

Detection of *Legionella* spp. in Cooling Tower Water by the Polymerase Chain Reaction Method

MICHIO KOIDE, ATSUSHI SAITO,* NOBUCHIKA KUSANO, AND FUTOSHI HIGA

First Department of Internal Medicine, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nisihara-cho, Okinawa 903-01, Japan

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The presence of *Legionella* spp. in cooling tower water was investigated by using the polymerase chain reaction. Total *Legionella* spp. detection was performed with 20-mer 5S rRNA complementary DNA sequence primers, and specific *Legionella pneumophila* detection was performed with 20-mer and then 21-mer macrophage infectivity potentiator gene sequence primers. Of 27 cooling tower water samples, 25 were positive for *Legionella* spp., and 14 of these contained *L. pneumophila*.

Detection of *Legionella* spp. by a polymerase chain reaction (PCR) method in clinical specimens or in water seeded artificially with cultured *Legionella* spp. has been studied by several workers (1, 4, 6, 11). However, there have been no reports of detection of *Legionella* spp. in cooling tower water by this method. We examined the ability of the PCR method to detect *Legionella* spp. in cooling tower water and compared the sensitivity of the PCR method with the sensitivity of the culture method.

Cooling tower water samples. A total of 27 samples of cooling tower water were collected from different hospitals or buildings in and near Naha City, Okinawa Prefecture, Japan, between September and December 1991. A 200-ml portion of each sample was centrifuged at room temperature at 6000 × g for 30 min. The resulting sediment was resuspended in 4 ml of supernatant, and 1 ml of the suspension was used for the PCR. Another 1 ml was used for culturing *Legionella* spp. by inoculating an equal volume of 0.2 M HCl-KCl buffer (pH 2.2) and allowing the mixture to stand at room temperature for 10 min. The buffer-treated suspension (0.1 ml) and a 10-fold dilution of the suspension (0.1 ml) were plated on MWY agar medium (Oxoid), and the preparations were incubated at 37°C for 7 days. *Legionella*-like colonies were selected and identified. Isolated *Legionella* spp. were identified by biochemical methods (long-wave UV fluorescence, hippurate hydrolysis, production of β-lactamase, production of pigment on TYE agar, gelatin liquefaction) serological methods, cellular fatty acid gas chromatography analysis, and the microplate DNA-DNA hybridization test (Kobayashi Pharmaceutical Co., Osaka, Japan). Twenty-five kinds of *Legionella* DNA were immobilized on a microplate as described by Ezaki et al. (3). The remaining 2 ml of suspension was used for counting total bacteria. Serial 10-fold dilutions of suspension (1.0 ml each) were mixed with 20-ml portions of standard method agar (Nissui Pharmaceutical Co., Tokyo, Japan), and the preparations were incubated at 37°C for 24 h and then at 30°C for 48 h. The number of colonies observed was counted.

Preparation of samples for the PCR. A 1-g portion of glass beads (diameter, 1.5 mm) was added to a bacterial suspension (1.0 ml) or concentrated cooling tower water (1.0 ml) in a polypropylene tube. Then each sample was vigorously vortexed for 1 min, and 100 μl of Tris-EDTA buffer (10 mM

Tris-HCl, 1.0 mM EDTA [pH 8.0]) and 100 μl of proteinase K (0.1 mg) were added. The preparations were incubated at 55°C for 1 h in a water bath and then boiled at 100°C for 5 min. Heat-treated samples (10 μl each) were added to microtubes containing 90-μl portions of PCR reaction mixture. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 10 mg of gelatin per liter, deoxynucleoside triphosphates (each at a concentration of 200 μM), primers (each at a concentration of 50 pM), and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus). The preparations in the microtubes (volume, 100 μl) were initially denatured at 94°C for 5 min and then subjected to 30 PCR cycles by using a three-temperature PCR system consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min.

PCR-amplified DNA was separated on either 1.5 or 2.0% horizontal agarose gels. The agarose gels were run in TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 2.0 mM EDTA [pH 8.3]) and contained a molecular size marker. The gels were stained with a TAE buffer solution containing 2.0 μg of ethidium bromide per ml and observed with a UV transilluminator.

