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Detection of Viable *Legionella pneumophila* in Water by Polymerase Chain Reaction and Gene Probe Methods

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Methods using polymerase chain reaction (PCR) and gene probes to detect viable *Legionella pneumophila* were investigated with cells exposed to biocide or elevated temperature. Exposure to hypochlorite caused viable nonculturable cells to form. Culturable and viable nonculturable cells showed positive PCR amplification, whereas nonviable cells did not. Viable cells were also specifically detected with *mip* mRNA as the target, reverse transcription (to form cDNA), and PCR amplification. After exposure to elevated temperature, only viable culturable cells were detected, which corresponded with positive PCR amplification.

While we have recently reported on the development of methods using polymerase chain reaction (PCR) and gene probes for the detection of legionellae in environmental waters (11), the question of whether such gene probe methodology detects dead and living bacteria has not been previously examined. Recognizing that the terms alive and viable are subject to different definitions (15), for the purpose of this study live or viable cells are considered those capable of cell division (colony-forming units [CFUs]), metabolism (respiration), or gene transcription (mRNA production). Viable nonculturable bacteria have been shown to cause infections in susceptible animals (15). *Legionella pneumophila* has been shown to form viable but nonculturable cells, which may be responsible for the failure to culture viable *L. pneumophila* from some environmental sources (8). In some cases, *L. pneumophila* cannot be cultured from cooling towers suspected to be environmental sources of infecting bacteria causing legionellosis, particularly when biocides have been used as disinfecting agents (4). As part of our studies to determine the efficacy of using PCR-gene probe approaches for environmental monitoring of legionellae, we investigated whether PCR-gene probe methods discriminate between viable and nonviable *L. pneumophila*.

To determine whether PCR amplification of the *mip* gene of *L. pneumophila* detects dead and living bacterial cells, *L. pneumophila* serogroup 3 (Bloomington) was grown at 37°C in a liquid medium consisting of 10 g of yeast extract (Difco), 10 g of ACES (*N*-2-acetamido-aminoethanesulfonic acid), 0.25 g of L-cysteine, 0.25 g of ferric pyrophosphate, and 1 liter of distilled H_2O, pH 7.0. Cells in 100-ml samples collected during the exponential growth phase were exposed to lethal conditions by raising the temperature to 70°C. The initial cell concentration was 10^5 CFU/ml. At 0, 1, 3, 5, and 10 min, serial dilutions of samples were plated on buffered charcoal-yeast extract agar (6), and samples were also analyzed by *L. pneumophila*-specific PCR-gene probe method (11).

For PCR, 1-ml samples were filtered through an ethanol-presoaked 13-mm-diameter Fluoropore membrane (0.5-μm pore size; FHLP; Millipore). The filter was rolled and transferred by using sterile forceps to a 0.6-ml Eppendorf tube with the cell-coated side facing inward. One hundred microliters of 0.1% diethylpyrocarbonate (DEPC) (Sigma)-treated autoclaved distilled water was added to the tube and vortexed vigorously for 5 to 10 s. Five freeze-thaw cycles were performed, using an ethanol-dry ice bath and warm water successively. Fifty microliters of reaction mix containing 1× PCR amplification buffer (10× buffer contains 50 mM KCl, 100 mM Tris hydrochloride [pH 8.13], 15 mM $MgCl_2$, and 0.01% [wt/vol] gelatin), 200 μM each of the deoxynucleoside triphosphates, 0.2 to 0.5 μM each of the primers, and 2.5 U of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus) were added to the tube. The template DNA was initially denatured at 96°C for 3 min, and then a total of 25 PCR cycles were performed by using a two-temperature PCR cycle with denaturation at 95°C for 0.5 min and primer annealing and extension at 50°C for 1 min.

