Applied and Environmental Microbiology, July 1997, p. 2489–2494 Vol. 63, No. 7
0099-2240/97/$04.00+0
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Development of a New Seminested PCR Method for Detection of *Legionella* Species and Its Application to Surveillance of Legionellae in Hospital Cooling Tower Water

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Received 27 January 1997/Accepted 28 April 1997

The presence of PCR inhibitors in water samples is well known and contributes to the fact that a practical PCR assay has not been developed for legionella surveillance. In this study, we devised a new seminested PCR assay for detection of *Legionella* spp. in water samples as a means of overriding the PCR inhibitors without loss of sensitivity. The seminested PCR assay utilized primers to amplify the 16S rRNA gene (LEG primers) of 39 *Legionella* spp. The assay was specific to legionellae, and the sensitivity was 1 fg of extracted *Legionella* DNA in laboratory examination. To evaluate the feasibility and sensitivity of the PCR assay in identifying the presence of legionellae, it was used to survey *Legionella* contamination in the water of 49 cooling towers of 32 hospitals. A commercially available EnviroAmp *Legionella* kit and a culture method were also used in the survey for comparison with the seminested PCR assay. The detection rates of legionellae in the samples were 91.8% (45 of 49) by the PCR assay and 79.5% (39 of 49) by the culture method. The EnviroAmp kit revealed that 30.6% of the water samples (15 of 49) contained inhibitors of the PCR amplification. However, the seminested PCR assay could produce the *Legionella*-specific DNA bands in 14 of the 15 samples. Although 8 of the 14 samples were positive in the first-step PCR, 6 of the 14 samples became positive in the second-step PCR. These results suggest that the effect of PCR inhibitors in samples, if any, can be reduced because of the dilution of the sample in the second-step PCR and that sensitivity of detection can be increased by the second-step PCR. Thus, the seminested PCR assay with LEG primers to amplify the 16S rRNA gene of 39 *Legionella* spp. was a practical and sensitive method to detect *Legionella* spp. in water samples.

Legionella pneumophila, the causative agent of Legionnaires' disease, was first recognized in 1977 following an epidemic of acute pneumonia in Philadelphia, Pa. (8). Since then, a total of 41 *Legionella* species containing 62 serogroups have been characterized (2, 10, 36). Twenty-one of the *Legionella* species have been reported as pathogenic in humans (7, 31, 36) (see Table 1). Exposure to and infection with *Legionella* species assume particular importance within hospitals, where patients with chronic pulmonary diseases, advancing age, and underlying immunosuppression are at an increased risk of legionellosis (9, 37). Outbreaks of nosocomial *Legionella* infection have been reported in both general hospital wards (6, 35) and intensive care and transplant units (22, 33). The degree of *Legionella* colonization in water supplies in hospitals has been shown to correlate with the incidence of nosocomial Legionnaires' disease (3, 22). Since *Legionella* contamination in the cooling tower water of hospitals has led to large nosocomial outbreaks (6, 9, 26, 37), surveillance of legionellae in the water and preventive measures against the contamination are needed, especially in hospitals. A sensitive and practical procedure for legionellae surveillance in cooling tower water should be established to prevent outbreaks of legionellosis.

To find effective methods for detection of legionellae in water samples from cooling towers and a river, we (38) previously examined colony counts, direct fluorescent-antibody staining, and a PCR assay with primers for amplification of the 16S rRNA gene (LEG primers) of 29 *Legionella* species. That study showed that the PCR assay with LEG primers was satisfactorily specific and sensitive for the survey of legionellae in cooling tower water and water from a river. During the study, we found that some water samples contained unknown inhibitors for PCR. To eliminate inhibitors completely, DNA should be purified. There are some reports on *Legionella* (20) and mycobacteria (34, 40) indicating that extraction procedures employing multiple steps to purify DNA from samples had lower rates of PCR inhibition than those with fewer steps. However, the DNA purification method with multiple steps is not suitable for surveillance of *Legionella* contamination in cooling tower water, a situation in which many samples must be examined. Although dilution of a sample is a simple and useful method to reduce the effect of inhibitors (29, 39), there is a risk of underestimation of *Legionella* contamination in the samples. The inhibition of the PCR assay still remains to be overcome. A practical PCR assay overriding the inhibitory effects is necessary for the survey of legionellae in cooling tower water.