Primers and probe. The 104-bp region of the *Legionella* 5S rRNA coding gene was amplified by using 20-mer primers L5SL9 (5'-ACTATAGCGATTGGAACCA-3') and L5SR93 (5'-GCGATGACCTACTTTTCGCAT-3') as described by Mahbubani et al. (10). Primer L5SL9 is located between 9 and 28 bp and primer L5SR93 is located between 93 and 112 bp on the 5S rRNA gene. The 30-mer biotinylated gene probe L5S1b (5'-biotin-GAAGTGAAACATTTCCGCGC CAATGATAGT-3'), which is located between 50 and 79 bp on the 5S rRNA gene, was used for identification of the amplified PCR products (9).

We acquired a 996-bp sequence of the *L. pneumophila* macrophage infectivity potentiator (*mip*) gene first reported by Engleberg et al., which covers the coding region (2, 5). We then amplified this sequence by first using 20-mer primers. Primer LmipL710 (5'-ATGATAGCTTATGACTG GTA-3') is located between 710 and 729 bp on the *mip* gene, and primer LmipR1686 (5'-TTCCTTTGTTCACTCAGTAT-3') is located between 1,686 and 1,705 bp on the *mip* gene.

As the second step (nested PCR), the 649-bp sequence-coding region was further amplified by using 21-mer primers as described by Mahbubani et al. (10). Primer LmipL920 (5'-GCTACAGACAAGGATAAGTTG-3') is located between 920 and 940 bp, and primer LmipR1548 (5'-GTTTTG

* Corresponding author.

TABLE 1. Comparison of bacterial culture and PCR methods performed with cooling tower water

Tower	Results with:		Total bacterial counts (CFU/100 ml)	<i>Legionella</i> counts (CFU/100 ml)	<i>Legionella</i> taxon(a) isolated
	<i>mip</i> PCR ^a	5S rRNA PCR ^b			
1	-	+	5.0 × 10 ⁵	5.0 × 10 ⁴	L.L.O. ^c
2	-	+	7.5 × 10 ³	6.2 × 10 ³	<i>L. anisa</i>
3	-	-	1.2 × 10 ³	ND ^d	
4	+	+	1.5 × 10 ⁶	2.7 × 10 ⁴	<i>L. pneumophila</i> serogroup 1, <i>L. pneumophila</i> serogroup 7, <i>L. anisa</i> , <i>L. erythra</i>
5	+	+	4.0 × 10 ²	3.2 × 10 ⁴	<i>L. pneumophila</i> UT, ^e L.L.O.
6	-	+	1.0 × 10 ⁵	1.2 × 10 ³	<i>L. anisa</i> , <i>L. erythra</i>
7	+	+	1.6 × 10 ³	6.4 × 10 ³	<i>L. pneumophila</i> serogroup 1, <i>L. erythra</i> , L.L.O.
8	-	+	2.0 × 10 ⁶	ND	
9	+	+	3.4 × 10 ⁴	5.8 × 10 ³	<i>L. pneumophila</i> serogroup 7
10	-	+	1.1 × 10 ⁴	ND	
11	+	+	1.6 × 10 ⁴	1.6 × 10 ²	<i>L. pneumophila</i> serogroup 7
12	+	+	4.8 × 10 ⁴	3.0 × 10 ³	<i>L. pneumophila</i> serogroup 1, <i>L. anisa</i> , <i>L. erythra</i>
13	-	+	3.0 × 10 ³	8.0 × 10 ²	<i>L. pneumophila</i> serogroup 1, <i>L. anisa</i>
14	-	-	4.1 × 10	ND	
15	+	+	2.0 × 10 ⁵	1.0 × 10 ³	<i>L. pneumophila</i> serogroup 1
16	+	+	5.8 × 10 ⁴	1.8 × 10 ²	<i>L. pneumophila</i> serogroup 1, <i>L. erythra</i>
17	+	+	2.0 × 10 ⁴	3.6 × 10 ²	<i>L. pneumophila</i> serogroup 1
18	-	+	1.8 × 10 ⁶	ND	
19	-	+	4.2 × 10 ²	2.0 × 10	<i>L. anisa</i>
20	-	+	2.7 × 10 ⁴	2.0 × 10	<i>L. anisa</i>
21	+	+	3.5 × 10 ³	4.0 × 10	<i>L. pneumophila</i> serogroup 1, L.L.O.
22	+	+	1.2 × 10 ⁴	3.2 × 10 ²	<i>L. anisa</i> , L.L.O.
23	+	+	5.1 × 10 ³	5.0 × 10 ³	<i>L. pneumophila</i> serogroup 1, <i>L. feeleii</i>
24	+	+	5.0 × 10 ³	6.8 × 10 ²	<i>L. pneumophila</i> serogroup 7, <i>L. anisa</i> , <i>L. erythra</i>
25	+	+	4.4 × 10 ²	1.4 × 10 ³	<i>L. pneumophila</i> UT, <i>L. feeleii</i> , L.L.O.
26	-	+	1.6 × 10 ²	2.0 × 10 ²	<i>L. pneumophila</i> serogroup 7, <i>L. erythra</i>
27	-	+	4.6 × 10 ²	4.2 × 10 ²	<i>L. pneumophila</i> serogroup 1, <i>L. erythra</i> , L.L.O.