The 21-mer primers *Lmip*L920 (5′-GCTACAGACAAG GATAAGTTG-3′) and *Lmip*R1548 (5′-GTTTTGTATGA CTTTAATTCA-3′) and the 50-mer oligonucleotide probe *Lmip*-1 (5′-TTTGGGGAAGAATTTTAAAAATCAAGGCA TAGATGTTAATCCGGAAGCAA-3′) used in this study have been described by Mahbubani et al. (11) and resulted in detection of a 650-bp sequence of the coding region of the *L. pneumophila* macrophage infectivity potentiator (*mip*) gene. Oligonucleotide probes for Southern blot hybridization were radiolabeled with [γ-^{32}P]ATP (>3,000 Ci/mmol) (New England Nuclear Corp., Boston, Mass.) at their 5′ ends, using the forward reaction described by Ausubel et al. (1). We also ran dot blot analyses using a ^{32}P-radiolabeled gene probe that hybridizes with the *mip* PCR-amplified DNA (11) and quantifying the amount of hybridized probe by cutting out the dots and using liquid scintillation counting to determine the counts per minute of hybridized probe.

The results of this experiment indicated that a PCR-gene probe-positive signal was seen only when viable cells were detectable by culture methods such as CFUs (Table 1). When cultures were negative (no CFUs detected), PCR-gene probe detection was also negative (no band in gel, no positive signal observed by Southern hybridization, and no counts above background in the dot blot analyses), indicating that the PCR-gene probe detection method appears to detect only living bacterial cells. Although PCR may not produce truly quantitative results, the dot blot analyses also showed a quantitative decrease in amount of probe that hybridized as the number of viable cells declined, indicating the apparent decrease in PCR target DNA as the number of viable cells declined (Table 1).

To further explore whether exposure to biocide, used to

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TABLE 1. Detection of *L. pneumophila* by plate count (viable culturable) and PCR-gene probe methods when cells were killed by exposure to elevated temperature

Time (min) after temperature raised to 70°C	Culturable organisms (CFU)	Intensity of DNA band by PCR-gene probe detection	Dot blot hybridization signal (cpm)
0	$>10^4$	++++	5×10^3
1	$>10^4$	++++	4×10^3
3	10^3	+++	7×10^2
5	10^1	++	2×10^3
10	0	−	0

TABLE 2. Detection of *L. pneumophila* by plate count (viable culturable), INT (total viable), and PCR-gene probe methods when cells were killed by exposure to biocide

Time (min) after biocide addition	Culturable organisms (CFU)	Viable organisms (% INT positive)	Intensity of DNA band by PCR-gene probe detection	Dot blot hybridization signal (cpm)
0	$>10^4$	100	++++	9×10^3
1	10^3	100	++++	5×10^3
2	0	85	++++	4×10^3
5	0	50	++	2×10^3
10	0	10	+	9×10^2
15	0	0	−	0
30	0	0	−	0

disinfect cooling towers, produces viable nonculturable cells, and if so whether such cells are detected by the PCR-gene probe detection method, we collected samples from an exponentially growing culture of *L. pneumophila* and added hypochlorite biocide to achieve a concentration of 100 ppm. At 0-, 1-, 2-, 5-, 10-, 15-, and 30-min intervals after biocide addition, 1-ml samples were collected, the hypochlorite was inactivated by adding 0.1% sterile sodium thiosulfate, and each sample was transferred to 10 ml of sterile water. Analyses were performed on these samples for total *L. pneumophila* by immunofluorescence microscopy (13, 16), for viable culturable *L. pneumophila* by plating on buffered charcoal-yeast extract agar (6), for total viable (metabolically active) *L. pneumophila* by the INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5 phenyltetrazolium chloride] (*p*-iodonitrotetrazolium violet; Sigma) method (17), and for *L. pneumophila* by PCR-gene probe detection of the *mip* gene (5), which we have shown to be diagnostic of *L. pneumophila* with single-cell detection sensitivity (11). For PCR, cells were collected by filtration and DNA amplification was performed as described earlier.