In this study, by selecting a new *Legionella*-specific sequence (LEG 225) from 16S rRNA genes and combining it with two other LEG primers, 448 (39) and 858, we devised a seminested PCR assay as a means of overriding the inhibition without loss of sensitivity. To evaluate the feasibility and sensitivity of the assay in identifying the presence of legionellae, it was used to survey *Legionella* contamination in water of 49 cooling towers of 32 hospitals. A culture method and a commercially available EnviroAmp *Legionella* kit (Perkin-Elmer Cetus, Norwalk,

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TABLE 1. Detection of the amplified DNA fragments from *Legionella* spp. by PCR with LEG primers

Species (serogroup)	Strain or source[a]	Detection of band of:	
		654 bp[b]	430 bp[c]
L. pneumophila[d] (1)	ATCC 33152	+	+
L. micdadei[d]	ATCC 33218	+	+
L. bozemanii[d] (1)	ATCC 33217	+	+
L. dumoffii[d]	ATCC 33279	+	+
L. feeleii[d] (1)	ATCC 35072	+	+
L. gormanii[d]	EY 3650	+	+
L. hackeliae[d] (1)	GIFU 10740	+	+
L. israelensis[d]	GIFU 11367	+	+
L. jordanis[d]	ATCC 33623	+	+
L. sainthelensi[d] (1)	GIFU 10392	+	+
L. longbeachae[d] (1)	GIFU 9245	+	+
L. maceachernii[d]	GIFU 10745	+	+
L. oakridgensis[d]	ATCC 33761	+	+
L. wadsworthii[d]	GIFU 10062	+	+
L. birminghamensis[d]	GIFU 11749	+	+
L. cincinnatiensis[d]	GIFU 12201	+	+
L. anisa[d]	GIFU 12075	+	+
L. tucsonensis[d]	GIFU 12656	+	+
L. lansingensis[d]	GIFU 13565	+	+
L. cherrii[d]	GIFU 10742	+	+
L. parisiensis[d]	GIFU 11745	+	+
L. santicrucis	GIFU 11746	+	+
L. adelaidensis	GIFU 13562	+	+
L. brunensis	GIFU 12655	+	+
L. erythra (1)	GIFU 11748	+	+
L. fairfieldensis	GIFU 13563	+	+
L. geestiana	GIFU 13568	+	+
L. gratiana	GIFU 13564	+	+
L. jamestownensis	GIFU 10741	+	+
L. londiniensis	GIFU 13635	+	+
L. moravica	GIFU 12649	+	+
L. nautarum	GIFU 13636	+	+
L. quateirensis	GIFU 13637	+	+
L. quinlivanii (1)	GIFU 12648	+	+
L. rubrilucens	GIFU 10743	+	+
L. shakespearei	GIFU 13566	+	+
L. spiritensis (1)	GIFU 11199	+	+
L. steigerwaltii	GIFU 11747	+	+
L. worsleiensis	GIFU 13638	+	+

[a] ATCC, American Type Culture Collection; EY, Eiko Yabuuchi; GIFU, Gifu University School of Medicine.
[b] PCR was performed with primers LEG 225 and LEG 858, and detection of the amplification product was on an ethidium bromide-stained gel.
[c] PCR was performed with primers LEG 225 and LEG 858, followed by primers LEG 448 and LEG 858. Detection was as described in footnote *b*.
[d] *Legionella* species associated with diseases (7, 31, 36).

Conn.), which is widely used for detection of legionellae in environmental (19, 27–29) and clinical (13, 21) samples, were also employed in the survey.

MATERIALS AND METHODS

Bacteria, culture, and control DNA extraction. Thirty-nine *Legionella* strains were used in this study (Table 1). All of the legionellae were cultured on buffered charcoal-yeast extract agar with α-ketoglutarate (BCYEα) at 37°C for 72 h. Nonlegionella strains (Table 2) were cultured on a nutrient agar (Nissui Pharmaceutical Co., Osaka, Japan). These bacteria are important pathogens in nosocomial infections and/or can produce acute pneumonia as well as legionellae can. To extract control genomic DNA, *L. pneumophila* Philadelphia-1 (ATCC 33152) was harvested from a BCYEα plate after 72 h of incubation at 37°C, washed twice, and resuspended in sterile phosphate-buffered saline (136.8 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). After adjusting the bacterial suspension to approximately 1.2×10^{10} cells/ml, the genomic DNA was extracted with the help of a DNA extraction reagent kit (SepaGene; Sanko Junyaku Co., Ltd., Tokyo, Japan). The DNA concentration was determined by measuring the optical density at 260 nm.

Collection of water samples from cooling towers in hospitals. A total of 49 samples of cooling tower water were collected from 32 different hospitals in Fukuoka Prefecture, Japan, between August and October 1995. The water sample (about 500 ml) was collected in a sterile plastic bottle from the basin of each cooling tower. Three portions of each sample, 200, 100, and 100 ml, were employed in a culture method, a seminested PCR assay, and EnviroAmp *Legionella* kits (Perkin-Elmer Cetus), respectively.

