# COMPARISON OF MULTIPLEX PCR AND CULTURE FOR DETECTION OF *LEGIONELLAE* IN COOLING TOWER WATER SAMPLES

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**Abstract.** We compared multiplex polymerase chain reaction (PCR) and culture for detecting the presence of *Legionella pneumophila* and *Legionella* spp in cooling tower water samples. Multiplex PCR was performed after phenol extraction of DNA from the samples. The set of primers for the PCR assay involved the 5S rRNA (*Legionella* spp) and the *mip* (macrophage infectivity potentiator gene, specific for *L. pneumophila*) genes as target sequences for amplification. Both the sensitivity and the specificity of the PCR assay were 100% when the 5S rRNA gene was used as target sequence. Isolation of *Legionellae* from the samples was observed only with the PCR-positive samples. We propose that PCR be used as a screening test before attempting to culture *Legionellae* from cooling tower water samples.

## INTRODUCTION

The causal agents of legionellosis, members of the genus *Legionella*, are ubiquitous in freshwater environments (Fliermans *et al*, 1981). The main means of contracting pneumonia appears to be the inhalation of aerosols containing virulent strains of *Legionella* species (Edelstein, 1993). Currently, culture is the standard method for detection of *Legionella* species in environmental water samples. However, this is a tedious and time-consuming procedure and isolation may not always be achieved. Other methods like direct fluorescent antibody testing (immunofluorescence microscopy) which requires skilled technical staff can give false positive results due to cross-reactivity and poor specificity may be a significant problem (Edelstein, 1993). Improved methods for detecting the presence of *Legionella* in environmental samples such as those based on PCR and nucleic acid probes have been developed (Mahbubani *et al*, 1990; Bej *et al*, 1991). In the study reported here we used a PCR assay to detect nucleic acid sequences of *L. pneumophila* and *Legionella* spp using a single pattern of amplification followed by hybridization with non-radioactive probes. The sensitivity and the specificity of this assay was compared to the culture method for the detection of *Legionellae* from cooling tower water samples.

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## MATERIALS AND METHODS

### Cooling tower water samples

Fifty-six water samples from cooling towers were collected prospectively from several locations in Singapore, from January to April 1994.

### Sample processing and culture

Five hundred ml of each water sample were filtered through a 0.45 μm membrane (Gelman Sciences, Michigan), the filter membrane was placed in 2 ml of sterile distilled water and vortexed briefly. Aliquots of the concentrated water sample were tested as follows: 1.0 ml for multiplex PCR assay; 0.1 ml plated directly onto buffered charcoal yeast extract medium with growth supplements (BCYEα) (Oxoid, Basingstoke, UK) and BCYEα medium supplemented with modified Wadowsky-Yee supplement (MWY) (Oxoid, Basingstoke, UK) (Wadowsky and Yee, 1981); the remainder treated with 0.2M KCl-HCl (Bopp *et al*, 1981) of which 0.1 ml was plated on BCYEα and MWY media. Plates were incubated in a candle-jar with moisture at 37°C for up to 10 days. Colonies were examined under a dissection microscope and screened for autofluorescence under UV light (365 nm). *Legionella* speciation and serogrouping were determined by direct immunofluorescence using monoclonal antibodies (MarDx Diagnostics Inc, Carlsbad, CA).

**DNA extraction**

One ml of bacterial suspension (for the preliminary studies) or cooling tower water sample was pelleted down, then resuspended in 50 mM Tris-HCl (pH 8.0), 0.7 M sucrose and lysed with SDS and EDTA. The lysate was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). To the aqueous layer, 5 M NaCl and 2 volumes of absolute ethanol were used to precipitate the DNA which was then washed with 70% ethanol, dried under vacuum and redissolved in TE buffer (pH 8).

