaA* genotype (*flaA*1) was detected in cooling tower isolates, whereas isolates from public spas and/or hot spring baths displayed a variety of *flaA* genotypes such as 2, 3, 4, 6, 7, 10, and 12. These observations have prompted us to propose that *flaA* typing of *L. pneumophila* serogroup 1 may be sufficient to determine general the origin of *L. pneumophila* isolates in Japan. In order to extend these previous results to other serogroups of *L. pneumophila* and to test this hypothesis, we collected independent isolates of *L. pneumophila* from bath water and cooling tower water.

A total of 295 independently isolated environmental strains of *L. pneumophila* mainly in the Kanto region in 2001 and 2005 (Table 1) were analyzed, including isolates from bath water ($n = 167$, the number of facilities was 122) and cooling tower water ($n = 128$, the number of facilities was 107). Most of the isolates were obtained from different facilities. Some were from the same facilities, but showed different serogroups. PCR targeted for the *mip* gene of *L. pneumophila* was used to distinguish *L. pneumophila* from other species (4). Further, serogrouping of the isolates was carried out by slide agglutination tests using a monovalent serum for *L. pneumophila* serogroups 1–15 (Denka Seiken, Tokyo, Japan). Genomic DNA was extracted using a High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany). PCR amplification was achieved using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reaction mixture and conditions used have been described previously (2, 3). Both strands of the amplicons were sequenced with a model 3100 ABI Sequencer (Applied Biosystems).

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List of Abbreviations: PCR, polymerase chain reaction; SBT, sequence-based typing

Table 1 *Legionella pneumophila* isolates typed by the sequencing of *flaA*

Year of isolation	Place of isolation	No. isolates		Total no. isolates
		Cooling tower water	Bath water	
2001	Tokyo Prefecture	20	4	24
	Kanto region except for Tokyo Prefecture	27	3	30
	Other areas	9	6	15
2005	Tokyo Prefecture	48	60	108
	Kanto region except for Tokyo Prefecture	22	75	97
	Other areas	2	19	21
	Total	128†	167‡	295

†Locations of cooling towers and no. isolates are listed as follows:
unknown ($n=31$); offices ($n=28$); schools ($n=14$); accommodations ($n=11$); public facilities ($n=11$); hospitals ($n=9$); multipurpose buildings ($n=7$); shopping centers ($n=6$); transportation stations ($n=4$); factories ($n=2$); nursing homes ($n=2$); cinema ($n=1$); condominium ($n=1$); sports stadium ($n=1$).
‡Locations of baths and no. isolates are listed as follows:
public baths ($n=107$); accommodations ($n=35$); dormitories ($n=8$); nursing homes ($n=7$); offices ($n=4$); condominiums ($n=2$); unknown ($n=2$); school ($n=1$); hospital ($n=1$).

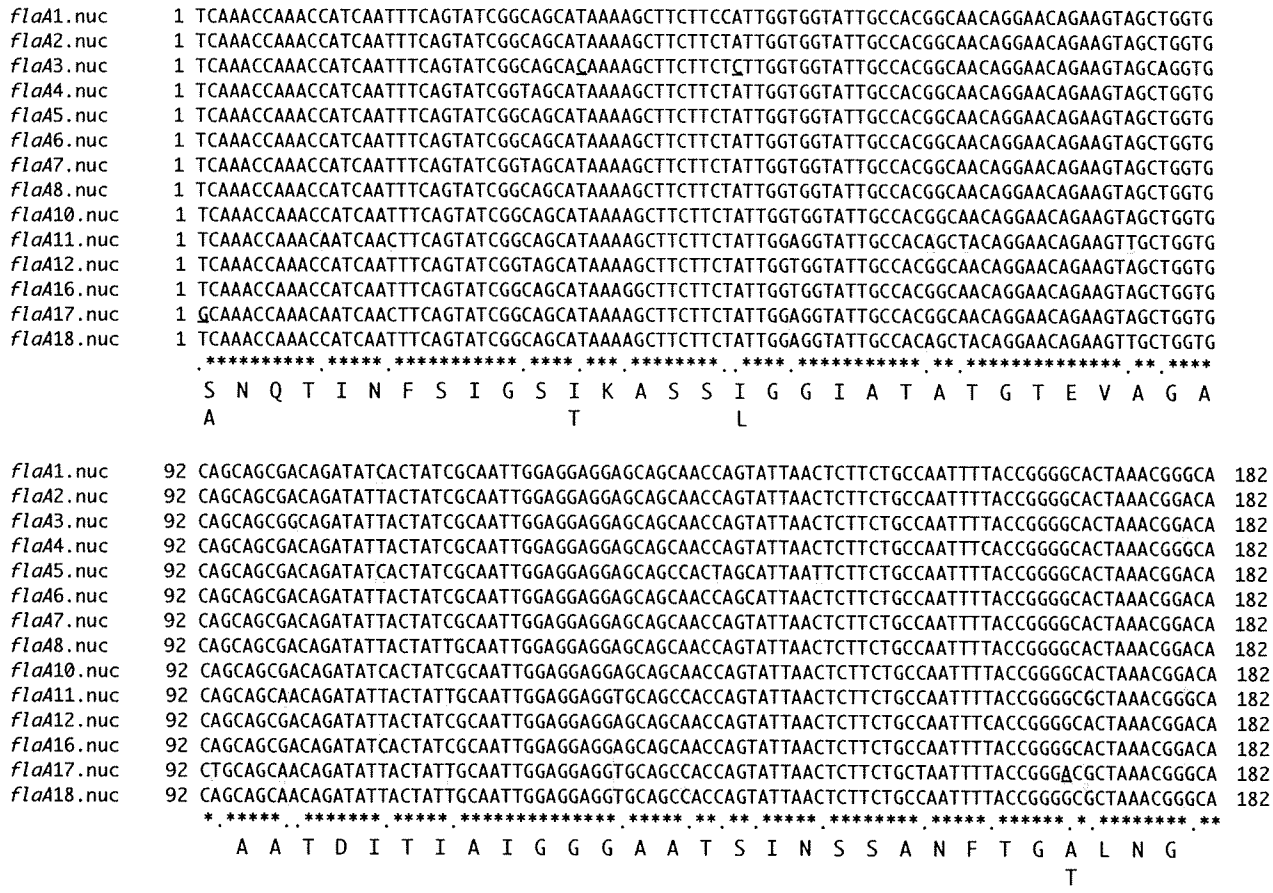

Fig. 1. Sequence of a part of the *flaA* gene used to determine *flaA* genotypes. The variable sites of nucleotides are shown as shaded letters. Non-synonymous changed letters are underlined: 35C and 49C (in *flaA*3), and 1G and 169A (in *flaA*17). Translated amino acid sequence and substituted amino acids by non-synonymous changed nucleotide bases are indicated on the bottom line.

The nucleotide sequences obtained were trimmed based on the data of *flaA* found in the EWGLI SBT database (http://www.ewgli.org); putative novel variants were submitted to the curators of this database for verification and assignment of new allelic numbers.

The 295 environmental isolates examined here comprised a total of 14 types of *flaA* genotypes, three of which were novel genotypes (*flaA*16, 17, and 18) (Fig. 1). Cooling tower water isolates comprised 10 types of *flaA* genotypes. Bath water isolates comprised nine types of *flaA* genotypes (Table 2).

Major genotypes of cooling tower water isolates were *flaA*1 (73%) and *flaA*11 (16%), and major genotypes of bath water isolates were *flaA*6 (34%), *flaA*3 (20%), *flaA*7 (18%), *flaA*2 (13%), and *flaA*10 (7.2%) (Table 2). These data clearly showed that *L. pneumophila* isolates from two water sources were distinctly different in the distribution of *flaA* genotypes (Fig. 2; χ^2-test, calculated as a 2 × 8 table [cooling tower water and bath water × *flaA*1, 2, 3, 6, 7, 10, 11, and others]; $P < 0.0001$). Such a difference was found in the isolates of 2001 and 2005, and in the isolates of different regions such as the Tokyo prefecture and the Kanto region (Table 1).

Differences of serogroups were also found between isolates from bath water and cooling tower water (Table 2). *L. pneumophila* serogroups 1 and 7 accounted for 67% and 23%, respectively, of the isolates obtained from cooling tower water, with the other serogroups being rarely isolated. In contrast, the isolates from bath water were more serotypically diverse, with *L. pneumophila* serogroups: 1,

Table 2 Relations of *flaA* genotypes and serogroups of isolates collected from cooling towers and bath water