Determination of viable count of legionellae. A 200-ml portion of each sample was centrifuged at 2,800 × *g* for 30 min. The resulting sediment was resuspended in 1 ml of sterile distilled water and mixed with an equal volume of 0.2 M KCl-HCl buffer (pH 2.2). After allowing the mixture to stand at room temperature for 20 min, the buffer-treated suspension (0.1 ml) and a 10-fold dilution of the suspension (0.1 ml) were inoculated onto Wadowsky-Yee-Okuda agar supplemented with α-ketoglutarate (WYOα) plates (Eiken Chemical Co., Ltd., Tokyo, Japan). The inoculated plates were then incubated for 7 to 10 days at 37°C, and the grayish-white, shiny colonies were counted as legionellae. The suspected *Legionella* colonies were subcultured onto BCYEα and BCYEα without L-cysteine for verification. If the isolate could grow only on BCYEα and the Gram stain was negative, it was determined to be a legionella. Several colonies isolated from each positive sample were used for determining the species and/or serogroups by slide agglutination tests with polyclonal antisera against *L. pneumophila* serogroups 1 to 6, *Legionella micdadei*, *Legionella bozemanii*, *Legionella dumoffii*, and *Legionella gormanii* (Denka Seiken Co., Ltd., Tokyo, Japan).

Sample preparation for PCR. A 100-ml portion of each water sample was centrifuged at 2,800 × *g* for 30 min, and the sediment was resuspended in 1 ml of autoclaved ultrapure water (Milli Q; Japan Millipore Co., Ltd., Tokyo, Japan). In the case of bacterial cells from a fresh culture, they were suspended at only about 10^8 cells/ml in autoclaved ultrapure water. To release the DNA directly from bacterial cells, the suspension was frozen at −80°C, heated in boiling water for 5 min, and supplemented with 1/10 volume of protease K (1 mg/ml) and lysis buffer (100 mM Tris-HCl [pH 8.3], 50 mM KCl, 15 mM $MgCl_2$, 0.1% Tween 20, 0.5% Nonidet P-40). After 1 h of incubation at 55°C, the solution was frozen at −80°C and then heated in boiling water for 5 min.

Primers and a probe. Oligonucleotide primers were designed from the partial sequences of a 16S rRNA gene of *Legionella* species which were obtained from the Ribosomal Database Project (18). For the first-step PCR, two oligonucleotides (LEG 225 and LEG 858) were used as amplimers enclosing a 654-bp fragment of the 16S rRNA gene. LEG 225 (5′-AAGATTAGCCTGCGTCCGAT-3′) was located at positions 225 to 244, and LEG 858 (5′-GTCAACTTATCGCGTTTGCT-3′) was complementary to positions 880 to 859 (numbers of universal position of *Escherichia coli* 16S rRNA sequence). LEG 225 was first selected from a large database of the 16S rRNA gene. Previously described LEG

TABLE 2. Detection of the amplified DNA fragments from nonlegionellae by PCR with LEG primers

Species	Strain or source[a]	Detection of band of:		Detection by hybridization[d]
		654 bp[b]	430 bp[c]	
Acinetobacter calcoaceticus	ATCC 19606	−	−	−
Alcaligenes xylosoxidans	Clinical isolate	−	−	−
Burkholderia cepacia	Clinical isolate	−	−	−
Chryseobacterium indologenes	Clinical isolate	−	−	−
Chryseobacterium meningosepticum	Clinical isolate	−	−	−
Chromobacterium violaceum	Clinical isolate	−	−	−
Comamonas acidovorans	ATCC 15668	−	−	−
Escherichia coli	Clinical isolate	−	−	−
Flavobacterium odoratum	Clinical isolate	−	−	−
Pseudomonas aeruginosa	Clinical isolate	−	−	−
Pseudomonas fluorescens	Clinical isolate	−	−	−
Pseudomonas stutzeri	Clinical isolate	−	−	−
Serratia marcescens	Clinical isolate	−	−	−
Shewanella putrefaciens	Clinical isolate	−	−	−
Sphingomonas paucimobilis	Clinical isolate	−	−	−
Staphylococcus epidermidis	Clinical isolate	−	−	−
Stenotrophomonas maltophilia	Clinical isolate	−	−	−

[a] ATCC, American Type Culture Collection.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] PCR was done as described in Table 1, footnote *c*, and detection was by Southern hybridization with the CP2 probe.

854 (38) was slightly modified and used as LEG 858 in this study. For the second-step PCR, LEG 448 (5′-GAGGGTTGATAGGTTAAGAGC-3′) (38) and LEG 858 were used as another set of amplimers enclosing a 430-bp fragment of the 16S rRNA gene. The 5′-digoxigenin-labeled 20-mer CP2 (5′-CAACCAG TATTATCTGACCG-3′), complementary to positions 649 to 630, was used as the probing oligomer (12).