**PCR assay and hybridization**

For the detection of all *Legionella* spp designated primers *L5S*L9 (5'-ACTATAGCGATTTGGAACCA-3') and *L5S*R93 (5'-GCGATGACCTACTTTCG-CAT-3') were used for the PCR, which synthesized a 104-bp segment of the coding region of the 5S rRNA gene sequence (MacDonnell and Colwell, 1987; Mahbubani *et al*, 1990). For the detection of *L. pneumophila*, designated primers *Lmip*L920 (5'-GCTACAGACAAGGATAAGTTG-3') and *Lmip*-R1548 (5'-GTTTTGTATGACTTTAATTCA-3') synthesizing a 650-bp segment of the coding region of macrophage infectivity potentiator (*mip*) gene were used (Engleberg *et al*, 1989; Mahbubani *et al*, 1990). The final volume of the PCR was 100 µl and contained 1x PCR amplification buffer (10X buffer: 50 mM KCl, 100 mM Tris HCl [pH 8.1], and 0.1% [wt/vol] gelatin), 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 1.5 µM each of primers *Lmip*L920 and *Lmip*R1548, and 0,1 µM each of primers *L5S*L9 and *L5S*R93, 1.5 µl of extracted DNA template, 2.5 U of *Taq* polymerase, and sterile double-distilled water. The reaction tube was layered with 50 µl of sterile mineral oil (Sigma, St Louis, MO). PCR was performed with a PTC-100™ Programmable Thermal Controller (MJ Research Inc, Watertown, MA). Templates were first denatured for 5 minutes at 94°C, followed by 40 cycles of 1 minute denaturation at 94°C, 1 minute of primer annealing at 52°C and 2 minutes extension at 72°C. The PCR products were examined by gel electrophoresis in 2% agarose with TBE buffer, stained with ethidium bromide and visualized by UV transillumination.

The probes used were internal to the amplification products whereby for the *mip* gene it was *Lmip*-1 (5'-TTTGGGGAAGAATTTTAAAAATCAAGGCAT-AGATGTTAATCCGGAAGCAA-3') and for the 5S rRNA it was *L5S*-1 (5'-CTCGAACTCAGAA-GTCAAACATTTCCGCGCCAATGATAGTG-TGAGGCTTC-3') (MacDonnell and Colwell, 1987; Engleberg *et al*, 1989; Mahbubani *et al*, 1990). Amplicons were transferred onto Hybond-N + nylon membranes (Amersham, Buckinghamshire, UK) using a manifold (Hybri-Slot, Life Technologies Inc., Gaithersburg, MD) and immobilized by UV light. Hybridization was done overnight at 42°C with the probes labeled with horseradish peroxidase and detected by enhanced chemiluminiscence (ECL kit, Amersham, Buckinghamshire, UK). The membranes were washed under stringent conditions and exposed to X-ray film (Hyperfilm-ECL, Amersham) at room temperature for 10-30 minutes.

**Sensitivity of PCR assay**

To determine the sensitivity of the assay, multiplex PCR was performed on extracted DNA of *L. pneumophila* (serogroup 1) NCTC 11192 with the amount of DNA assessed ranging from 1 ng to 100 ag. Negative controls included samples without DNA and samples with extracted DNA of *Escherichia coli* ATCC 25922. Slot blots prepared with the amplification products were hybridized with probes for *L. pneumophila* and *Legionella* spp.

## RESULTS

We observed that with our multiplex PCR protocol, up to 1 pg DNA of *L. pneumophila* (649-bp amplicon, Fig 1A) could be detected, whereas for *Legionella* spp, detection was up to 100 fg (104-bp amplicon, Fig 1A). The results of the slot blot hybridization analysis using specific oligonucleotide probes internal to the amplified gene fragments showed positive reactions with samples containing as few as 100 fg and 10 fg of DNA of *Legionella pneumophila* (Fig 1B) and *Legionella* spp (Fig 1C), respectively.

From the 56 cooling tower water samples studied, a total of 22 samples (39.3%) produced PCR positive (5S rRNA) bands (Tables 1 and 2). All PCR-positive (gel electrophoresis and sample banding) samples were also positive for *Legionella* culture (Table 1). None of the *Legionella* spp gave positive mip bands. The calculated sensitivity and specificity of the PCR test (5S rRNA) as compared with the gold standard