Controls run to confirm that PCR was not inhibited by sodium thiosulfate-inactivated hypochlorite showed that whereas no amplified DNA was formed in the presence of hypochlorite alone, a strong PCR signal was seen when pure *L. pneumophila* DNA was amplified in the presence of sodium thiosulfate-treated hypochlorite. Another control run by using pure (cell-free) *L. pneumophila* DNA showed no detectable signal for the filter, whereas a positive signal was found for the filtrate, indicating that if DNA had leaked from dead cells, it would not have been detected by the PCR-gene probe detection method employed for detecting cells of *L. pneumophila*.

The results of the three detection methods (plate count, INT, and PCR-gene probe) as a function of time after biocide addition are shown in Table 2. Southern blot analyses indicated that *L. pneumophila* was detectable by the PCR-gene probe method for 10 min after addition of biocide. No PCR-gene probe signal for *L. pneumophila*, however, was found 15 min after biocide treatment. Although viable culturable *L. pneumophila* (CFUs) was detectable only for 1 min after biocide was added, suggesting that the PCR-gene probe method might have been detecting dead bacteria for a prolonged period, the INT method indicated that viable but nonculturable bacteria remained for 10 min and that only after a 15-min exposure to biocide were metabolically active bacteria absent (Table 2). Thus, when the INT method indicated an absence of living bacteria, there was no PCR-gene probe signal, and when there was a positive gene probe signal, there were viable bacteria present. Further, the intensity of the PCR-amplified DNA as detected by gel electrophoresis declined with time as the proportion of viable cells decreased, suggesting that the PCR-gene probe method detected only living bacteria. These observations were confirmed by running dot blot analyses using a ^{32}P-radiolabeled gene probe that hybridizes with the *mip* PCR-amplified DNA (11) and quantifying the amount of hybridized probe by cutting out the dots and using liquid scintillation counting to determine the counts per minute of hybridized probe. There was a quantitative decrease in the amount of probe that hybridized as the number of viable cells declined (Table 2). Hence, at least for *L. pneumophila*, in environmental waters where biocide treatment occurs, positive detection by PCR indicates the presence of viable cells.

However, it is known that in other situations in which DNA is preserved, dead cells are detected by PCR-gene probe methods; PCR is used in forensic testing (7), has been shown to be capable of detecting DNA in cells from mummies (cells that have been nonviable for centuries) (14), and can be used to detect Formalin-preserved cells (9). Thus, while direct PCR amplification of *L. pneumophila* DNA did not detect dead cells which had died as a result of heat exposure or treatment with hypochlorite biocide, if the cells had died by other means, such as by Formalin treatment, which preserves DNA within cells, they might have been detected by PCR amplification of target DNA. Therefore, we designed a procedure for detecting viable bacterial cells using mRNA as the target. The underlying principle of this approach is that most of the bacterial mRNAs have half-lives of <2 min (3). When rapid RNA extraction methods were used, only fragments of the 650-bp *mip* cDNA could be detected, indicating that *mip* mRNA has a very short half-life (10); therefore, detecting *mip* mRNA would indicate the presence of metabolically active living *L. pneumophila* or that living cells had been present within the last few minutes.

Initially we attempted to recover *mip* mRNA by pouring cells in a 500-ml culture of exponential-phase *L. pneumophila* containing approximately 10^5 cells per ml over chloramphenicol-ice mix (100 μg of chloramphenicol per ml) using the procedure described by Engleberg et al. (5) for total RNA extraction. However, only a smear of RNA fragments of much less than 650 bp in size that hybridized with *Lmip*-1 oligonucleotide probe could be detected in Northern (RNA) blots, indicating probable rapid degradation of *mip* mRNA by cellular nuclease. Therefore, the procedure for recovery and detection of *mip* mRNA was modified by adding chloramphenicol (100 μg/ml), which inhibits protein synthesis (including production of RNase), to the culture of *L. pneumophila* 10 min prior to RNA extraction. To eliminate any