PCR conditions and gel electrophoresis. Heat-treated samples (5 μl each) were added to microtubes containing 45-μl portions of PCR mixture. The PCR mixture contained 2.5 U of *Taq* polymerase (TaKaRa Biochemicals, Shiga, Japan), 20 pM each primer (LEG 225 and LEG 858), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, and 0.02% (wt/vol) gelatin. By use of the Program Temp Control System PC700 (ASTEC Co., Ltd., Fukuoka, Japan), the preparations in the microtubes were initially denatured at 95°C for 90 s and then subjected to 30 cycles of denaturation at 95°C for 10 s, annealing at 64°C for 1 min, and extension at 74°C for 1 min. A 1-μl portion of the first-step PCR products was added to 49 μl of the PCR mixture containing primers LEG 448 and LEG 858. The second-step (seminested) PCR was performed with an initial denaturation step at 95°C for 90 s followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 66°C for 1 min, and extension at 74°C for 1 min. PCR-amplified DNA fragments (8 μl each) were separated in 2 or 3% agarose gels (NuSieve 3:1 agarose; FMC BioProducts, Rockland, Maine) with TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 2.0 mM EDTA [pH 8.3]) and visualized by ethidium bromide staining. A 100-bp DNA ladder (Pharmacia Biotech Co., Ltd., Tokyo, Japan) was also used as a DNA size marker.

Southern hybridization. DNA fragments from gels containing PCR products separated by electrophoresis were transferred to a nylon membrane (Hybond-N; Amersham Japan, Ltd., Tokyo, Japan) as described previously (23). Prehybridization, hybridization with 12 nM CP2 (at 53°C overnight), and chemiluminescent detection of the nylon membrane blots were done as described in the instructions of the manufacturer (DIG DNA labeling and detection kit; Boehringer GmbH, Mannheim, Germany).

The EnviroAmp *Legionella* kit. The EnviroAmp *Legionella* kit (Perkin-Elmer Cetus) is composed of three kits (the *Legionella* sample preparation kit, the *Legionella* PCR amplification kit, and the *Legionella* PCR detection kit) that are designed to be used successively to specifically detect DNA from *Legionella* species. They utilize PCR amplification on concentrated water samples and a nonradioactive reverse dot blot hybridization. These kits were kindly donated by Perkin-Elmer Applied Biosystems Inc.

DNA extraction with the *Legionella* sample preparation kit. DNA extraction was performed as described in the manufacturer's instructions. Briefly, a 100-ml portion of each sample was concentrated by filtration through a 0.45-μm-pore-size Durapore HV membrane. The filter was vortexed for 30 s and boiled for 10 min with DNA extraction reagent supplied by the manufacturer. After a brief centrifugation, the supernatant was transferred to another tube, and DNA was precipitated with 100% isopropanol and carrier reagent. The pellet was resuspended in 160 μl of kit-provided PCR water, and 20 μl of the extracted DNA was subjected to PCR amplification with the EnviroAmp kit.

PCR with the EnviroAmp *Legionella* PCR amplification kit. With the EnviroAmp PCR amplification kit, the genus *Legionella* is identified by amplifying DNA sequences with specific biotinylated primers (L primers) complementary to conserved regions in the 5S rRNA gene (17). Identification of *L. pneumophila* is achieved by biotinylated primers (P primers) complementary to sequences of the macrophage infectivity potentiator (*mip*) gene (5, 16), incorporating an internal positive control (IPC). The IPC is a synthetic DNA sequence that is coamplified with the P primers and is included in the *Legionella* PCR mixture. The IPC is a sensitive indicator of poor amplification which may be caused by PCR inhibitors present in the samples.

PCR amplification was performed as described in the manufacturer's recommendations by adding 20 μl of the extracted DNA and 15 μl of 25 mM $MgCl_2$ solution of the reaction tubes containing the *Legionella* PCR mixture with uracil *N*-glycosylase. Samples were subjected to 30 cycles of amplification (30 s at 95°C and 60 s at 63°C) after holding programs of 10 min at 95°C with a DNA Thermal Cycler 480 (Perkin-Elmer Cetus).

Hybridization with the EnviroAmp *Legionella* PCR detection kit. Biotinylated PCR products generated by amplification with biotinylated primers specifically hybridize to the immobilized probes on a nylon membrane (detection strips). One probe hybridizes to positions 66 to 82 of the *Legionella* 5S rRNA DNA sequence, and the other probe hybridizes to positions 1012 to 1036 of the *L. pneumophila mip* sequence (reverse blot). The EnviroAmp PCR detection kit also includes positive and negative control probes. The positive control probe is complementary to the IPC sequence, and the negative control probe has a 1-base mismatch with the IPC sequence. Detection of the hybridized PCR products is performed with a streptavidin-horseradish peroxidase conjugate. The appearance of a blue dot on the nylon membrane indicates the presence of a bound PCR product.