	No. isolates with *flaA* genotype														Total no.
Origin and serogroup	1	2	3	4	5	6	7	8	10	11	12	16	17	18	(%)
Cooling tower, SG1	64				2	2	1			15		1		1	86 (67)
Bath, SG1	4	6	3	2		19	13		2		2				51 (31)
Cooling tower, SG3							1			1			1		3 (2.3)
Bath, SG3		3	2	1		2			4						12 (7.2)
Cooling tower, SG4															0
Bath, SG4						2			1						3 (1.8)
Cooling tower, SG5								2							2 (1.6)
Bath, SG5		7	4			7	8								26 (16)
Cooling tower, SG6			1												1 (0.8)
Bath, SG6		1	18			7	4				1				31 (19)
Cooling tower, SG7	28											1			29 (23)
Bath, SG7															0
Cooling tower, SG8	1														1 (0.8)
Bath, SG8		2													2 (1.2)
Cooling tower, SG9															0
Bath, SG9									3						3 (1.8)
Cooling tower, SG10						1									1 (0.8)
Bath, SG10						2									2 (1.2)
Cooling tower, SG11															0
Bath, SG11											1				1 (0.6)
Cooling tower, SG12															0
Bath, SG12						1									1 (0.6)
Cooling tower, SG13												1			1 (0.8)
Bath, SG13															0
Cooling tower, SG14															0
Bath, SG14						1									1 (0.6)
Cooling tower, SG15															0
Bath, SG15									1						1 (0.6)
Cooling tower, SG UT										4					4 (3.1)
Bath, SG UT		2	7			16	5		1				2		33 (20)
Total no. cooling tower water isolates	93	0	1	0	2	3	2	2	0	20	0	3	1	1	128
(%)	(73)		(0.8)		(1.6)	(2.3)	(1.6)	(1.6)		(16)		(2.3)	(0.8)	(0.8)	(100)
Total no. bath water isolates	4	21	34	3	0	57	30	0	12	0	4	0	2	0	167
(%)	(2.4)	(13)	(20)	(1.8)		(34)	(18)		(7.2)		(2.4)		(1.2)		(100)

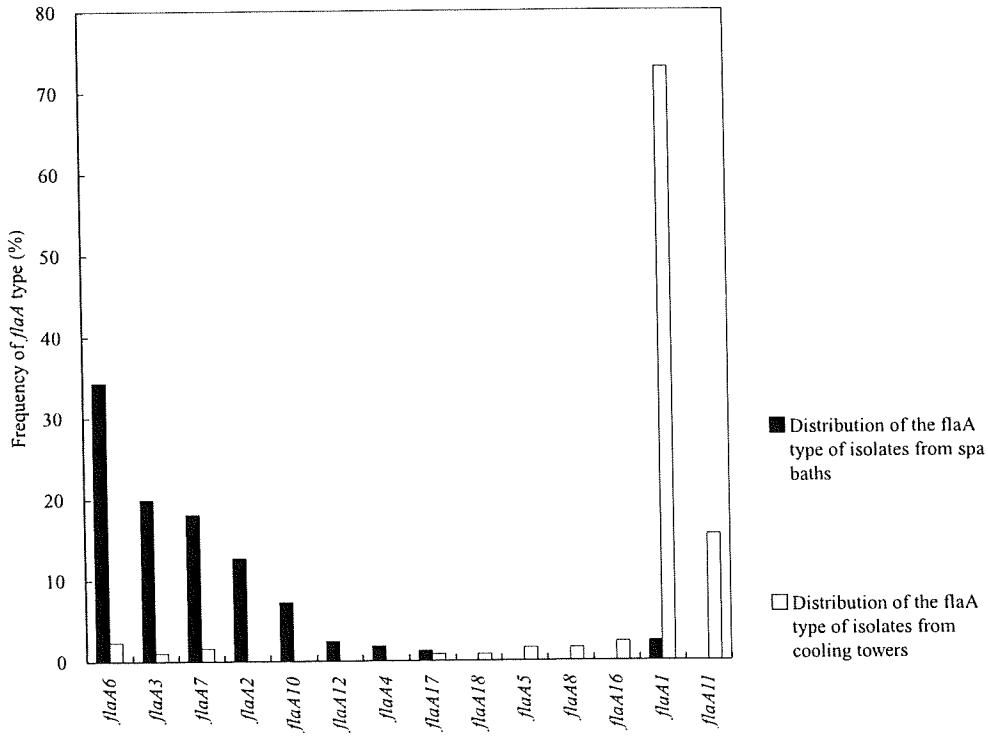

Fig. 2. Distribution of *flaA* genotypes of *L. pneumophila* isolates from cooling tower water and bath water. The percentage of each *flaA* genotype referred to in this figure is shown in the last line (bath water isolates) and in the third line from last (cooling tower water isolates) in Table 2.

untypable, 6, and 5 accounting for 31%, 20%, 19%, and 16%, respectively, of these isolates. While most common serogroup isolate derived from bath water was serogroup 1, which was the same as that of isolates derived from cooling tower water, the distribution of serogroups of isolates derived from bath water was more diverse than that from cooling tower water.

The present study showed a distinct difference of *flaA* genotypes and serogroups between isolates from cooling tower water and bath water in Japan. Isolates from bath water were more diverse in the *flaA* genotype and serogroup than isolates from cooling tower water. This diversity might reflect the sources and properties of water. Water for public baths in Japan is often obtained from hot springs where the chemical composition, pH, and temperature of the water are highly variable. This source diversity might be reflected in the genetic diversity of isolates from bath water. In contrast, tap water, which is the typical source of cooling tower water, is chemically more homogeneous.

Certain *flaA* genotypes were predominant in the isolates of cooling tower water and bath water; *flaA*1 and *flaA*11 in cooling tower water and *flaA*6, *flaA*3, *flaA*7 and *flaA*2 in bath water. A reference strain, Dalas 1E (ATCC33216), which was isolated from cooling tower water was also *flaA*11, although it was serogroup 5. This indicates that determination of the *flaA* genotype from the isolate may be available for estimating the water source. According to The National Epidemiological Surveillance of Infectious Diseases in Japan, in only approximately 50% of reported legionellosis cases is the source of the infection known, with ≥75% of cases associated with the use of bath water (1).

It is not easy to distinguish between clinical and environmental isolates of *L. pneumophila* serogroup 1 by sequence analysis of genes encoding several surface and housekeeping proteins (5). However, a comparison of virulence genes, *lvh* and *rtxA*, is more readily available to differentiate between clinical and environmental isolates (6). Environmental isolates from cooling tower water and circulating hot water in public baths are reported to be different in the genetic properties of both *rpoB* and *dotA* sequences (7). However, the sensitivity of discrimination by *rpoB* and *dotA* genotyping seems not to be very high. The *flaA* genotyping presented here may provide a more sensitive tool for the differentiation between isolates from cooling tower water and bath water.

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Specific Detection of Viable *Legionella* Cells by Combined Use of Photoactivated Ethidium Monoazide and PCR/Real-Time PCR[▽]

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***Legionella* organisms are prevalent in manmade water systems and cause legionellosis in humans. A rapid detection method for viable *Legionella* cells combining ethidium monoazide (EMA) and PCR/real-time PCR was assessed. EMA could specifically intercalate and cleave the genomic DNA of heat- and chlorine-treated dead *Legionella* cells. The EMA-PCR assay clearly showed an amplified fragment specific for *Legionella* DNA from viable cells, but it could not do so for DNA from dead cells. The number of EMA-treated dead *Legionella* cells estimated by real-time PCR exhibited a 10^4- to 10^5-fold decrease compared to the number of dead *Legionella* cells without EMA treatment. Conversely, no significant difference in the numbers of EMA-treated and untreated viable *Legionella* cells was detected by the real-time PCR assay. The combined assay was also confirmed to be useful for specific detection of culturable *Legionella* cells from water samples obtained from spas. Therefore, the combined use of EMA and PCR/real-time PCR detects viable *Legionella* cells rapidly and specifically and may be useful in environmental surveillance for *Legionella*.**

Legionellae are gram-negative, rod-shaped bacteria that are ubiquitous inhabitants of aquatic environments and moist soil, replicating as intracellular parasites of protozoa (6, 22, 23). The bacterium causes legionellosis in humans. Hot springs, public baths, and cooling towers are the most probable sources of legionellosis. In Japan, several legionellosis outbreaks caused by *Legionella pneumophila* have been reported (13, 16, 17, 19, 29, 33). To prevent this infectious disease, surveillance investigations of manmade water systems, such as cooling towers, showerheads, and water distribution pipelines, should be carried out regularly. Because it takes 4 to 7 days to isolate viable *Legionella* organisms from environmental and clinical samples, the development of a rapid detection and isolation method is indispensable for identification of sources and routes of incidents at an early stage.

PCR/real-time PCR is the most widely applied technology for direct detection and quantification of pathogens in foods and environmental or clinical samples. PCR/real-time PCR assays targeting the 16S rRNA or 5S rRNA genes specific for *Legionella* and the macrophage infectivity potentiator (*mip*) gene specific for *L. pneumophila* have been developed for detection and identification of the bacterium (14, 27, 32; EnviroAmp *Legionella* kit package insert [Perkin-Elmer Corporation]). However, a lack of differentiation of DNAs from living and dead *Legionella* cells has seriously hampered the implementation of DNA diagnostics in routine applications. Since chlorine is routinely added to water distribution systems to kill *Legionella* and other bacteria, the resulting bacterial death and lysis release copious amounts of genomic DNA into the water. DNAs from dead *Legionella* strains act as a major obstacle in PCR/real-time PCR detection of viable bacteria. It is conceivable that PCR/real-time PCR can be utilized more extensively for detection if this problem can be cleared up.

Ethidium monoazide (EMA) is a dye that allows microscopic differentiation between viable and dead cells (1, 21). Specifically, the phenanthridinium DNA/RNA-intercalating agent enters only those bacteria that have compromised cell walls and membranes and subsequently covalently links to the DNA within the cells (2, 4, 9, 31). Photolysis of EMA by visible light produces a nitrene that covalently links to genomic DNA, cleaving it into small pieces upon photoactivation (9, 24, 26). Contrastingly, unbound EMA, which remains free in solution, is simultaneously inactivated by reaction with water molecules and no longer capable of covalently binding to DNA (4, 10). Thus, DNA from viable cells, protected from reactive EMA by an intact cell wall/cell membrane, should not be affected by the inactivated EMA after cell lysis during DNA extraction.