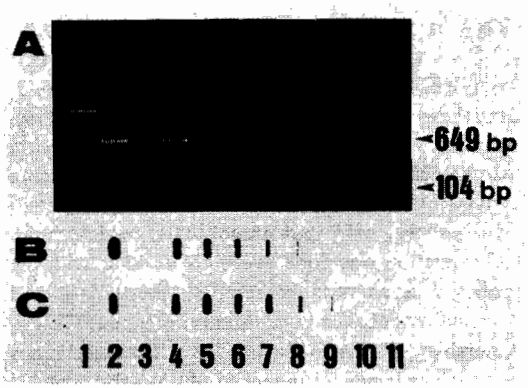


Fig 1–(A) 2% agarose gel electrophoresis of multiplex PCR amplified DNA samples using 5S rRNA primers *L5S*L9 and *L5S*R93 (104 bp) for *Legionella* spp, and *mip* primers *Lmip*L920 and *Lmip*R1548 (649 bp) for *Legionella pneumophila*. (B) Southern (slot) blot analysis using the probe *Lmip*-1 for *L.pneumophila*. (C) Southern blot analysis uing the probe *L5S*-1 for *Legionella* spp. Lane 1, ϕX174 *Hae* III DNA digest molecular marker. Lane 2, positive control for both 5S RNA and mip genes using 1 μg *L. pneumophila* serogroup 1 (NCTC 11192) DNA. Lane 3, Negative control. Lanes 4 to 11, *L. pneumophila* serogroup 1 (NCTC11192) DNA in amounts ranging from 1 ng to 100 ag.

of culture method were 100% (Table 2). All PCR positive tests were analyzed by Southern (slot) hybridization using internal probes to the PCR amplified gene segments. No false positive PCR test was observed as determined by slot hybridization. We noticed that three samples (number 2, 13 and 30, Table 1) which contained small number of colonies of *L. pneumophila* (80 CFU/100 ml or less) upon culturing were negative for amplification of the *mip* gene but were positive for amplification of the 5S rRNA gene fragment. Thus, of the 12 samples from which *L. pneumophila* was isolated, 9 (83.3%) were PCR-positive and 3 (16.7%) were negative. One of the PCR negative samples for *L. pneumophila* was positive for slot blot hybridization when using an internal probe to the *mip* amplicon; the other two remained negative.

## DISCUSSION

Few studies have been performed to assess the value of the PCR assay to detect the presence of Legionellae in environmental water samples and in particular cooling tower water samples (Koide *et al*, 1993; Maiwald *et al*, 1994a). The results of our study clearly demonstrate that multiplex PCR is an assay as sensitive as the traditional culture on selective media for determination of the presence of Legionellae from cooling tower water samples.

Under our experimental conditions the PCR assay allowed us to detect as low as 1 pg DNA of *L. pneumophila* and 100 fg DNA of Legionella spp. The PCR assay with the 5S rRNA primers was more sensitive than the mip primers for detection of *Legionella* DNA. This is in agreement with previous observations (Koide *et al*, 1993) and may be due to a higher copy number of the 5S rRNA gene sequence as compared with the mip gene. However, in our study the possibility that some material was lost during the DNA extraction procedure from samples containing small number of *L. pneumophila* cannot be excluded.

No false PCR-positive results were observed during our study. Studies performed by other investigators have described problems with PCR false-positive bands which did not hybridize with the internal oligonucleotide probe for the 5S rRNA gene (Maiwald *et al*, 1994a). However, the same group of workers has since found that those bands were due to the presence of contaminating DNA in the *Taq* polymerase used (Maiwald *et al*, 1994b). This episode underscores the importance of ensuring that each batch of *Taq* polymerase used is free from any contaminating DNA.

In our study we overcome the problem of the presence of potential inhibitory substances in our samples by using a DNA extraction method. No previous study has used a protocol with full DNA extraction from the samples. In previous studies, solubilization of the entire sample pellet using Chelex 100 or a freeze-thaw procedure together with the addition of proteinase K (Koide *et al*, 1993; Maiwald *et al*, 1994a) have been used before performing the PCR assay. Although the use of those procedures simplifies sample processing, there is the potential of producing inhibition of the PCR assay due to substances remaining in the unextracted samples. Particularly, rust present in the cooling tower water samples may pose a problem (Maiwald *et al*, 1994a).