Hybridization was done as described in the manufacturer's instructions. Briefly, the detection strips were incubated successively with the hybridization solution and the denatured PCR products at 55°C for 20 min and with the enzyme conjugate at 55°C for 12 min. The strips were washed twice for 10 min at 55°C with a washing solution and developed with development solution for 30 min at room temperature. When no blue dots appeared, which indicates a noninterpretable sample, the undenatured PCR-amplified samples were electrophoresed with 4% agarose gels to determine whether the samples contained PCR products and thus contained inhibitors of the PCR amplification or the hybridization and color development steps did not work correctly. No product on the gels was considered to show the presence of inhibitors of PCR amplifications in the samples.

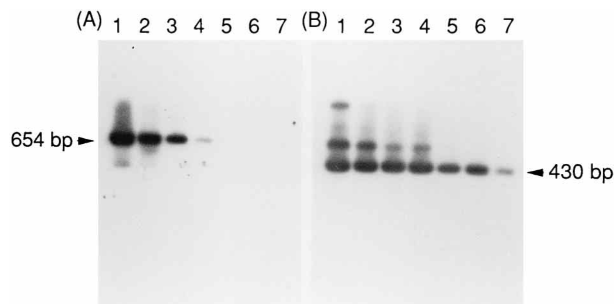

FIG. 1. Southern blot analysis of the first-step PCR products (A) and the second-step PCR products (B) from *L. pneumophila* DNA with the CP2 probe. The first-step PCR was performed with primers LEG 225 and LEG 858, and a 1-μl portion of the first-step PCR products was subjected to the second-step PCR with primers LEG 448 and LEG 858. These PCR products were separated by electrophoresis with 2% gels, transferred to nylon membranes, and hybridized with the CP2 probe. Extracted *L. pneumophila* DNA was used as a template DNA. Lanes: 1, 1 ng; 2, 100 pg; 3, 10 pg; 4, 1 pg; 5, 100 fg; 6, 10 fg; 7, 1 fg. The sizes of the DNA bands amplified by the PCR and hybridized with the CP2 probe are indicated on the left (A) and right (B).

RESULTS

Specificity of the seminested PCR. The specificity of the PCR method was investigated for 39 *Legionella* species (Table 1) and 14 other bacterial species (Table 2). The first amplifications of the seminested PCR with primers LEG 225 and LEG 858 produced 654-bp DNA bands from all 39 *Legionella* species (Table 1) and 10 reference strains belonging to *L. pneumophila* serogroups 2 to 11 (data not shown). The second amplifications of the seminested PCR with the primers LEG 448 and LEG 858 produced 430-bp DNA bands from all *Legionella* strains (Table 1). Neither a 654-bp nor a 430-bp DNA band was produced from 17 nonlegionella strains (Table 2), but faint and dim images of 600-bp amplified DNA were observed in the seminested PCR from the samples of *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* (data not shown). However, these 600-bp DNA bands could be easily distinguished from the 654-bp bands from *Legionella* species. It was confirmed that the 600-bp bands did not hybridize with the CP2 probe by Southern hybridization (data not shown).

Sensitivity of the seminested PCR. The sensitivity of the seminested PCR was assessed with purified DNA from *L. pneumophila* SG 1 (ATCC 33152). Although the lowest detection threshold for the first-step PCR was 1 pg of the DNA per PCR (Fig. 1A, lane 4), a specific 430-bp DNA fragment amplified by primers LEG 448 and LEG 858 was observed in 1 fg of *Legionella* DNA per PCR by the seminested PCR (Fig. 1B, lane 7).

Viable counts of legionellae by the culture method. Legionellae were isolated in 79.5% (39 of 49) of the cooling tower water samples (Table 4), with viable counts of 10 CFU/100 ml (Table 3, tower 31) to 10^5 CFU/100 ml (Table 3, tower 9). Of the 43 isolates from the cooling tower water, 33 strains (76.7%) were *L. pneumophila* serogroup 1. The remaining 10 strains were 1 strain each of *L. pneumophila* serogroups 4, 5, and 6 and 7 serologically unidentified strains (Table 3).