EMA can selectively enter the cytoplasm of dead cells and cleave the DNA via photoactivation (24, 26). Therefore, cleaved DNA from damaged and/or dead cells cannot be amplified by PCR/real-time PCR. Thus, the combination of EMA and PCR/real-time PCR may potentially distinguish the DNA of viable cells from the DNA of dead cells. In the present study, we assessed this potential for the specific detection of DNA of viable *Legionella*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *Legionella* strains used in this study are listed in Table 1. All strains were grown at 37°C on buffered charcoal yeast extract (BCYE) agar (Becton Dickinson, Sparks, MD), using standard protocols. *Pseudomonas aeruginosa* strain PAO1, *Escherichia coli* K-12 strain

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TABLE 1. Summary of *Legionella* strains used in this study[a]

Strain	Species	Serogroup	Alternate strain name or source
80-045	*L. pneumophila*	1	Clinical isolate
Philadelphia-1	*L. pneumophila*	1	ATCC 33152; clinical isolate
NIIB0733	*L. pneumophila*	1	Bathtub
NIIB0805	*L. pneumophila*	1	Bathtub
NIIB0744	*L. pneumophila*	1	Cooling tower
NIIB0802	*L. pneumophila*	1	Cooling tower
NIIB0784	*L. pneumophila*	5	Bathtub
NIIB0797	*L. pneumophila*	5	Bathtub
NIIB0792	*L. pneumophila*	6	Bathtub
NIIB0864	*L. pneumophila*	6	Bathtub
NIIB0794	*L. pneumophila*	7	Cooling tower
NIIB0806	*L. pneumophila*	7	Cooling tower
NIIB0008	*L. micdadei*		ATCC 33218; clinical isolate
NIIB0009	*L. bozemanii*	1	ATCC 33217; clinical isolate
NIIB0010	*L. dumoffii*		ATCC 33343; clinical isolate
NIIB0012	*L. longbeachae*	2	ATCC 33484; clinical isolate
NIIB0052	*L. feeleii*	2	ATCC 35849; clinical isolate
NIIB0234	*L. gormanii*		ATCC 33297; soil isolate

[a] ATCC, American Type Culture Collection; NIIB, National Institute of Infectious Diseases, Department of Bacteriology.

XL1-Blue, *Serratia marcescens* strains E1 and E46, and *Brevundimonas nasdae* NIIB2318 were incubated at 37°C overnight on LB plates (Becton Dickinson). *Sphingomonas paucimobilis* (JCM 7516), *Caldimonas manganoxidans* (JCM 10698), *Porphyrobacter sanguineus* (JCM 20691), *Microbacterium lactium* (JCM 1379), *Bacillus megaterium* (JCM 2506), *Tepidimonas arfidensis* (JCM 13232), *Methyloversatilis universalis* (JCM 13912), and *Rhizobium radiobacter* (JCM 20371) strains were purchased from the Japan Collection of Microorganisms (JCM) (RIKEN BioResource Center, Saitama, Japan) and were incubated as recommended by JCM. After incubation, separate suspensions of each strain were made in sterile normal saline. Bacterial counts were determined by plating cells on appropriate plates after serial 10-fold dilutions.

Heat and chlorine treatments for *Legionella* strains. Dead *Legionella* cells were prepared by treatment with heat or sodium hypochlorite (Sigma-Aldrich, St. Louis, MO). Heat treatment was performed at 95°C for 2 min. Sodium hypochlorite treatment was performed at an available chlorine concentration of 0.5 or 1.0 ppm, followed by incubation for 30 min at room temperature. The residual chlorine concentration was assayed using Rapid DPD liquid (Kanto Chemical, Tokyo, Japan). After either treatment, *Legionella* cells were pelleted and resuspended in the original volume of normal saline before being subjected to EMA treatment. Death of the heat- and chlorine-treated *Legionella* cells was confirmed by using a BacLight Live/Dead bacterial viability kit (Molecular Probes, Leiden, The Netherlands).

EMA treatment and visible light irradiation of *Legionella* strains. EMA purchased from Sigma-Aldrich was prepared at a concentration of 10 mg/ml. EMA was added to *Legionella* suspensions at various concentrations and kept at 4°C for 10 min in the dark. Subsequently, each suspension was set on ice and exposed to visible light for 5 min (24).

Preparation of a mock sample of environmental conditions. To prepare a mock sample for use as an environmental model, isolated *Legionella* cells were added to tap water along with sodium thiosulfate (0.05%) to inactivate the chlorine already present in the tap water. Two hundred milliliters of water to which viable or chlorine-treated *Legionella* cells had been added was centrifuged for 15 min, and the pellets were resuspended in 2 ml normal saline. One milliliter of the suspension was treated with a low-pH buffer (0.2 M KCl-HCl buffer, pH 2.2) to reduce the number of environmental bacteria other than *Legionella*, and 100 μl of each dilution was plated on BCYE agar to determine the number of living *Legionella* cells. The genomic DNA of the remaining sample, with or without EMA treatment, was purified and used for real-time PCR.

Collection of water samples from public and model spas. A total of 25 samples, 9 from public spas and 16 from a model spa system (18, 28), were collected. In the model spa, no chlorine disinfection was performed for 10 days after men took baths to allow for *Legionella* contamination and growth in the bathtub and filter tank. Water samples (samples 10 to 17) from the bathtub were collected from day 3 to day 10 after the bath. On day 10, after one sample (sample 18) was obtained from the filter tank, a high concentration of chlorine was swiftly added into and circulated reversely throughout the filter tank to prepare chlorine-treated *Legionella* cells (18, 28). Water samples (samples 19 to 25) were separately collected from the filter tank 0, 1, 2, 3, 5, 6, and 7 min after the addition of chlorine. A solution of 500 milliliters of each sample was collected, and chlorine was inactivated by sodium thiosulfate. The samples were centrifuged at 7,500 rpm for 15 min, and the pellets were resuspended in 5 ml normal saline. One milliliter of the suspension was treated with 0.2 M KCl-HCl buffer (pH 2.2) to reduce the number of environmental bacteria other than *Legionella* and then plated on GVPC agar (Oxoid, Hampshire, United Kingdom) to determine the number of living *Legionella* cells. Five hundred microliters of each sample, with or without EMA treatment at 1, 5, 10, and 20 μg/ml, was exposed to visible light as described above. After photoactivation, the bacteria were collected by centrifugation and their genomic DNAs were purified for real-time PCR.

PCR. The genomic DNAs of bacteria were purified using a High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany). Oligonucleotide primers LEG448A and LEG854B, targeting the 16S rRNA gene (32), an EnviroAmp primer targeting the 5S rRNA gene (EnviroAmp *Legionella* kit package insert; Perkin-Elmer, Waltham, MA), and primers LmipL920 and LmipR1548, targeting the *L. pneumophila mip* gene (14), were used for PCR amplifications. The amplifications were carried out with Ex *Taq* polymerase (Takara Bio, Otsu, Japan), using a GeneAmp PCR system 9700 instrument (Applied Biosystems, Foster City, CA). A 20-μl PCR preparation was subjected to 30 cycles of denaturation at 98°C for 10 s, annealing at 63.5°C for 30 s, and extension at 72°C for 60 s. Ten-microliter solutions with the PCR-amplified DNA fragments were separated in 2% agarose gels (Takara Bio).

Real-time PCR. Real-time PCR targeting the 16S rRNA gene of *Legionella* was performed using an ABI Prism 7000 machine (Applied Biosystems). The 25-μl reaction volume contained 2 μl of DNA purified from each sample. Real-time quantification for SYBR green detection was performed with SYBR green PCR master mix (Applied Biosystems). The primers used were LEG427F (5′-GTAAAGCACTTTCAGTGGGGAG-3′) and LEG880R (5′-GGTCAACTTATCGCGTTTGCT-3′). The amplification reaction was performed with an initial 10-min denaturation step at 95°C followed by 40 cycles of repeated denaturation at 95°C for 15 s and annealing and polymerization at 63.5°C for 60 s. Premix Ex *Taq* (Takara Bio) was used for fluorescent probe-based real-time PCR. The quantification was performed with primers LEG427F and LEG880R and the molecular beacon probe P1 (5′-6-carboxyfluorescein-ACTGGACGTTACCCACAGAAGAAG-6-carboxytetramethylrhodamine-3′) (Takara Bio), designed for detection of the *Legionella* 16S rRNA gene. The amplification reaction was performed with 40 cycles of repeated denaturation at 95°C for 10 s and annealing and polymerization at 63.5°C for 60 s after a denaturation step at 95°C for 30 s.

Purified genomic DNA from 2×10^8 CFU of *L. pneumophila* 80-045 was used as an external standard. For each real-time PCR, the purified DNA was thawed and serially diluted to prepare four to six dilution points ranging from 1×10^7 to 1×10^2 *Legionella* cells as an external standard. A negative extraction control (PCR-grade water), a positive control, and the test samples were run in duplicate.

Statistical analyses. All experiments were carried out more than twice. The significance of the results was analyzed using Student's *t* test. Differences were considered significant at *P* values of <0.05.

RESULTS

Heat and chlorine treatment of *Legionella* strains. Eighteen *Legionella* strains, comprising 12 *L. pneumophila* and 6 non-*L. pneumophila* strains, were used (Table 1). The 12 *L. pneumophila* strains, which belonged to serogroups 1, 5, 6, and 7 (data not shown), were isolated from patients, water from cooling towers, or bathtubs. The six non-*L. pneumophila* strains comprised different *Legionella* species (Table 1) that are known as human pathogens.