^a PCR performed with the 996-bp *mip* probe, followed by PCR performed with the 649-bp *mip* probe.

^b PCR performed with the 104-bp 5S rRNA probe, followed by PCR performed with the 104-bp 5S rRNA probe and then Southern hybridization.

^c L.L.O., *Legionella* strain that does not belong to any of the 25 known *Legionella* species.

^d ND, not detected (less than 200 CFU/100 ml).

^e *L. pneumophila* UT, *L. pneumophila* strain that does not belong to serogroups 1 to 7.

TATGACTTTAATTCA-3') is located between 1,548 and 1,568 bp.

For detection of all *Legionella* spp., repeated PCR amplifications were performed by using primers L5SL9 and L5SR93. A 1- μ l portion of the first-cycle PCR products was added to 90 μ l of reaction mixture containing the same L5SL9 and L5SR93 primers. The resulting two-cycle PCR products were identified with L5S1b probes by Southern hybridization.

For detection of *L. pneumophila*, PCR amplifications were performed by using primers LmipL710 and LmipR1686 as the first step. A 1- μ l portion of the first-step PCR products was then added to 90 μ l of reaction mixture containing primers LmipL920 and LmipR1548.

Southern hybridization. The gels containing PCR products separated by electrophoresis were immersed in a denaturing solution containing 0.2 N NaOH and 0.6 N NaCl for 1 h. The gels were then neutralized with 0.6 N NaCl and 0.2 N Tris-HCl (pH 7.3) for 1 h and transferred to an Immobilon-N transfer membrane (Millipore) by using 10 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015M sodium citrate [pH 7.0]) for 18 h. The membranes were treated with a nonradioactive nucleic acid detection system kit (Bethesda Research Laboratories) as described by Leary et al. (8).

The specificity of the PCR method was investigated by using 27 *Legionella* strains (8 reference strains belonging to *L. pneumophila* serogroups 1 to 8 and 19 type strains of other *Legionella* spp.) and 13 other bacterial strains. PCR

amplifications performed with the primers for 5S rRNA at bacterial concentrations of 10⁸ CFU/ml produced positive 104-bp DNA bands with all 27 *Legionella* strains tested. Positive bands were also observed for *Pseudomonas fluorescens* and *Acinetobacter calcoaceticus*, as well as *Pseudomonas aeruginosa*, as reported by Mahbubani et al. (10). However, Southern blotting with probe L5S1b revealed hybridization only with *Legionella* amplified DNA.

PCR amplifications performed with primers for the *mip* gene produced positive bands with the eight *L. pneumophila* strains tested. No non-*L. pneumophila* species (none of the other *Legionella* species tested and no member of the other genera tested) showed DNA amplification.

The sensitivity of the two-cycle PCR performed with 5S rRNA primers and serial 10-fold dilutions of *L. pneumophila* was 0.13 cell, and the sensitivity with *mip* primers was 1.3 cells, which is nearly the same as the sensitivity of the single-step PCR-gene probe method described by Mahbubani et al. (10).

Of 27 cooling tower water samples subjected to two-cycle PCR amplification with primers L5SL9 and L5SR93 followed by Southern hybridization with the L5S1b probe, 25 showed positive hybridization reactions (Table 1 and Fig. 1). Two samples showed negative reactions; one of these samples (sample 14) was negative after PCR amplification, while the other (sample 3) was negative as determined by the Southern hybridization reaction despite producing a positive band after PCR amplification. Of the 25 positive samples, 3