Detection of legionellae by the EnviroAmp *Legionella* kit. Legionellae were detected with the EnviroAmp kit in 67.3%

TABLE 3. Results of survey of water samples from cooling towers in hospitals

Tower no.	Detection of *Legionella* by:				
	PCR methods			Culture method	
	PCR[a]	Seminested PCR[b]	EnviroAmp *Legionella* kits	*Legionella* counts (CFU/100 ml)	*Legionella* species and serogroup (SG)
1	+	+	*L. pneumophila*	2×10^4	*L. pneumophila* SG 1
13	+	+	*L. pneumophila*	1×10^2	*L. pneumophila* SG 1
14	+	+	*L. pneumophila*	4×10^4	*L. pneumophila* SG 1
16	+	+	*L. pneumophila*	9×10^1	*L. pneumophila* SG 1
17	+	+	*L. pneumophila*	2×10^1	*L. pneumophila* SG 1
18	+	+	*L. pneumophila*	2×10^3	*L. pneumophila* SG 1
19	+	+	*L. pneumophila*	4×10^1	*L. pneumophila* SG 1
20	+	+	*L. pneumophila*	4×10^4	*L. pneumophila* SG 1
21	+	+	*L. pneumophila*	4×10^4	*L. pneumophila* SG 1
22	+	+	*L. pneumophila*	2×10^3	*L. pneumophila* SG 4, SG 6
24	+	+	*L. pneumophila*	1×10^4	*L. pneumophila* SG 1
25	+	+	*L. pneumophila*	6×10^3	*L. pneumophila* SG 1
26	+	+	*L. pneumophila*	6×10^3	*L. pneumophila* SG 1
32	+	+	*L. pneumophila*	4×10^3	*L. pneumophila* SG 1
35	+	+	*L. pneumophila*	4×10^3	*L. pneumophila* SG 1
38	+	+	*L. pneumophila*	2×10^3	*L. pneumophila* SG 1
41	+	+	*L. pneumophila*	4×10^2	*L. pneumophila* SG 1
42	+	+	*L. pneumophila*	2×10^4	*L. pneumophila* SG 1
44	+	+	*L. pneumophila*	2×10^3	*L. pneumophila* SG 5
46	+	+	*L. pneumophila*	6×10^4	*L. pneumophila* SG 1
47	+	+	*L. pneumophila*	2×10^2	*L. pneumophila* SG 1
36	+	+	*L. pneumophila*	6×10^3	*L. pneumophila* SG 1, NA[c]
48	+	+	*L. pneumophila*	6×10^3	*L. pneumophila* SG 1, NA
3	+	+	*L. pneumophila*	2×10^3	NA
45	+	+	*L. pneumophila*	8×10^3	NA
33	+	+	*Legionella* spp.[d]	2×10^4	*L. pneumophila* SG 1, NA
37	+	+	*Legionella* spp.	4×10^3	NA
11	+	+	*L. pneumophila*	ND[e]	
2	+	+	*Legionella* spp.	ND	
5	+	+	*Legionella* spp.	ND	
7	+	+	IPC(−)[f]	2×10^3	*L. pneumophila* SG 1
9	+	+	IPC(−)	1×10^5	*L. pneumophila* SG 1
10	+	+	IPC(−)	2×10^4	*L. pneumophila* SG 1
15	+	+	IPC(−)	2×10^4	*L. pneumophila* SG 1
31	+	+	IPC(−)	1×10^1	NA
34	+	+	IPC(−)	2×10^4	*L. pneumophila* SG 1
23	+	+	IPC(−)	ND	
27	+	+	IPC(−)	ND	
8	−	+	*Legionella* spp.	ND	
12	−	+	IPC(−)	2×10^3	*L. pneumophila* SG 1
40	−	+	IPC(−)	3×10^2	*L. pneumophila* SG 1
43	−	+	IPC(−)	1×10^2	*L. pneumophila* SG 1
49	−	+	IPC(−)	2×10^4	*L. pneumophila* SG 1
4	−	+	IPC(−)	1×10^2	*L. pneumophila* SG 1
28	−	+	IPC(−)	ND	
39	−	−	*L. pneumophila*	8×10^3	*L. pneumophila* SG 1
30	−	−	*Legionella* spp.	ND	
29	−	−	IPC(−)	ND	
6	−	−	−	ND	

[a] PCR with primers LEG 225 and LEG 858.
[b] PCR with primers LEG 225 and LEG 858, followed by primers LEG 448 and LEG 858.
[c] NA, nonagglutinable against polyclonal antisera to *L. pneumophila* serogroups 1 to 6, *L. micdadei*, *L. bozemanii*, *L. dumoffii*, and *L. gormanii*.
[d] *Legionella* spp., *Legionella* species other than *L. pneumophila*.
[e] ND, not detected (less than 10 CFU/100 ml).
[f] IPC(−), negative reaction to the kit-provided internal positive control (IPC).

TABLE 4. Comparison of results with bacterial culture, seminested PCR[a], and EnviroAmp *Legionella* kit test of water samples from cooling towers in hospitals

Type of *Legionella* samples	No. of samples				
	Seminested PCR		EnviroAmp *Legionella* kit		
	Positive	Negative	Positive	Negative	IPC(−)[b]
Culture positive	38	1	28	0	11
Culture negative[c]	7	3	5	1	4
Total	45	4	33	1	15

[a] See Table 1, footnote *c*.
[b] See Table 3, footnote *f*.
[c] Samples contaminated with bacteria at less than 10 CFU/100 ml.