All *Legionella* strains were suspended in sterile normal saline at approximately 1×10^7 CFU/ml and treated with heat or

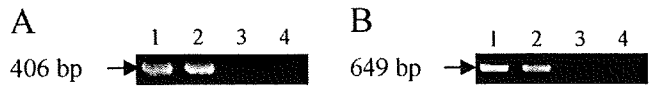

FIG. 1. PCRs targeting the 16S rRNA (A) and *mip* (B) genes of *Legionella* strain 80-045. The sizes of the amplified fragments from the 16S rRNA and *mip* genes are 406 and 649 bp, respectively. Lanes 1, viable *Legionella* cells without EMA treatment; lanes 2, viable *Legionella* cells with EMA treatment; lanes 3, heat-killed *Legionella* cells with EMA treatment; lanes 4, chlorine-killed *Legionella* cells with EMA treatment. EMA was used at a concentration of 20 μg/ml.

chlorine. After heat treatment at 95°C for 2 min, no colonies were detected in any suspensions of the *Legionella* strains plated on BCYE agar (data not shown). Chlorine treatment was initially performed at a concentration of 0.5 ppm of free residual chlorine. After 30 min of incubation at room temperature, the residual chlorine concentration became 0.3 ppm. No colonies were cultured, with the exception of strain 80-045, where approximately 5×10^4 cells remained culturable. Use of 1.0 ppm chlorine, which produced a residual concentration of 0.6 ppm, resulted in no detectable growth of strain 80-045. Therefore, 1.0 ppm chlorine was used in further experiments. By using a BacLight Live/Dead bacterial viability kit, >99% of the heat- and chlorine-treated *Legionella* cells were determined to be in a nonviable state (dead), while >99% of the *Legionella* cells without treatment were in a viable state.

Combined use of EMA and PCR to detect viable *Legionella* cells. We examined whether the combined use of EMA and PCR could specifically detect viable *Legionella* cells. Viable, heat-treated, and chlorine-treated *Legionella* cells were treated with 10 μg/ml, 20 μg/ml, and 50 μg/ml of EMA. Viable *Legionella* cells that were not treated with EMA were used as a control. Genomic DNA was purified and used as a template for PCR.

The results of the EMA-PCR assay using 20 μg/ml EMA are depicted in Fig. 1. PCR products targeting the 16S rRNA (Fig. 1A) and *mip* (Fig. 1B) genes in genomic DNA from viable cells of *L. pneumophila* strain 80-045, with or without treatment of EMA, displayed similar agarose gel electrophoretic patterns (Fig. 1, lanes 1 and 2). However, no PCR products for DNAs from the heat- and chlorine-killed cells with EMA treatment were observed (Fig. 1, lanes 3 and 4). Amplified fragments of *Legionella* DNA from the heat- and chlorine-killed cells without EMA treatment were detected and were similar to those from viable cells (data not shown). Similar results were also obtained by the use of the other 11 *L. pneumophila* strains for the detection of 16S rRNA and *mip* genes and the 6 non-*L. pneumophila* strains for detection of the 16S rRNA gene (data not shown). When 10 μg/ml of EMA was used, the intensity of the amplified fragments from the heat- and chlorine-killed *Legionella* cells was weaker than that for the viable cells, although amplified bands were still visible on the gel (data not shown). Conversely, the amplified fragments from the heat- and chlorine-killed *Legionella* cells were undetectable when 50 μg/ml EMA was used, while the intensity of fragments from EMA-treated viable *Legionella* cells was significantly weaker than that for untreated cells (data not shown). Therefore, 20 μg/ml EMA was used in further experiments, except for treatment of water samples from spas.

PCR targeting the 5S rRNA gene was also performed. Although the intensity of PCR fragments for the DNAs from the heat- and chlorine-treated cells became weaker than that for the viable cells with EMA treatment, the bands were clearly observed on the gel (data not shown).

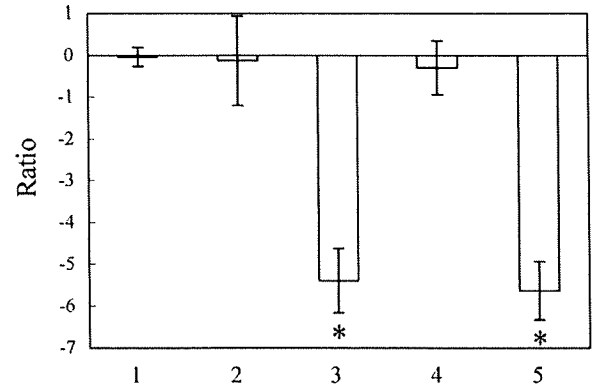

FIG. 2. EMA and real-time PCR combined analyses of viable or dead cells of *L. pneumophila* strain 80-045. The number of bacteria was estimated from the amount of DNA detected by real-time PCR with SYBR green as the reporter dye. The number of viable cells without EMA treatment was set as 1. The numbers of EMA-treated viable cells (bar 1), untreated heat-killed cells (bar 2), EMA-treated heat-killed cells (bar 3), untreated chlorine-killed cells (bar 4), and EMA-treated chlorine-killed cells (bar 5) are described as ratios against the number of untreated viable cells [i.e., ratio = $\log_{10}$ (number of test cells/number of untreated viable cells)]. The error bars represent standard deviations from more than three independent experiments. Asterisks indicate significant decreases in the numbers of EMA-treated samples compared to those of untreated samples.

Combined use of EMA and real-time PCR to detect viable *Legionella* cells. To quantify the DNA purified from the bacteria treated with EMA, real-time PCR targeting the 16S rRNA gene was performed with SYBR green PCR master mix. Approximately 1×10^6 to 1×10^7 CFU/ml of strains 80-045, Philadelphia-1, NIIB0008, and NIIB0009 was used. The detection limit for *L. pneumophila* was 1 CFU/reaction (data not shown).

Results of real-time PCR are shown in Fig. 2. When the amount of DNA detected by real-time PCR was calculated as the cell count, the number of viable bacteria treated with EMA, heat-killed bacteria with or without EMA treatment, or chlorine-killed bacteria with or without EMA treatment was compared to the number of viable bacteria without EMA treatment and expressed as a ratio. The ratio of viable to EMA-treated 80-045 cells was -0.04 ± 0.23 $\log_{10}$ (Fig. 2, bar 1). No significant difference was evident between the amounts of DNA of untreated and EMA-treated viable *Legionella* cells, because EMA could not intercalate and cleave the genomic DNA of viable *Legionella* cells. The numbers of heat- and chlorine-killed *Legionella* cells without EMA treatment, estimated by real-time PCR, did not obviously decrease compared to that of the viable cells. The ratios of heat- and chlorine-killed 80-045 cells were -0.13 ± 1.06 $\log_{10}$ (Fig. 2, bar 2) and -0.30 ± 0.64 $\log_{10}$ (Fig. 2, bar 4), respectively. These results indicate that the heat and chlorine treatments performed here left the DNAs intact. Conversely, after EMA treatment, the amounts of amplifiable DNA in heat- and chlorine-killed *Legionella* cells significantly decreased compared to that in the

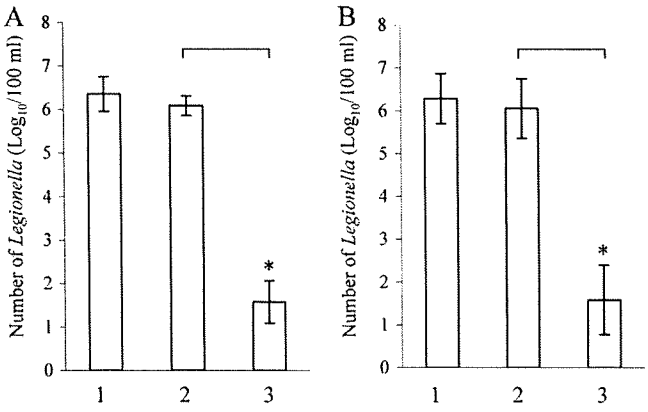

FIG. 3. Number of *L. pneumophila* strain 80-045 cells (A) and *L. bozemanii* strain NIIB0009 cells (B) isolated from 100-ml mock samples, determined by incubation or estimated by real-time PCR. 6-Carboxyfluorescein was used as the reporter dye for real-time PCR. Bars 1, real numbers of viable *Legionella* cells determined by plating on BCYE plates; bars 2, numbers of EMA-treated viable *Legionella* cells determined by fluorescent probe-based real-time PCR; bars 3, numbers of EMA-treated chlorine-killed *Legionella* cells determined by real-time PCR. No viable cells of chlorine-killed 80-045 and NIIB0009 were detected by plating on BCYE agar. The experiments were repeated separately more than five times. Asterisks indicate significant decreases in the number of EMA-treated chlorine-killed *Legionella* cells determined by real-time PCR.

viable cells. The ratios of heat- and chlorine-killed 80-045 cells were -5.39 ± 0.77 $\log_{10}$ (Fig. 2, bar 3) and -5.64 ± 0.70 $\log_{10}$ (Fig. 2, bar 5), respectively. Comparable results were also yielded when the Philadelphia-1, NIIB0008, and NIIB0009 strains were used (data not shown). Altogether, these results are consistent with the ability of EMA to cleave genomic DNA and to decrease the amount of intact DNA of the heat- and chlorine-treated *Legionella* cells to the level of approximately 4 to 5 $\log_{10}$ fewer cells.