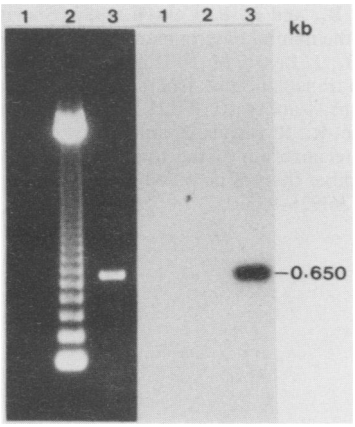

FIG. 1. Ethidium bromide-stained 1% agarose gel (left) and Southern blot DNA-DNA hybridization (right) analyses of PCR-amplified *mip* cDNA, which was synthesized by reverse transcription from total RNAs. The hybridization was performed by using radiolabeled 50-mer *Lmip*-1 oligonucleotide probe. Lanes 1, 15 μg of total RNA was amplified without cDNA synthesis (no contamination of DNA in the sample). Lanes 2, 123-bp DNA ladder as size standard. Lanes 3, PCR amplification of the *mip* cDNA, which was synthesized from 15 μg of total RNA.

contamination with DNA, several aliquots of the total RNA sample were treated with RNase-free DNase I (U.S. Biochemicals, Cleveland, Ohio), extracted twice with equal volumes of phenol-chloroform-isoamyl alcohol (24:24:2), precipitated with 2.5 M ammonium acetate and 2.5 volumes of ethyl alcohol, washed with cold 70% alcohol, and resuspended in TE (10 mM Tris hydrochloride [pH 7.2], 1 mM EDTA) buffer (10).

cDNA was synthesized by a modified version of the procedure described by Murakawa et al. (12) using a 20-μl reaction mixture containing 30 to 40 μg of total RNA extracted from *L. pneumophila*, 2 μl of 10× PCR reaction buffer, 3 μl of 10 mM deoxynucleoside triphosphates, 6 μl of distilled H_2O, 1 μl of RNAsin (Promega Biotech Inc., Madison, Wis.), 1 μl (200 U) of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories Inc., Gaithersburg, Md.), and 40 pmol (approximately 2 μl) of *Lmip*R1548 primer (10). PCR amplification of the *mip* cDNA was performed by adding the PCR reaction mixture as follows: in a total 100-μl reaction volume, 8 μl of 10× PCR reaction buffer, 40 pmol of the *Lmip*L940 primer, 5 μl of 10 mM deoxynucleoside triphosphate mix, and 2.5 U of Ampli*Taq* polymerase, and using a PCR program as described earlier. Southern blot analysis was performed by using Zetaprobe nylon membrane (BioRad) and radiolabeled *Lmip*-1 oligonucleotide probe.

When viable cells were present, a 650-bp amplified DNA band that hybridized with *Lmip*-1 oligonucleotide probe was detected, indicating amplification of the cDNA of the *mip* gene of *L. pneumophila* (Fig. 1). RNA extracted from heat-killed (70°C for 10 min) *L. pneumophila* cultures, which were also treated with chloramphenicol (100 μg/ml), consistently showed no *mip* mRNA (on the basis of detection of PCR-amplified DNA following cDNA synthesis), indicating that nonviable cells were not detected by the mRNA-based procedure. Thus the detection of *mip* mRNA was specific for viable *L. pneumophila*. The level of sensitivity for detecting *mip* mRNA was 1,000 cells (10) compared with single-cell detection by direct DNA PCR amplification (2, 11). The lower sensitivity of the mRNA-PCR method compared with that of direct DNA-PCR detection is probably due to the low efficiency of mRNA extraction and protection.

