Applied and Environmental Microbiology, Jan. 2009, p. 147–153 Vol. 75, No. 1
0099-2240/09/$08.00+0 doi:10.1128/AEM.00604-08
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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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Received 13 March 2008/Accepted 6 October 2008

***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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▽ Published ahead of print on 31 October 2008.

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).

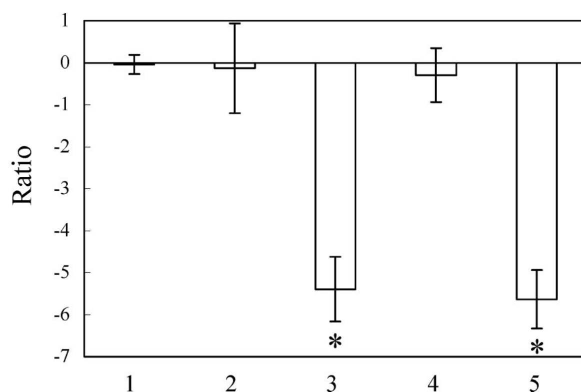

FIG. 2. EMA and real-time PCR combined analyses of viable or dead cells of *L. pneumophila* strain 80-045. The number of bacteria was estimated from the amount of DNA detected by real-time PCR with SYBR green as the reporter dye. The number of viable cells without EMA treatment was set as 1. The numbers 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 $-0.04 \pm 0.23 \log_{10}$ (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 $-0.13 \pm 1.06 \log_{10}$ (Fig. 2, bar 2) and $-0.30 \pm 0.64 \log_{10}$ (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

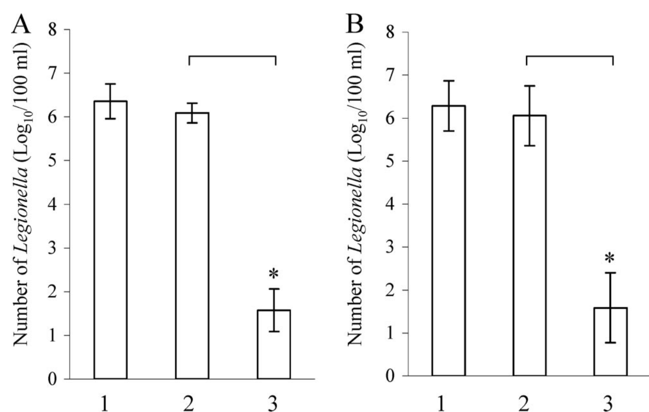

FIG. 3. Number of *L. pneumophila* strain 80-045 cells (A) and *L. bozemanii* strain NIIB0009 cells (B) isolated from 100-ml mock samples, determined by incubation or estimated by real-time PCR. 6-Carboxyfluorescein was used as the reporter dye for real-time PCR. Bars 1, real numbers of viable *Legionella* 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.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (no. 18790316) and from the Ministry of Health, Labour and Welfare (H19-kenki-014 to F.K.).

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