Discrimination of viable and chlorine-treated *Legionella* cells from mock environmental samples by the combined use of EMA and real-time PCR. In order to investigate the possible utility of EMA and real-time PCR for the detection of viable *Legionella* cells in environmental samples, we prepared a mock sample by the suspension of *Legionella* strains in tap water. Approximately 1×10^6 to 1×10^7 CFU of viable or chlorine-treated *Legionella* were added to tap water. The number of bacteria was determined by plating on BCYE agar and estimated by the combined EMA and real-time PCR assay. To avoid the possible contamination of the assay by nonlegionella bacteria present in tap water, fluorescently probed real-time PCR targeting the 16S rRNA gene was performed with the LEG427F and LEG880R primers and probe P1. The detection limit was 10 CFU/reaction (data not shown).

The numbers of *Legionella* cells detected in 100 ml water are shown in Fig. 3. Approximately 6.35 ± 0.40 $\log_{10}$ CFU of viable 80-045 cells (Fig. 3A, bar 1) and 6.29 ± 0.58 $\log_{10}$ CFU of viable NIIB0009 cells (Fig. 3B, bar 1) were detected by plating. After treatment with 20 μg/ml of EMA, 6.09 ± 0.22 $\log_{10}$ 80-045 cells (Fig. 3A, bar 2) and 6.06 ± 0.70 $\log_{10}$ NIIB0009 cells (Fig. 3B, bar 2) were detected by real-time PCR. When the chlorine-treated above *Legionella* solution (after chlorine treatment, the number of cultivable cells became <10 CFU/100 ml by plating) was treated with EMA, only 1.57 ± 0.49 $\log_{10}$ 80-045 cells (Fig. 3A, bar 3) and 1.59 ± 0.81 $\log_{10}$ NIIB0009 cells (Fig. 3B, bar 3) were detected by real-time PCR. The number of EMA-treated dead *Legionella* cells, estimated by real-time PCR in this experiment, was approximately 4.5 $\log_{10}$ less than that of the viable cells.

Specificity of real-time PCR. Surveillance performed in Japan has detected over 20 species of nonlegionella bacteria in bathtub water by using a PCR-denaturing gradient gel electrophoresis method (5). In addition to these bacterial species, *E. coli*, *S. marcescens*, and *Brevundimonas* species have been reported to usually be present in water and/or soil of the external environment (3, 8, 11, 15, 25). Thirteen appropriate nonlegionella strains (Table 2) were used to assess PCR specificity. Genomic DNAs purified from these strains were used as templates, and real-time PCR was performed with primers LEG427F and LEG880R and probe P1. After 40 PCR cycles, no amplification signals could be detected (Table 2). The results indicate that real-time PCR has a high specificity for *Legionella* species.

Detection of *Legionella* in water samples collected from spas by the combined use of EMA and real-time PCR. In order to investigate the utility of the proposed method for the specific detection of culturable *Legionella* from the environment, 25 water samples were analyzed in this study. Samples 1 to 9 were collected from public spas, whereas samples 10 to 25 were from a model spa (18, 28), as described in Materials and Methods.

The results are shown in Table 3. Samples 1 and 2 were collected from jetted and outdoor bathtubs, respectively, at the same public spa facility. The number of *Legionella* cells in sample 1, as estimated by real-time PCR assay, was higher than

TABLE 2. Nonlegionella bacteria used to test the specificity of real-time PCR[a]

Species	Strain or source[b]	No. of bacteria (CFU/ml)[c]
Pseudomonas aeruginosa	Clinical isolate	1.6×10^6
Escherichia coli	K-12	1.3×10^6
Serratia marcescens E1	Environmental isolate	8.6×10^5
Serratia marcescens E46	Environmental isolate	3.8×10^5
Brevundimonas nasdae	Environmental isolate	1.6×10^7
Sphingomonas paucimobilis	JCM7516; environmental isolate	1.0×10^6
Caldimonas manganoxidans	JCM10698; environmental isolate	3.1×10^6
Porphyrobacter sanguineus	JCM20691; environmental isolate	1.5×10^6
Microbacterium lactium	JCM1379; environmental isolate	1.0×10^6
Bacillus megaterium	JCM2506; environmental isolate	7.0×10^5
Tepidimonas arfidensis	JCM13232; clinical isolate	8.0×10^5
Methyloversatilis universalis	JCM13912; environmental isolate	1.0×10^6
Rhizobium radiobacter	JCM20371; environmental isolate	1.2×10^6

[a] Real-time PCR was performed with primers LEG427F and LEG880R and probe P1. No products were detected for any of the organisms tested.
[b] JCM, Japan Collection of Microorganisms.
[c] The number of bacteria of each strain was determined by plating cells on suitable plates, and the number indicated was used for real-time PCR.

TABLE 3. Comparison of results of plating and real-time PCR for water samples from spas

Sample no.[a]	Free chlorine concn (ppm)	No. of *Legionella* cells by plating (log_{10} CFU/100 ml)[b]	No. of *Legionella* cells estimated by real-time PCR (log_{10} CFU/100 ml)[c]				
			No EMA treatment	Treatment with EMA			
				1 μg/ml	5 μg/ml	10 μg/ml	20 μg/ml
1	1.0	1.3	3.2	ND	ND	ND	1.4
2	0.1	2.4	2.2	ND	ND	ND	1.3
3	0.5	1.3	2.8	1.9	1.3	—	—
4	0	3.0	3.2	2.9	2.4	2.2	1.9
5	0.1	1.3	—	—	—	—	—
6	3	<1	—	—	—	—	—
7	2	<1	—	—	—	—	—
8	0	1	—	—	—	—	—
9	2.4	<1	—	—	—	—	—
10	0	<1	—	—	—	—	—
11	0	<1	—	—	—	—	—
12	0	<1	—	—	—	—	—
13	0	<1	—	—	—	—	—
14	0	2.9	3.0	3.1	3.0	2.7	1.9
15	0	3.8	4.0	3.9	3.8	3.1	3.0
16	0	4.8	5.2	4.7	3.8	3.1	—
17	0	4.8	5.4	4.3	3.5	2.9	2.3
18	0	4.6	5.2	4.4	3.5	2.5	2.2
19	0.01	5.4	5.9	5.5	5.5	4.0	3.0
20	2.5	3.6	5.5	4.8	3.8	3.1	2.8
21	3.5	1.8	5.2	3.3	2.6	2.1	1.6
22	6.4	1.5	4.0	3.6	3.4	1.6	—
23	8.1	<1	3.3	1.4	1.3	0.7	0
24	8.2	<1	3.0	1.2	1.3	0.7	—
25	8.3	<1	3.8	3.6	1.1	1	—

[a] Samples 1, 2, and 7 to 9 were obtained from bathtubs, samples 3 to 5 were from filter tanks, and sample 6 was from a pipeline of public spas. Samples 10 to 17 were obtained from the bathtub and samples 18 to 25 were from the filter tank of a model spa.
[b] The number of bacteria was determined by plating cells on GVPC plates.
[c] Real-time PCR was performed with primers LEG427F and LEG880R and probe P1. ND, not done; —, not detected.

that determined by plating on GVPC agar. After treatment with EMA at 20 μg/ml, the number of *Legionella* cells estimated by real-time PCR was similar to that determined by plating. These results indicate that sample 1 contained DNA and/or uncultivable cells of *Legionella* which are sensitive to treatment with EMA. On the other hand, the number of *Legionella* cells in sample 2, as estimated by real-time PCR, was similar to that determined by plating. This result suggested that almost all of the *Legionella* cells in the sample were culturable. After treatment with 20 μg/ml of EMA, the number of *Legionella* cells estimated by real-time PCR was smaller (1.1 log_{10} CFU/100 ml) than that determined by plating. It seems that EMA cleaves a part of the genomic DNA of viable cells under environmental conditions. All of these results suggested that the appropriate concentrations of EMA were different among water samples. Therefore, EMA was used at 1, 5, 10, and 20 μg/ml for the other 23 samples.

Among the 25 water samples, no amplification signals were detected in 9 samples (samples 5 to 9 and 10 to 13), with or without treatment of EMA, by using real-time PCR (Table 3). By plating on GVPC agar, no colonies were isolated from seven of the nine samples. Only a few colonies (10 CFU/100 ml and 20 CFU/100 ml) were detected in the other two samples (samples 5 and 8), which almost corresponded to the results obtained by real-time PCR (Table 3). More than 2 log_{10} CFU/100 ml of *Legionella* cells, estimated by real-time PCR, existed in the remained 16 water samples (Table 3). The numbers of *Legionella* cells in nine samples (samples 3, 4, and 14 to 20), estimated by the combined EMA and real-time PCR assay, were similar to those determined by plating when EMA treatment was performed at 1 or 5 μg/ml (Table 3). When these samples were treated with EMA at 10 and 20 μg/ml, the numbers of *Legionella* cells estimated by real-time PCR were smaller than those determined by plating. Meanwhile, 10 or 20 μg/ml of EMA treatment was needed for the remaining six samples (samples 1 and 21 to 25) in order to obtain similar results between the numbers of *Legionella* cells estimated by real-time PCR and those determined by plating (Table 3). The numbers of *Legionella* cells, estimated by real-time PCR, in the water samples (samples 19 to 25) without EMA treatment were gradually decreased after the addition of chlorine (Table 3). Although the precise cause has not yet been elucidated, it may be attributed to breaking of the genomic DNA from the uncultivable *Legionella* cells by chlorine.