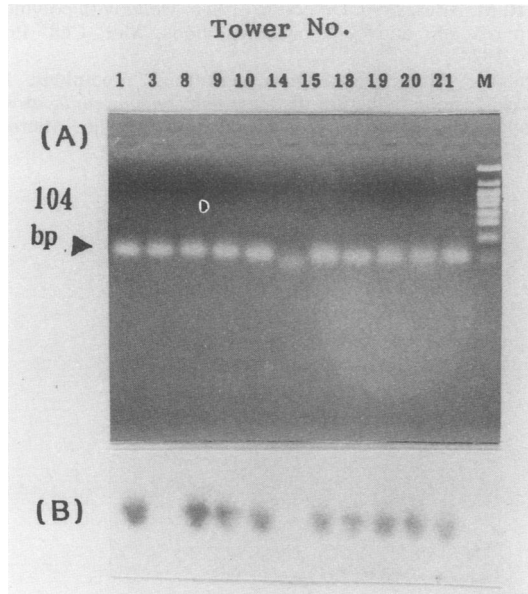


FIG. 1. (A) Agarose (2%) gel electrophoresis of amplified DNA from cooling tower water samples performed with primers L5SL9 and L5SR93. The samples were from cooling towers 1, 3, 8, 9, 10, 14, 15, 18, 19, 20, and 21, and *Hind*III-digested pHY300PLK and *Hae*III-digested pHY300.2PLK were used as molecular weight markers (lane M). (B) Southern blot analysis performed with gene probe L5S1b after electrophoresis as shown in panel A.

were negative for *Legionella* spp. in culture but positive in PCR-Southern hybridization determinations.

A total of 14 cooling tower water samples produced positive bands when we used primers LmipL710 and LmipR1686 and then primers LmipL920 and LmipR1548 (Fig. 2). Of these 14 samples, 1 was negative for *L. pneumophila* in culture (sample 22). Conversely, 3 of 13 PCR-negative samples were found to be positive for *L. pneumophila* in culture (samples 13, 26, and 27).

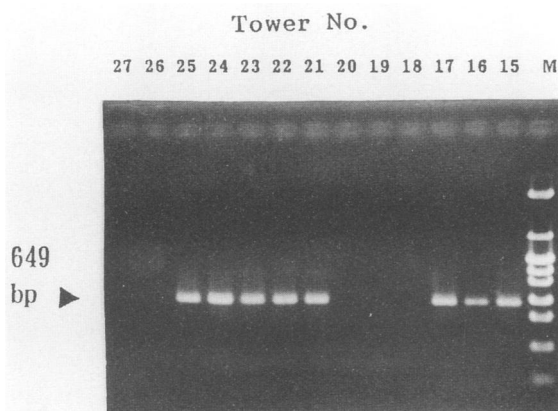


FIG. 2. Agarose (1.5%) gel electrophoresis of amplified DNA from cooling tower water samples performed with primers LmipL710 and LmipR1686 and with primers LmipL920 and LmipR1548. The samples were from cooling towers 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, and 15, and *Hind*III-digested pHY300PLK and *Hae*III-digested pHY300.2PLK were used as molecular weight markers (lane M).

Four *Legionella* species were isolated by the culture method from water collected from a single cooling tower (sample 4), and three *Legionella* species were obtained from each of five other samples (samples 7, 12, 24, 25, and 27). Among the strains isolated, the predominant taxa were *L. pneumophila* serogroup 1, *Legionella anisa*, and *Legionella erythra*. The first two of these exhibited the same pattern of distribution as that reported by us in Kinki District, Central Japan, but the last, *L. erythra*, was isolated for the first time in Japan (7). The reason for this is probably that in Japan only Okinawa District has a semitropical climate.

On the *L. pneumophila* whole chromosome, there may be several copies of the 5S rRNA complementary DNA sequence, in contrast to only one copy of the *mip* DNA sequence, which means that the PCR performed with 5S rRNA primers is more sensitive than the PCR performed with *mip* primers.

A comparison of the PCR method and the culture method by using cooling tower water showed that the 5S rRNA primer PCR method has a higher level of sensitivity than the culture method and that the *mip* primer PCR method and the culture method have equal levels of sensitivity. There were seven samples in our study that contained 10^2 CFU of *L. pneumophila* per ml of concentrated water. *L. pneumophila* was not detected in three of these seven water samples by the *mip* primer PCR, because only 10 μ l of each 1.0-ml sample prepared was used, which is at the limit of sensitivity. However, one water sample that contained 10 CFU of *L. pneumophila* per ml produced a positive reaction in the *mip* primer PCR. If the phenol extraction method for sample preparation was used, the entire 1.0-ml sample could be used for PCR, in which case the sensitivity would be enhanced 100-fold and easily surpass the sensitivity of the culture method.

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