Effect of Amplicon Size on PCR Detection of Bacteria Exposed to Chlorine

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The effect of amplicon size on the PCR detection of *Legionella pneumophila* after chlorine inactivation was investigated. Two amplicons specific to the *L. pneumophila* *mip* gene were used for the PCR analyses: a 650-bp amplicon and smaller 168-bp amplicon within the 650-bp amplicon; a 108-bp amplicon specific to species rRNA coding sequence also was used. After exposure to chlorine, viable agar grown cells were not detected by plate counts or direct counts with *p*-iodonitrotetrazolium (INT) after 1 min for treatment at 10 mg/l, after 2 min for treatment at 5 mg/l, and after 4 min for treatment at 2.5 mg/l; viable water grown cells were present at least 4 min after biocide addition even with a chlorine dose of 5 mg/l. At the 10-mg/l dosage, PCR products from the 168-bp amplicon were detected on agarose gels up to 16 min after chlorination; even after 24 hr of PCR the 168-bp products were detectable using a capture probe hybridization assay. However, the 650-bp target was not detected after 4 min chlorine contact time at the same biocide dosage using agarose gels, and PCR products could not be detected by hybridization after 32 min. At lower chlorine concentrations, a similar pattern was seen with the 168-bp amplicon detectable longer after biocide addition than the 650-bp *mip* amplification target. On the basis of these data, larger amplicons appear to correlate better with viability of *L. pneumophila* in water samples.

Several methods for the detection of microorganisms in environmental samples using PCR have been developed.⁽¹⁾ Roche Molecular Systems and the Perkin-Elmer Corporation have developed a commercial PCR kit for the detection of the water-associated pathogen *Legionella pneumophila*, the EnviroAmp *Legionella* kit, which allows the semiquantitative detection of the genus *Legionella* and species *L. pneumophila* in under 6 hr.⁽²⁾ The amplicon for *Legionella* detection is a 108-bp region of a 5S rRNA coding gene, and the amplicon for *L. pneumophila* detection is a 168-bp region of the macrophage infectivity potentiator (*mip*) gene.

The public health significance of a PCR positive result rests with distinguishing viable from nonviable organisms, because PCR procedures can detect DNA trapped inside dead or even preserved cells.^(3,4) PCR positive results may require confirmation using conventional procedures like plate counts or animal infectivity studies, unless it can be demonstrated that a particular PCR procedure detects only viable cells. Studies performed using histological samples preserved for PCR examination have shown that the signals from longer amplicons are lost more rapidly than smaller amplicons.⁽⁴⁾ A PCR-based assay to assess DNA damage induced by the anticancer drug cisplatin has been developed on the basis of loss of signal from long amplicons (750 and 2000 bp) after drug treatment.⁽⁵⁾ In the same assay a smaller amplicon (150 bp) showed no appreciable loss of signal following cisplatin treatment. If the likelihood of DNA damage in any defined sequence is proportional to the length, a relationship between amplicon length and persistence in dead cells may exist. Particu-

larly during chlorine disinfection—the most common treatment for the destruction of pathogens in drinking water and a process demonstrated to produce chromosomal lesions⁽⁶⁾—longer amplicons may be more reflective of inactivation than smaller amplicons. Treatment with bleach (chlorine) of glassware, equipment, and benchtops is routinely recommended for laboratories performing PCR to prevent contamination with DNA that may give unwanted amplification; adequate chlorine treatment eliminates DNA that can be amplified by PCR. In this study we determined the effect of amplicon length on the PCR detection of bacteria after chlorine disinfection of water; chlorination is the method most commonly used for disinfection of potable waters and has been used to treat cooling to decontaminate them from *Legionella* species.