In conclusion, the direct PCR-gene probe method detects viable *L. pneumophila*, including viable but nonculturable cells, in waters—even following biocide treatment. An alternate but less sensitive method for detecting viable bacterial cells is accomplished by synthesizing cDNA from an mRNA target, such as from *mip* mRNA of *L. pneumophila*, followed by PCR amplification and gene probe detection. While for the detection of legionellae in waters the direct DNA-PCR detection approach corresponds to the detection of viable bacterial cells, and thus the technically more difficult and less sensitive mRNA method is not needed for monitoring legionellae in environmental waters and cooling towers, in other situations the mRNA-based detection method may be needed to establish the presence of viable bacterial cells.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Sideman, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Bej, A. K., R. J. Steffan, J. DiCesare, L. Haff, and R. M. Atlas.** 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Appl. Environ. Microbiol. **56**:307–314.
3. **Belasco, J. G., and C. F. Higgins.** 1988. Mechanisms of mRNA decay in bacteria: a perspective. Gene **72**:15–23.
4. **England, A. C., D. W. Fraser, G. F. Mallison, D. C. Mackel, P. Skaliy, and G. W. Gorman.** 1982. Failure to predict culture results from disinfectant-treated air conditioning cooling towers. Appl. Environ. Microbiol. **43**:240–244.
5. **Engleberg, N. C., C. Carter, D. R. Weber, N. P. Cianciotto, and B. I. Eisenstein.** 1989. DNA sequence of *mip*, a *Legionella pneumophila* gene associated with macrophage infectivity. Infect. Immun. **57**:1263–1270.
6. **Felley, J. C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Resheed, D. C. Mackel, and W. B. Baine.** 1979. Charcoal yeast extract agar: primary isolation medium for *Legionella pneumophila*. J. Clin. Microbiol. **10**:437–441.
7. **Higuchi, R., C. H. von Beroldingen, G. F. Sensabaugh, and H. A. Erlich.** 1988. DNA typing from single hairs. Nature (London) **332**:543.
8. **Hussong, D., R. R. Colwell, M. O. O'Brien, E. Weiss, A. D. Pearson, R. M. Wiener, and W. D. Burge.** 1987. Viable *Legionella pneumophila* not detectable by culture on agar media. Bio/Technology **5**:947–950.
9. **Impraim, C. C., R. K. Saiki, H. A. Erlich, and R. L. Teplitz.** 1987. Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridization with sequence-specific oligonucleotides. Biochem. Biophys. Res. Commun. **142**:710–716.
10. **Mahbubani, M., A. Bej, J. DiCesare, R. Miller, L. Haff, and R. M. Atlas.** 1990. Detection of bacterial mRNA using PCR. BioTechniques **10**:48–49.
11. **Mahbubani, M. H., A. K. Bej, R. Miller, L. Haff, J. DiCesare, and R. M. Atlas.** 1990. Detection of *Legionella* with polymerase chain reaction and gene probe methods. Mol. Cell. Probes **4**:175–187.
12. **Murakawa, G. J., J. A. Zaia, P. A. Spallone, D. A. Stephens, B. E. Kaplan, R. B. Wallace, and J. J. Rossi.** 1988. Direct

detection of HIV-1 RNA from AIDS and ARC patient samples. DNA **7**:287–295.
13. **Orrison, L. H., W. F. Bibb, W. B. Cherry, and L. Thacker.** 1983. Determination of antigenic relationships among legionellas and nonlegionellas by direct fluorescent antibody and immunodiffusion tests. J. Clin. Microbiol. **17**:132–137.
14. **Paabo, S., J. A. Gifford, and A. C. Wilson.** 1988. Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Res. **16**:9775–9787.
15. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51**:365–379.
16. **Tobin, J. O., J. Beare, M. S. Dunnill et al.** 1980. Legionnaire's disease in a transplant unit: isolation of the causative agent from shower baths. Lancet **ii**:118–121.
17. **Zimmerman, R., R. Iturriaga, and J. Becker-Birck.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36**:926–935.