Taking all of these results together, EMA treatment could selectively amplify the genomic DNA of the culturable *Legionella* cells in the water samples by real-time PCR assay. However, EMA concentrations that were effective were different among environmental samples and seemed to be related to the sensitivity of *Legionella* cells to EMA or the chlorine concentrations of the samples. A low concentration of EMA (1 or 5 μg/ml) was enough for eight samples (samples 3, 4, and 14 to 19) in which chlorine was not detected or detected at a low concentration (≤0.5 ppm). On the other hand, a high concentration of EMA (10 or 20 μg/ml) was needed for six samples (samples 1 and 21 to 25) in which chlorine was detected at high

concentrations (≥1.0 ppm). A large number of uncultivable cells killed by chlorine in the six samples may be one of the reasons that EMA must be used at high concentrations for the number of *Legionella* cells determined by plating to match that estimated by real-time PCR. All of the results suggested that a high concentration of EMA is needed to cleave genomic DNA of uncultivable *Legionella* cells treated with chlorine at high concentrations. In the case of sample 20, the water sample was collected immediately after chlorine treatment, so 5 μg/ml of EMA was probably sufficient.

DISCUSSION

In the present study, we demonstrate the combined use of EMA and PCR/real-time PCR for rapid detection of viable *Legionella* cells. The results reveal that EMA can specially enter and cleave the genomic DNA of heat- and chlorine-treated *Legionella* cells. After treatment with EMA, PCR could not detect the DNA present in the dead cells and the amount of DNA significantly decreased compared to that for the viable cells in the real-time PCR assay. The assay was also useful for detection of culturable *Legionella* in water samples. These results show that the combined use of EMA and PCR/real-time PCR is sufficient to detect viable *Legionella* cells.

Approximately 1×10^6 to 1×10^7 CFU/ml of *Legionella* strains was used in the EMA real-time PCR assay. The detected decrease of DNA in the dead cells by EMA treatment was approximately 4 to 5 $\log_{10}$ at 20 μg/ml EMA (Fig. 3); therefore, part of the genomic DNA, corresponding to approximately 10^2 CFU/ml, still remained. However, the number of uncultivable *Legionella* cells estimated by real-time PCR with 68 environmental samples was $<10^5$ CFU/100 ml, and that with 66 samples (97%) was $<10^4$ CFU/100 ml in our preliminary experiment (unpublished data). In Japan, the guideline by the Ministry of Health, Labor, and Welfare for prevention of Legionnaires' disease specifies that the detection limit of culturable *Legionella* from bath water must be <10 CFU/100 ml. Therefore, the decrease of 4 to 5 $\log_{10}$ CFU with EMA treatment is theoretically sufficient to place environmental samples with low levels of putative viable but uncultivable *Legionella* in a low-risk category according to Japanese guidelines. However, plating for detection of viable *Legionella* cells should be performed at the same time because the possibility of false-positive results cannot entirely be eliminated.

Part of the genomic DNA, corresponding to approximately 10^2 CFU/ml, of the heat- and chlorine-treated *Legionella* cells still remained when the combined EMA (20 μg/ml) and real-time PCR assay was performed (see above) (Fig. 2 and 3), which may be due to the limit of EMA activity at that concentration. When the EMA concentration was increased, the intensity of PCR-amplified fragments in the viable *Legionella* cells became lower, which would be due to the damage of viable cells caused by EMA. The concentration of EMA used seems to be critical for the maximum discrimination of viable cells from dead cells. The 5S rRNA, 16S rRNA, and *mip* genes could be amplified by PCR as fragments of 108 bp, 406 bp, and 649 bp, respectively, in our experiment. If EMA randomly binds and cleaves the DNA sequence, a smaller DNA region would not be affected after EMA treatment and could be amplified by PCR. Amplification of the 16S rRNA gene was most effective for the discrimination of viable *Legionella* cells from dead cells, although we do not know the exact reason that amplification of the 16S rRNA gene was most available in our experiment. EMA might recognize the DNA region of the 16S rRNA gene most effectively, but the problems with amplification should be resolved in the future.

Twenty-five water samples were tested in this study, and the combined EMA–real-time PCR assay was confirmed to be useful for specific detection of viable *Legionella* cells in these environmental samples. In order to avoid false-positive or -negative results by combined use of EMA and real-time PCR, the concentration of EMA used for water samples may be most critical. The concentration of EMA needed was shown to be related to the residual chlorine concentration in the water samples in this study. Because the investigation in this study was done on a small number of water samples, further confirmation will be required by the use of a large number of water samples from public spas. It is probable that EMA at the high concentration used in some water samples (samples 3, 4, and 14 to 19) (Table 3) could also enter viable *Legionella* cells and cleave their genomic DNA. It was recently shown that propidium monoazide (PMA) is superior to EMA for avoiding entrance into and/or cleavage of genomic DNA of viable bacterial cells (20). We are planning to compare the effects of EMA and PMA on detection of viable *Legionella* cells in water samples in our next experiment.

After the first outbreak of legionellosis caused by *L. pneumophila* in Philadelphia (7), much research was conducted on the behavior and life cycle of *Legionella*. It is now clear that monitoring and removal of *Legionella* from waters that come into contact with humans, particularly water from distribution systems, are an effective way to prevent infections caused by *Legionella*. In recent years, disinfection and cleaning of man-made water systems have been strictly observed in Japan. Surveillance investigations on the water systems in the Kanto area of Japan showed that the number of *Legionella*-positive samples and the number of *Legionella* isolates from such samples have been decreasing annually since 2003 (12, 30). We hope that the rapid detection method described here is useful for the control and monitoring of water systems, especially for continuous environmental surveillance at certain points.

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Short Communication

An Improved Protocol for the Preparation and Restriction Enzyme Digestion of Pulsed-Field Gel Electrophoresis Agarose Plugs for the Analysis of *Legionella* Isolates

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SUMMARY: Pulsed-field gel electrophoresis (PFGE), which determines the genomic relatedness of isolates, is currently used for the epidemiological investigation of infectious agents such as bacteria. In particular, this method has been used for the epidemiological investigation of *Legionella* outbreaks. However, it takes 4 days to complete a *Legionella*-PFGE analysis. Due to partial digestion and DNA damage, the reproducibility of the obtained fragment digestion patterns is poor for this pathogen. In this study, we report an improved protocol that takes only 2 days to complete and that allows clear discrimination of the restriction profile with higher reproducibility than that previously achieved.

Each bacterial strain has genomic diversity in its nucleotide sequence. Restriction enzyme digestion of genomic DNA produces a distinctive restriction profile that is useful for epidemiological analyses. The standard gel electrophoresis protocol was improved by introducing an alternating voltage gradient to obtain better resolution of larger DNA fragments; this technique is known as pulsed-field gel electrophoresis (PFGE) (1). The PFGE method is very useful for discriminating between bacterial genomes.

Legionella are ubiquitous inhabitants of aquatic environments and moist soil, and they replicate as intracellular parasites of protozoa (2,3). Hot springs, public baths, and cooling towers are the most common sources of legionellosis in humans. PFGE has been used to identify sources and routes of *Legionella* infections (4,5). The conventional PFGE protocol was modified by De Zoysa and Harrison; according to this modification, no lysozyme treatment was performed, and the concentrations of proteinase K (2 mg/ml) and *Sfi*I (60 U/plug) were increased (6). However, that protocol required at least 4 days to yield results, and reproducibility remained poor (4,6). Given these disadvantages associated with this method, we conducted the present study to improve the PFGE protocol.

A total of 16 *Legionella* strains belonging to 6 species were used in this study (Table 1). We collected 10 *L. pneumophila* strains isolated from patients, bath water, and cooling tower water, and these strains belonged to serogroups 1, 5, 6, and 7. Strain K6 possessed a Km^r Laz^+ cassette gene on the chromosome of strain Philadelphia-1 (8). Five non-*L. pneumophila* strains isolated from patients were also collected, and these belonged to different *Legionella* spp. (Table 1). All strains were grown at 37°C on buffered charcoal yeast extract (BCYE) agar (Becton Dickinson, Sparks, Md., USA). After a 2-day incubation period, *Legionella* cells were suspended in sterile water at an optical density at 600 nm (OD_{600}) of 0.5. The suspensions were then mixed with the same volume of 1% SeaKem® Gold Agarose (Cambrex Bio Science Rockland, Maine, USA) and were used for casting plugs. A conventional protocol (4,9) and the improved protocol discussed below were both used to process the agarose plugs for PFGE.

Table 1. Summary of *Legionella* strains used in the study

Strains no.	Species	Serogroup	Strain, source or reference
80-045	*L. pneumophila*	1	Clinical isolate (7)
K6	*L. pneumophila*	1	(8)
Philadelphila-1	*L. pneumophila*	1	ATCC33152[1], Clinical isolate
NIIB0733[2]	*L. pneumophila*	1	Bathtub
NIIB0805	*L. pneumophila*	1	Bathtub
NIIB0744	*L. pneumophila*	1	Cooling tower
NIIB0802	*L. pneumophila*	1	Cooling tower
NIIB0784	*L. pneumophila*	5	Bathtub
NIIB0792	*L. pneumophila*	6	Bathtub
NIIB0794	*L. pneumophila*	7	Cooling tower
NIIB0806	*L. pneumophila*	7	Cooling tower
NIIB0008	*L. micdadei*	–	ATCC33218, Clinical isolate
NIIB0009	*L. bozemanii*	1	ATCC33217, Clinical isolate
NIIB0010	*L. dumoffii*	–	ATCC33343, Clinical isolate
NIIB0012	*L. longbeachae*	2	ATCC33484, Clinical isolate
NIIB0052	*L. feeleii*	2	ATCC35849, Clinical isolate

[1]: ATCC, American Type Culture Collection.
[2]: NIIB, National Institute of Infectious Diseases, Department of Bacteriology.