MATERIALS AND METHODS

Growth and Maintenance of *L. pneumophila* Cultures

The *L. pneumophila* strain used for this investigation was isolated from a cooling tower and confirmed using a fluorescent antibody test (Genetic Systems Corp., Seattle, WA). This strain was maintained on BCYE agar (BDMS, Inc., Cincinnati, OH). For chlorination experiments, 10 BCYE agar plates were inoculated and incubated at 37°C for 5 days. The surface of the plates was then flooded with dechlorinated filter-sterilized (DCFS) tap water and placed on an orbital shaker for 20 min at 200 rpm to loosen the cells. DCFS tap water was prepared using powered activated carbon, filtered through a 0.2- μ m membrane, and then autoclaved; the

effectiveness of elimination of residual chlorine was confirmed by chemical analysis. The cells were then transferred to a sterile centrifuge tube and pelleted by centrifugation at 1500g for 30 min. The pellet was then washed three times in DCFS and resuspended in 20 ml of DCFS water. The cell density of the suspension was determined using acridine orange direct counts (AODC) and stored at 4°C before use.

Water-grown cells were prepared by inoculating 4×10^8 cells of the same *L. pneumophila* strain into two liters of DCFS tap water (cell density, 2×10^5 cells/ml). Plate counts were performed to track the growth of this strain. After 35 days, the culturable cell density was 4×10^5 cells/ml; there may have been additional viable, but nonculturable, cells. This cell suspension was used directly for chlorination experiments.

Chlorination of *L. pneumophila*

DCFS tap water (800 ml at pH 8.6) was placed in a chlorine-demand-free beaker and placed on a magnetic stirrer. The stirrer was set to create a 5-cm vortex in the center of the solution. Agar-grown *L. pneumophila* cells were added to achieve a final density of $\sim 10^7$ cells/ml. Water-grown cells were used directly. The cells were stirred at room temperature for 30 min. A 75-ml aliquot was removed before the addition of chlorine. A measured dose (10, 5, or 2.5 mg/l) of sodium hypochlorite solution (Fisher Scientific, Cincinnati, OH) was added, and aliquots were removed for analyses at 1, 2, 4, 8, 16, and 32 min and at 24 hr. The dosages used are typical of the range of concentrations and exposure times used to disinfect potable waters. As samples were collected, the chlorine residual was neutralized using 0.1 ml of 10% (wt/vol) sodium thiosulfate and placed on ice before analyses. An additional sample was collected at 5 min, and the chlorine residual determined using a Hach colorimetric test kit (Loveland, CO). Although the chlorine residual was not measured after 24 hr, all samples had pungent chlorinous odors. Each experiment with the agar-grown cells was performed in triplicate for each chlorine dose. The experiment using the water-grown cells was performed in triplicate using a chlorine dose of 5 mg/l. Controls with no chlorine added were also run.

Assessment of *L. pneumophila* Viability

Viability was assessed using plate counts and direct counts using *p*-iodonitrotetrazolium (INT) (Sigma Chemical Co., St. Louis, MO). For plate counts dilutions of the samples were plated onto BCYE agar (BDMS, Cincinnati, OH) and were then incubated for 10 days at 37°C. Direct INT

counts were performed by placing 9 ml of the sample into a well of a six-position microtiter plate; 1 ml of a 1% (wt/vol) aqueous INT solution was added, and the microtiter plate was then incubated at 37°C for 6 hr. The INT-stained cells were preserved using 0.1 ml of formalin and stored at 4°C before enumeration. INT-stained cells were stained with a *L. pneumophila*-specific FAb (Genetic Sys-

FIGURE 1 Results of chlorine disinfection and agarose gel analysis of PCR products. (◆) *L. pneumophila* percent survival based on viable plate count determination; (△) percent of initial PCR signal from the 108-bp amplicon; (□) percent of initial PCR signal from the 168-bp amplicon; (○) percent of initial PCR signal from the 650-bp amplicon. (A,B,C) Agar-grown cells chlorinated with 10, 5, and 2.5 mg/l, respectively; (D) water-grown cells chlorinated at 5 mg/l. Percent survival of 10^{-6} indicates no viable cells. Controls with no chlorine treatment still showed strong PCR signals (no loss of intensity) at 24 hr for the 108-, 168-, and the 650-bp amplicons.

tems, Seattle, WA), and the percentage of actively respiring cells was determined using a Nikon Labophot microscope with an epifluorescence attachment.

PCR Analyses

Ten milliliters of each sample was filtered through a Millipore Durapore HVHP membrane filter (25-mm-diam., 0.45- μ m pore size). The filter was placed into a tube containing 2 ml of DNA extraction reagent (Perkin-Elmer Corp., Alameda, CA) with the trapped cells facing into the tube, and it was then vortexed for 30 sec. The tube was boiled for 10 min to extract the DNA, and 20 μ l was used as the template for PCR reactions.