(33 of 49) of the cooling tower water samples (Table 4), and *L. pneumophila* was present in 81.8% (27 of 33) of the legionella-positive samples (Table 3). In 30.6% (15 of 49) of the water samples, no blue dots with IPC appeared in the detection kit (Tables 3 and 4). These samples contained inhibitors of the PCR amplification because we could not observe any PCR products with the IPC after electrophoresis with 4% gels (data not shown).

Detection of legionellae by the seminested PCR assay. In 30 cycles of the first-step PCR, legionellae were detected in 77.6% (38 of 49) of the cooling tower water samples (Table 3). By cycle 20 of the second-step (seminested) PCR, 7 of 11 first-step PCR-negative samples were found to be positive (Table 3) and the detection rate rose to 91.8% (45 of 49 samples) (Table 4). Fifty cycles of the first-step PCR could detect legionellae in one of the seven seminested PCR positive samples (Fig. 2A, lane 2). However, the remaining six samples remained negative in the 50 cycles of the first-step PCR (Fig. 2A, lanes 3 to 8).

The seminested PCR assay amplified the 430-bp DNA fragments from 14 IPC-negative samples in the EnviroAmp kits (Table 3). It was confirmed that the 430-bp bands hybridized with the CP2 probe by Southern hybridization (data not shown).

DISCUSSION

We developed a seminested PCR assay to override inhibitors for PCR amplification in samples without loss of sensitiv-

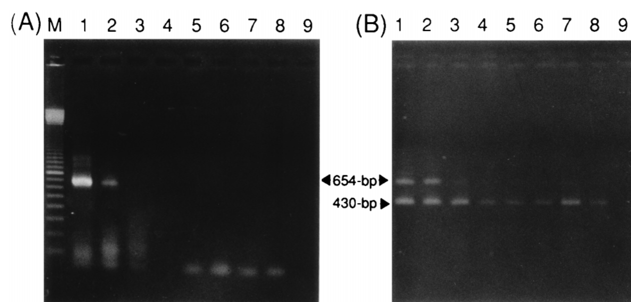

FIG. 2. Agarose gel electrophoresis of the first-step PCR products (A) and the seminested PCR products (B) of water samples from cooling towers. (A) Fifty cycles of the first-step PCR were performed with primers LEG 225 and LEG 858. (B) Thirty cycles of the first-step PCR with primers LEG 225 and LEG 858 were performed, and a 1-μl portion of the first-step PCR product was subjected to 20 cycles of the second-step PCR with primers LEG 448 and LEG 858. These PCR products were separated in 2% agarose gels and visualized by ethidium bromide staining. The water samples are from cooling towers 7 (lane 1), 8 (lane 2), 12 (lane 3), 40 (lane 4), 43 (lane 5), 49 (lane 6), 4 (lane 7), 28 (lane 8), and 6 (lane 9). In lane M, a 100-bp DNA ladder (Pharmacia Biotech) was used as a DNA size marker. The 800-bp band appears at twice the intensity of the other bands.

ity. The seminested PCR assay was specific to the 39 *Legionella* species tested, and the sensitivity of the assay was 1 fg of *Legionella* DNA per PCR. This assay was used to assess its usefulness in the surveillance of contamination by legionellae of cooling tower water. Although EnviroAmp *Legionella* kits showed that 30.6% (15 of 49) of the water samples contained inhibitors of the PCR amplification, the seminested PCR assay could reduce the effect of inhibitors to produce the *Legionella*-specific DNA bands in 14 of the 15 samples. The detection rates of legionellae in the samples were 91.8% (45 of 49) by the PCR assay or 79.5% (39 of 49) by the culture method. These results suggest that the PCR assay is a practical and sensitive method and that the seminested PCR assay with LEG primers is useful for the survey of legionellae in water samples.

The presence of PCR inhibitors in water samples is well known (14, 19, 27, 38). By use of the EnviroAmp *Legionella* kits and subsequent electrophoresis of the IPC-negative samples, 15 samples were revealed to contain PCR inhibitors (Tables 3 and 4). Since 8 of the 15 samples were positive in the first-step PCR (Table 3), the sample concentration method by filtration used in the kit may increase the concentration of PCR inhibitors in the sample. In six of the remaining seven samples, the specific 430-bp DNA fragments could be first observed in the seminested PCR assay (Fig. 2). Although a reason why the inhibitors did not affect the seminested PCR could not be elucidated in this study, dilution of the sample may reduce the concentration of the inhibitors. If the seminested PCR assay is employed, the simplified sample preparation for the PCR used here will be sufficient for overriding inhibitors of PCR amplification because the inhibitors in samples are automatically diluted to 50-fold in the second-step PCR.