The conventional protocol is shown in Fig. 1; *Legionella* cells in the agarose plugs were first lysed with lysozyme (Wako Pure Chemical Industries, Osaka, Japan). Subsequently, the plugs were treated overnight with 1 mg/ml of proteinase K (Roche Diagnostics, Mannheim, Germany) and 1% N-lauroylsarcosine (Sigma-Aldrich, St. Louis, Mo., USA) in 0.5 M EDTA (pH 8.0) at 50°C. After washing the plugs with Pefabloc SC (Roche Diagnostics), TE Buffer (10 mM Tris: 1 mM EDTA, pH 8.0), and 1 × M buffer (TaKaRa Bio, Otsu, Japan), DNA in the plugs were digested overnight with

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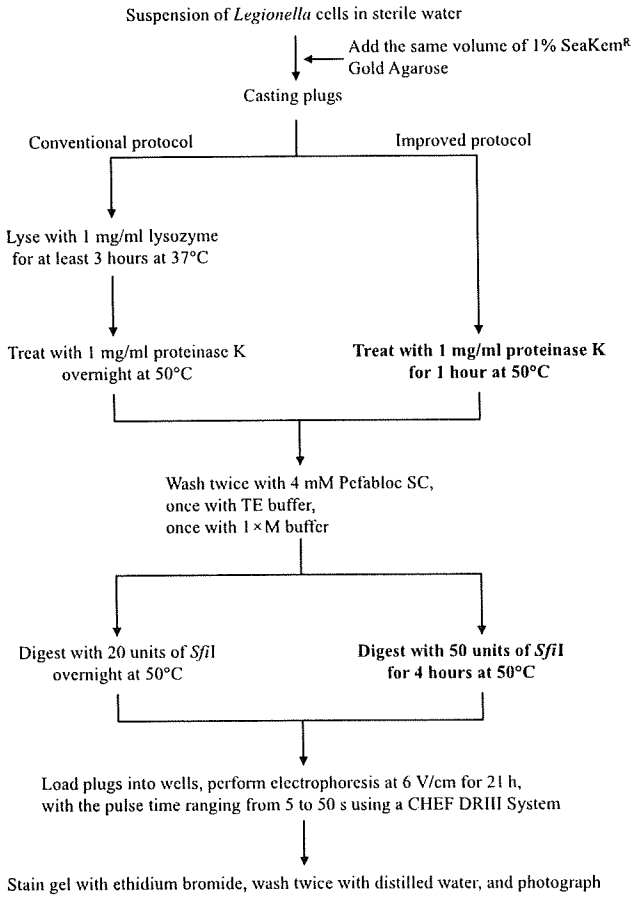

Fig. 1. Experimental overview of the conventional and improved PFGE protocols for *Legionella* strains. The main modified steps are shown in bold.

20 units of *Sfi*I (10 U/μl; TaKaRa Bio). Subsequently, electrophoresis was carried out using a CHEF DRIII System (Bio-Rad Laboratories, Richmond, Calif., USA). Thereafter, the gels were stained and photographed. This conventional PFGE protocol took 4 days to complete. The electrophoresis profiles of 16 *Legionella* strains yielded by the conventional protocol are shown in Fig. 2A. A white smeary band at the bottom of the gel (e.g., lanes 3, 5, 9, and 10) was observed, suggesting that some damage to the DNA had occurred. Furthermore, some faint bands possibly attributable to incomplete restriction enzyme digestion were visible in the profiles of all strains. As the incompletely digested bands appeared at different positions in various experiments (data not shown), it was difficult to obtain reproducible profiles and to compare sample data between gels. Similar observations have also been reported by De Zoysa and Harrison (6).

In order to overcome these disadvantages, we modified the conventional protocol for the preparation and restriction enzyme digestion of agarose plugs. To reduce plug preparation time and decrease damage to the genomic DNA, the lysozyme treatment was eliminated. Furthermore, the time required for treatment with proteinase K and N-lauroylsarcosine at 50°C was reduced from overnight to 1 h. Next, a high concentration of *Sfi*I (40 U/μl; Roche Diagnostics) of 50 units/plug was used in order to complete the digestion of *Legionella* DNA. The electrophoresis profiles of the 16 strains analyzed by this improved method are shown in Figs. 2B and 2C. With the improved protocol, no white smeary band was obtained, and the bands of digested DNA fragments were significantly clearer than those shown in Fig. 2A. Fewer digested DNA fragments from *Legionella* strains were seen as compared to the number of those from the corresponding strains presented in Fig. 2A; this difference may be attributed to the disap-

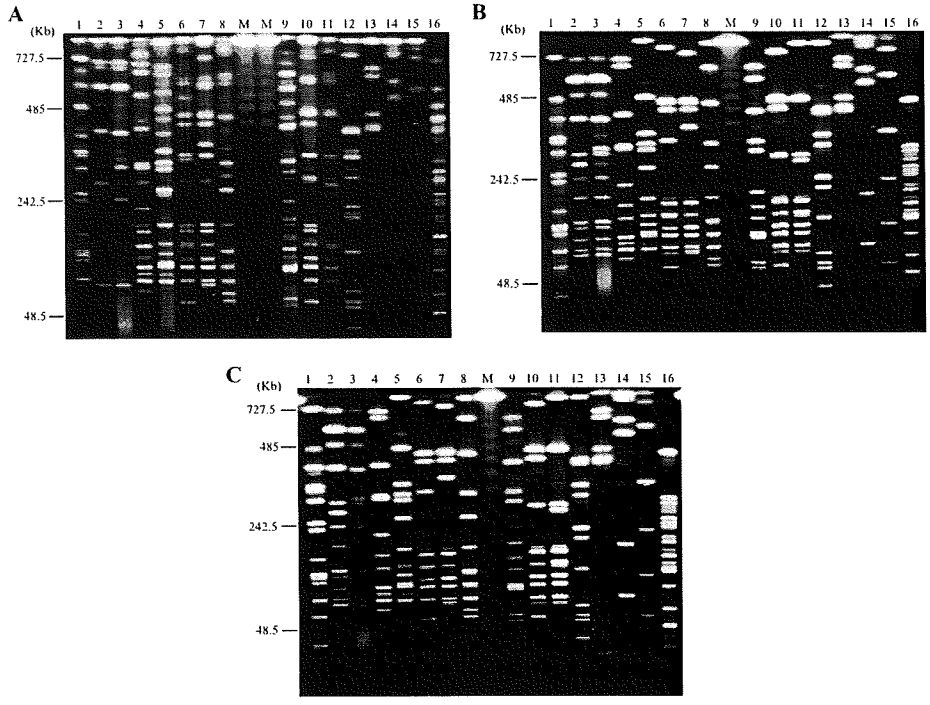

Fig. 2. PFGE profiles obtained with the conventional (A) and improved (B and C) protocols for *Legionella* strains. Lambda ladder marker (Bio-Rad) was used as a DNA size standard and molecular sizes are indicated on the left. Lane 1, 80-045; lane 2, K6; lane 3, Philadelphila-1; lane 4, NIIB0733; lane 5, NIIB0805; lane 6, NIIB0744; lane 7, NIIB0802; lane 8, NIIB0784; lane 9, NIIB0792; lane 10, NIIB0794; lane 11, NIIB0806; lane 12, NIIB0008; lane 13, NIIB0009; lane 14, NIIB0010; lane 15, NIIB0012; lane 16, NIIB0052; M: lambda ladder marker. A white smeary band at the bottom of the gel A (e.g., in lanes 3, 5, 9, and 10) indicates DNA damage.

pearance of incompletely digested fragments (Fig. 2B). The modified protocol of PFGE took only 2 days, as compared to the 4 days required to carry out the original protocol. A time saving of 2 days would exert a significant positive impact on the genotyping of *Legionella* infections in the context of medical diagnostics services.

To examine the reproducibility of the profiles obtained by the improved treatment, PFGE of these *Legionella* strains was separately performed at least 3 times. Figures 2B and 2C show two profiles of independently prepared plugs of *Legionella* isolates produced using different CHEF DRIII System machines. The profiles of the respective strains, with the exception of NIIB0010 (lane 14), were the identical between agarose gels run on different CHEF DRIII System machines. The 3 bands located at the top of lane 14 were separated in Fig. 2B, whereas only imperfect separation is shown in Fig. 2C. When the same machine was used, no differences were observed between the profiles of NIIB0010 (data not shown). Therefore, the difference noted above might be attributable to the different conditions required for the use of different machines. The present results demonstrated that reproducible profiles could be obtained with the improved PFGE method.