The 108- and 168-bp amplicons were amplified using the EnviroAmp *Legionella* kit, whereas the 650-bp amplicon was amplified using a previously published protocol with slight modifications.⁽⁷⁾ The EnviroAmp *Legionella* kit supplied the master mix for PCR, and 65 μ l of this mix was aliquoted into a GeneAmp Reaction Tube (Perkin-Elmer Corp.) and overlaid with mineral oil. The extracted sample was added, and 15 μ l of 25 mM $MgCl_2$ was added for a total reaction volume of 100 μ l; PCR was then performed with the step-cycle recommended by the manufacturer with a DNA Thermal Cycler (Perkin-Elmer model 480). After PCR the amplification products were stored at -20°C .

For the 650-bp amplicon, the PCR reaction mix contained 1 $\times$ PCR reaction buffer (10 $\times$ buffer contains 500 mM KCl, and 100 mM Tris-HCl at pH 8.3), 200 μ M each of the deoxynucleoside triphosphates, 0.35 μ M each of the primers, and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) and was overlaid with mineral oil. Twenty microliters of extracted template was added, along with 15 μ l of 25 mM $MgCl_2$. PCR was performed using the following step-cycle: one cycle of 3 min at 96°C , followed by 25 cycles of a two-temperature PCR cycle with denaturation at 95°C for 0.5 min, followed by primer annealing and extension at 50°C for 1 min. Following PCR the products were stored at -20°C . These conditions have been found previously to be optimal and to permit even single-cell detection.⁽⁷⁾

Agarose Gel Analysis of PCR Products

Ten microliters each of the 650-, 168-,

and 108-bp amplicon PCR products was mixed, 5 μ l of gel-loading dye was added, and the samples were electrophoresed on a 3% agarose gel in Tris-acetate EDTA buffer. The gel was then stained with ethidium bromide (EtBr) and photographed under a UV light transilluminator. The signal intensity of replicate samples was determined using a commercially available densitometer program (Biomed Instruments, Inc., Ful-

erton, CA). The signal intensity was standardized against the intensity before chlorine disinfection.

Hybridization Analysis of PCR Products

For the 108- and 168-bp amplicons, the EnviroAmp *Legionella* kit detection system was used. The 650-bp amplification products were detected using a 50-mer

FIGURE 2 Results of chlorine disinfection and hybridization analysis of PCR products. (◆) *L. pneumophila* percent survival based on viable plate count determination; (Δ) percent of initial PCR signal from the 108-bp amplicon; (□) percent of initial PCR signal from the 168-bp amplicon; (○) percent of initial PCR signal from the 650-bp amplicon. (A,B,C) Agar-grown cells chlorinated with 10, 5, and 2.5 mg/l, respectively; (D) water-grown cells chlorinated at 5 mg/l. Percent survival of 10^{-6} indicates no viable cells. Controls with no chlorine treatment still showed strong PCR signals (no loss of intensity) at 24 hr for the 108-, 168-, and the 650-bp amplicons.

single-stranded oligonucleotide probe (5'-TTTGGGGAAGA ATTTAAAAATCAAG-GCATAGATGTTAATCCGAAGCAA-3') labeled with digoxigenin and detected using a chemiluminescent detection system according to the manufacturer's instructions (Genius System, Boehringer Mannheim, Indianapolis, IN). The signal intensity was determined using the same commercially available software program used for the agarose gels, and all signals were standardized against the initial signal obtained before chlorination.