Several primer and probe systems for amplification of *Legionella*-specific sequences have been described previously (1, 14, 30, 32). Choosing the target gene for the PCR assay is an important factor in the efficiency and specificity of the detection. The LEG primers for the 16S rRNA gene in the seminested PCR or the L primers for the 5S rRNA gene in the EnviroAmp kit are a suitable choice for detection and identification of *Legionella* species because the rRNA sequence has been used as a fundamental molecular marker in bacterial taxonomy and phylogenetic analysis (18, 25). Another superior factor for using the rRNA gene as the PCR target is its higher sensitivity than that of other target genes, as demonstrated in a previous study (38). Even if 1 fg of *Legionella* DNA per PCR was used, a faint 430-bp DNA band amplified with primers LEG 448 and LEG 858 could be observed in an ethidium bromide-stained gel (data not shown). Although Southern blot analysis with digoxigenin-labeled probe CP2 increased the sensitivity about fourfold, and made the specific band clear (Fig. 1), the sensitivity of the PCR assay without the Southern blot analysis was sufficient to detect legionellae in cooling tower water. The sensitivity of the seminested PCR with one nested primer (LEG 448) and one original primer (LEG 858) may be the same level as that of a two-stage (30-20) cycle of the PCR with the first set of primers (LEG 225 and LEG 858). However, since the specificity of a nested approach is higher than that of a two-stage cycle of PCR with a first set of primers, the seminested approach may be more useful for legionella surveillance than the two-stage cycle of PCR with the first set of primers.

The EnviroAmp kit employing P primers for the *mip* gene, which is unique to *L. pneumophila*, identified environmental *L. pneumophila* in 27 samples (Table 3). In 24 of the 27 samples, *L. pneumophila* could be isolated by the culture method (Table 3). This shows that the kit is well designed to detect and identify *Legionella* species and *L. pneumophila*. Further studies about genetic identification of each *Legionella* species by use of 16S rRNA sequences (10) are necessary for our PCR assay.

As shown in Table 4, there was a difference in the detection rate of legionellae in the water samples between the culture method (79.5%) and the seminested PCR assay (91.8%). Although 10 of 49 water samples (20.4%) were negative by the culture method, 7 of the 10 water samples were positive by the seminested PCR assay (Table 4). EnviroAmp *Legionella* kits also showed that 5 of the 10 culture-negative samples were positive (Table 4). The culture method is a standard method for the determination of viable legionellae, but there may be a risk of underestimation in the number of viable cells. Pretreatment with acid buffer to kill other microbes in the samples may also kill some legionellae in the samples (4). In addition, some *Legionella* species are sensitive to selective agents in the medium (15, 24). Another reason why the detection rate by the culture method was lower (79.5%) than that by the seminested PCR assay (91.8%) may be due to the viability of cells. The seminested PCR assay would detect dead cells as well as viable cells. Culture methods would detect only viable (and culturable) cells (11). Therefore, the difference in the detection rate may be due, at least in part, to these phenomena. The increased sensitivity of detection by the second-step PCR may also be associated with this difference. Conversely, one of four seminested PCR-negative samples was positive by the culture method (Table 3, tower 39; Table 4). The sample did not contain PCR inhibitors because EnviroAmp *Legionella* kits worked well in the sample (Table 3, tower 39). The cause still remains to be determined.

The seminested PCR assay with LEG primers to amplify the 16S rRNA gene of *Legionella* species is a practical and sensitive method to detect *Legionella* species in water samples. In addition, cultivation of legionellae from environmental and clinical samples takes at least 2 to 10 days for incubation while the PCR assay can be completed within 1 day. These facts show that the PCR assay developed here is a suitable and useful method for legionella surveillance. Use of the PCR assay may ultimately lead to the determination and decontamination of relevant sites in the hospital and thus may promote a decline in the nosocomial infection rate. Since PCR-detectable DNA does not prove that bacteria are viable (39), a combination of the PCR assay and colony count may be a more practical protocol to assess the risk of infection and dissemination.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research 08670328 from the Ministry of Education, Science, Sports and Culture, Japan, and by a UOEH Research Grant for Promotion of Occupational Health in 1995.

We are grateful to Eiko Yabuuchi and Tadao Tanabe for kindly providing *L. gormanii* EY3650 and clinical isolates of nonlegionellae, respectively. We thank Christine Paszko-Kolva of Perkin-Elmer Cetus for her arrangement to provide the kit. We also thank Midori Ogawa and Yukiko Suzuki for their technical assistance.

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