In this study, we attempted to overcome some of the disadvantages associated with the conventional PFGE method used for the analysis of *Legionella* strains. The improved protocol was found to be quicker and easier than the former approach. Lysozyme treatment for the preparation of PFGE plugs of Gram-negative bacteria (*Campylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella*, *Shigella*, *Vibrio cholerae*, and *Yersinia pestis*) has already been omitted from the protocols recommended by Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/PULSENET/protocols.htm). De Zoysa and Harrison also demonstrated that the use of lysozyme is not necessary for treating *Legionella* plugs (6). Therefore, lysozyme treatment was omitted in the present study as well. The profiles of the isolates examined were identical with and without lysozyme treatment (data not shown). In addition, the duration of proteinase K treatment was reduced in the present study. No differences between profiles of corresponding samples treated with proteinase K for 1, 8, and 24 h (data not shown) were observed. Treatment with 0.5 mg/ml of proteinase K for 1 h was sufficient to produce the same results (data not shown). Thus, the present results demonstrated that *Legionella* cells in plugs can be efficiently lysed by treatment with 0.5 - 1 mg/ml of proteinase K for 1 h. Overnight digestion with *Sfi*I at 50°C gave a white smeary band at the bottom of the gel (data not shown), which was suggestive of damage to the *Legionella* DNA due to extended digestion with the restriction enzyme. Use of the improved protocol described in this study rendered the fragment bands more clearly visible, and thus comparison of the profiles between different isolates was facilitated, as was analysis of the linkage between environmental isolates and the clinical isolates of *Legionella*. This new protocol will therefore be recommended for use in epidemiological investigations of *Legionella* isolates.

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〈原　著〉

分離集落の特徴を利用したレジオネラ属菌分別法の有用性

森本　洋

Usefulness of Selection of Legionella by Colony Appearance

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要　旨

レジオネラ属菌の培養検査を行う場合，検査対象試料によっては，さまざまな雑菌が混在しているため，酸，熱による前処理や選択分離培地の使用が必要となる．しかしながら，これら前処理や選択分離培地を使用しても，それら雑菌の発育を抑制できない場合が往々にしてある．多くのレジオネラ属菌検査マニュアルには，「レジオネラ属菌分離集落の特徴として，大小不同の灰白色湿潤集落で特有の淡い酸臭がある」と記載されているが，実際の検査においては，このような集落が多数存在し，雑菌との分別が困難な場合が多い．そこで本研究では，より正確で簡便な集落観察方法を検討したので報告する．

分離培地上の発育集落に斜光を当て，実体顕微鏡で観察すると，レジオネラ属菌は，特徴的な外観構造(カットグラス様，モザイク様)を呈した．この観察法を用いると，レジオネラ属菌と雑菌を効率良く分別，釣菌することができ，菌数測定も簡便に，極めて正確に行えることが明らかとなった．

本研究では，環境試料から *L. pneumophila* の13血清群およびその他17種類のレジオネラ属菌が，この観察法で効率良く検出された．また，レジオネラ肺炎の患者から最も高率に検出されている *L. pneumophila* 血清群1においても，この特徴が観察されたことから，本観察法は，定期的な環境水の自主検査および感染源や汚染源を調査するにあたり有効な検査法の一つであると思われた．

Key words：レジオネラ集落，斜光，カットグラス様，モザイク様

はじめに

レジオネラ症の感染源・感染経路は，レジオネラ属菌が生息する土壌の砂塵や，それらが混入し本菌が増殖した温泉などの浴槽水およびビルの冷却塔水など人工環境水からのエアロゾルの吸入が挙げられている．そのため，これらの環境試料が検査対象となる場合が多い．分離培地上では，特有の淡い酸臭のある灰(青)白色湿潤集落を釣菌することとなっている(以下従来法)[1~6]．しかしながら本検査では，分離培地上に，前述のような特徴の集落が，レジオネラ属菌，非レジオネラ属菌を問わず多数存在していることが多い．その結果，分離培地上での分別が難しいため，確認には多数のレジオネラ属菌様集落を釣菌して，同定しなければならない．

1981年に竹田らは，当時確認されていたレジオネラ症菌血清型1～3は，分離培地上の集落に斜光を当てると，その外観がカットグラス様の構造を呈すると紹介している[7]．*Manual of* CLINICAL MICROBIOLOGY[8,9]においても，レジオネラ属菌集落に対し同様の記載がなされている．

そこで我々は，現在一般的に行われているレジオネラ属菌検査法の中に，この形態的特徴を利用する方法を取り入れることで，効率的にレジオネラ属菌の確認，釣菌および菌数の測定などができるかを検討し，その有用性を明らかにしたので報告する．

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材料と方法

1. 基準株による確認

1) 供試菌株

5種類のGIFU strain (*Legionella pneumophila* GTC 296 (ATCC 33152), GTC 297 (ATCC 33216), *L. micdadei* GTC 299 (ATCC 33218), *L. oakrigensis* GTC 319 (ATCC 33761), *L. spiritensis* GTC 401 (ATCC 35249))を供試菌株とした.

2) 使用分離培地

現在広く普及している市販分離生培地である，6社(栄研化学㈱，極東製薬工業㈱，日研生物医学研究所，OXOID, BD, BIOMÉRIEUX)のBCYEα寒天生培地，5社(極東製薬工業㈱，日研生物医学研究所，OXOID, BIOMÉRIEUX, MERCK)のGVPC寒天生培地，栄研化学㈱のWYOα寒天生培地，OXOID社のMWY寒天生培地を使用した．また，OXOID社の製品については他に，レジオネラCYE寒天基礎培地にサプリメントを指示通り溶解，添加し調製(以下自家調製)したBCYEα, BMPAα, MWY寒天培地も使用した.

3) 分離集落の観察

5種類の供試菌株を，各分離培地に画線塗抹し，37℃で培養した．培養後の各分離集落の観察は，実体顕微鏡(ニコンSMZ-10, SMZ-1000)で行った．暗室中に実体顕微鏡を設置し，分離培地を顕微鏡のステージに載せ，光源(ニコンまたはオリンパス製コールドライト)を設置し，分離培地に斜光を当てることによって分離集落の観察を行った(以下斜光法)．装置の構成を図1に示した．観察は10日間，毎日行った.

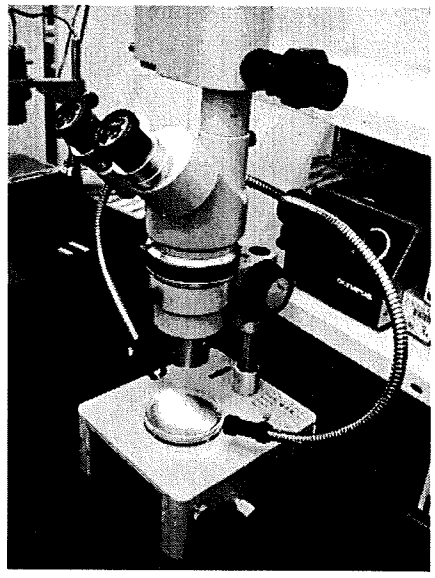

図1　分離培地上の集落観察(斜光法：暗所で行う)

2. 環境試料に対する検討

1) 対象試料

浴槽水764試料，冷却塔水17試料，腐葉土2試料の計783試料に対し，レジオネラ属菌検査を行った.

2) 検査法

浴槽水および冷却塔水は，非濃縮試料と100倍濃縮試料に分けた．試料の濃縮は，ろ過濃縮法で行った．ろ過濃縮法は，以下のとおりである．試料500 mLを，直径47 mm，孔径0.2 μmのポリカーボネート製メンブランフィルター(ADVANTEC)を使用したろ液受フラスコ付きポリサルフォン製のろ過器(NALGENE)で吸引ろ過した．ろ過終了後，メンブランフィルターを50 mLの滅菌遠心管中で5 mLのろ液にひたし，10分間手で強く振り洗浄した．この洗浄液を100倍濃縮試料とした．非濃縮およびろ過濃縮法にて調製した接種試料は，それぞれ1 mLを熱処理区，酸処理区に使用した．熱処理区は，接種試料を50℃，20分間処理してから100 μLを，酸処理区は0.2M HCl・KCl buffer(pH2.2)を等量加え，室温にて4分間放置してから200 μLを，さらに，未処理区として接種試料100 μLを，それぞれ自家調製したMWY寒天培地(OXOID)に滅菌コンラージ棒で塗布した．それらを37℃で10日間培養した．培養期間中は，毎日分離培地を斜光法で観察し，発育集落において，レジオネラ属菌と他の細菌との分別が可能か検討を行った．斜光法および従来法によって，レジオネラ様集落と判断した集落を釣菌し，区画した自家調製のBCYEα寒天培地(OXOID)と血液寒天培地に画線培養した．培養後，BCYEα寒天培地にのみ発育したものをレジオネラ属菌と推定した．レジオネラ属菌の同定は，PCR法[10,11]，レジオネラ免疫血清「生研」，レジオネララテックステスト(OXOID)，DDHレジオネラ(極東)によるDNA-DNAハイブリダイゼーション，16S rRNA遺伝子の塩基配列の確認[12]により行った.

腐葉土は，微生物検査必携 細菌・真菌検査[13]に従って希釈液を調製した．前処理として，レジオネラの発育に影響を及ぼす雑菌が相当量含まれていることを想定し，50℃ 20分の加熱後，酸処理(0.2M HCl・KCl buffer pH2.2を等量加え室温で4～30分間放置)したものを接種試料とした．分離培養は前述の環境水の培養に準じて行った.

結　果

1. 基準株による確認

斜光法による観察の結果，5種類のレジオネラ基準株の集落は，今回使用したすべてのレジオネラ用分離培地上で，カットグラス様(モザイク様)，または集落の辺縁部がカットグラス様で中心部が綿様といった特徴的な外観構造を呈した(図2)．このことは，斜光法によって観