From the 22 positive samples, seven were positive for *Legionella* species for which we could not do further speciation as they did not react with our available panel of monoclonal antibodies. It is also possible that those *Legionella* may represent new

Table 1

Comparison of multiplex PCR and culture method for detection of *Legionellae.*

| Sample | Multiplex PCR | | *Legionella* count (CFU/10 ml) | Identification of *Legionellae* |
|---|---|---|---|---|
| | *mip* (*L. pneumophila*) | 5S rRNA (*Legionella* spp) | | |
| 1 | + | + | 77,800 | *L. longbeacheae* ser 1-2 |
| 2 | - | + | 50 | *L. pneumophila* ser 5 |
| 3 | - | - | 0 | - |
| 4 | - | - | 0 | - |
| 5 | - | - | 0 | - |
| 6 | - | - | 0 | - |
| 7 | + | + | 340 | *L. pneumophila* ser 6 |
| 8 | - | - | 0 | - |
| 9 | - | - | 0 | - |
| 10 | - | - | 0 | - |
| 11 | - | - | 0 | - |
| 12 | - | - | 0 | - |
| 13 | - | + | 80 | *L. pneumophila* ser 8-14 |
| 14 | - | + | 40 | *Legionella* spp |
| 15 | - | - | 0 | - |
| 16 | - | - | 0 | - |
| 17 | - | - | 0 | - |
| 18 | + | + | 1,000 | *L. pneumophila* ser 6 |
| 19 | + | + | 1,400 | *L. pneumophila* ser 6 |
| 20 | - | + | 5,000 | *Legionella* spp |
| 21 | - | - | 0 | - |
| 22 | - | - | 0 | - |
| 23 | - | - | 0 | - |
| 24 | - | - | 0 | - |
| 25 | - | - | 0 | - |
| 26 | + | + | 1,680 | *L. pneumophila* ser 8-14 |
| 27 | + | + | 720 | *L. pneumophila* ser 8-14 |
| 28 | + | + | 640 | *L. pneumophila* ser 8-14 |
| 29 | + | + | 1,200 | *L. pneumophila* ser 7 |
| 30 | - | + | 40 | *L. pneumophila* ser 8-14 |
| 31 | - | + | 2,320 | *Legionella* spp |
| 32 | - | + | 1,160 | *Legionella* spp |
| 33 | - | + | 400 | *Legionella* spp |
| 34 | - | - | 0 | - |
| 35 | - | - | 0 | - |
| 36 | - | - | 0 | - |
| 37 | - | - | 0 | - |
| 38 | - | - | 0 | - |
| 39 | - | - | 0 | - |
| 40 | - | - | 0 | - |
| 41 | - | - | 0 | - |
| 42 | - | - | 0 | - |
| 43 | - | + | 4,400 | *Legionella* spp |
| 44 | - | - | 0 | - |
| 45 | - | - | 0 | - |
| 46 | - | - | 0 | - |
| 47 | - | - | 0 | - |
| 48 | - | - | 0 | - |
| 49 | - | + | 1,493 | *Legionella* spp |
| 50 | - | - | 0 | - |
| 51 | - | - | 0 | - |
| 52 | + | + | 853 | *L. pneumophila* ser 8-14 |
| 53 | + | + | 1,387 | *L. pneumophila* ser 8-14 |
| 54 | - | + | 2,346 | *L. longbeacheae* ser 1-2 |
| 55 | - | + | 1,973 | *L. longbeacheae* ser 1-2 |
| 56 | - | - | 0 | - |

Table 2

Detection of Legionellae by PCR (5S rRNA gene target sequence) and culture.

| PCR result for *Legionella* | Culture | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 22 | 0 | 22 |
| Negative | 0 | 34 | 34 |
| Total | 22 | 34 | 56 |

*Legionella* spp or species for which we do not have specific antibodies.

The PCR assay takes less than 24 hours to perform and results are obtained much earlier than culture. However, we do not think that the PCR could replace the culture method entirely as the isolation of *Legionella* is still necessary to fully establish their identity and for epidemiological studies. For instance, the PCR assay described here cannot discriminate between the different species of *Legionella* spp and the different serogroups of *L. pneumophila* and samples containing both *L. pneumophila* and *Legionella* spp. As the culture method yields positive results only when PCR-positive results were obtained we propose that the PCR assay described here be used as a "screening test" before any attempts to culture *Legionella* from cooling tower water samples. This could provide a substantial reduction in labor and cost for reference laboratories which provide services for detectiom of *Legionella* from cooling tower samples. Whether the PCR assay described here can be applied to accurately detect *Legionellae* from other types of environmental samples requires further study.

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