RESULTS AND DISCUSSION

After chlorine disinfection of *L. pneumophila*, the PCR signals on agarose gels and blots using the 108- and 168-bp amplicons were significantly more persistent than the signals seen when the 650-bp amplicon was used (Figs. 1 and 2). At higher chlorine dosages (10 and 5 mg/l) using agar-grown cells, no PCR signals were detected after 24 hr of chlorine contact time. At lower chlorine dosages or using water-grown cells, PCR signals were obtained after 24 hr of contact time with the 168-bp amplicon only. Agar-grown cultures of *L. pneumophila* were inactivated in <8, 2, and 1 min, with chlorine dosages of 2.5, 5, and 10 mg/l, respectively (Figs. 1 and 2). Water-grown cells of the same strain were significantly more resistant to chlorine disinfection, and complete inactivation was obtained in under 8 min using a chlorine dose of 5 mg/l. Increased chlorine resistance in water-grown cultures has been reported previously.⁽⁸⁾ Survival of viable nonculturable cells of *L. pneumophila* has been reported for water stored for 35 days without chlorination.⁽⁹⁾ No actively respiring cells (INT positive) were detected using FAB–INT counts in any sample after the addition of chlorine. As seen in Figures 1 and 2, the decline in the signal obtained using the larger amplicon correlated better with chlorine inactivation than the corresponding decrease seen in the smaller amplicon signal. Loss of detectable PCR amplification signal has also been reported for an 800-bp amplicon of *Legionella*.⁽⁹⁾

The differences seen in the results obtained using agarose gel and hybridization analysis of PCR products is related to the quantitative reliability of each procedure; hybridization is several orders of magnitude more sensitive than agarose gel visualization. Using agarose

TABLE 1 The Predicted Number of Chromosomal Lesions per 10⁶ bp Required to Give Rise to the Corresponding Decrease in PCR Product Intensity Based on the Poisson Distribution, Assuming One Lesion in an Amplicon Inhibits Amplification

Decrease in signal intensity (%)	Amplicon size (bp)			
	168	650	1000 ^a	2000 ^a
10	625 ^b	161	105	53
20	1,327	343	223	112
40	5,452	1,409	916	458
80	9,577	2,475	1,609	805
90	13,707	3,542	2,303	1,152
99	27,409	7,082	4,605	2,303
99.9	41,116	10,695	6,908	3,454
99.99	54,818	14,165	9,210	4,605
99.999	68,448	17,687	11,500	5,750

^aNot analyzed in this study; presented for comparison purposes only.

^bCalculated using the following formula:

$$\text{Number of lesions}/10^6 \text{ bp} = -[\ln(1 - x)]/(10^6/n)$$

where x is the proportion of intact amplicons (i.e., 10% decrease in signal intensity equals 90% intact amplicons), and n is bp/amplicon.

gel analysis of PCR products we were only able to detect changes in product intensity when there was a 30% difference in starting template number, whereas using hybridization analysis we could detect 10% changes in starting amplicon copy number (data not shown).

In most PCR procedures for environmental monitoring small amplicons are used (<500 bp) to optimize sensitivity.^(1,2,10–12) Data presented here indicate that larger amplicons (>500 bp) are better correlated with viability after chlorine disinfection and may provide more relevant information. Statistical analysis supports this hypothesis; the number of chromosomal lesions necessary to produce a detectable reduction in signal intensity is inversely proportional to the amplicon length (Table 1); a single lesion should block PCR amplification regardless of amplicon size.

When monitoring microorganisms ubiquitous in aquatic environments, like *Legionella* species, the use of a two-tiered PCR approach is warranted, with one tier of tests being used to detect the presence or absence of specific microorganisms and the second tier being used to determine whether these microorganisms are viable. A test using a smaller amplicon could be used for screening samples, and positive samples could be evaluated using a larger amplicon. Ideally, nested amplicons should be used in these assays, because studies with carcinogens have shown that DNA damage occurs in a nonrandom manner.⁽¹³⁾ Those sam-

ples in which large amplicons are found could trigger remedial actions, such as periodic flushing or chlorination. At the very least, larger amplicons could be used to determine when plate counts should be performed.

In summary, we found that the 650-bp amplicon of the *L. pneumophila* *mip* gene failed to amplify more rapidly after chlorine disinfection than a 168-bp amplicon within the 650-bp amplicon or a distinct 108-bp amplicon. The loss of signal from the 650-bp amplicon appeared to correlate with contact time and chlorine dosage. This behavior is consistent with expectations for a viability assay. The loss of the signal from the 650-bp amplicon was independent of the method of cultivation for the test strain before disinfection. This would suggest that an amplicon sequence in environmental bacteria would be lost in a similar fashion after chlorine disinfection.

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