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LEGIONELLA PNEUMOPHILA OCCURRENCE IN WATERS OF EAST CENTRAL INDIANA

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ABSTRACT. Drinking water is a potential means of transmission for the opportunistic bacterial pathogen *Legionella pneumophila*. This study evaluated the presence of *Legionella pneumophila* in source water supplies, granular activated carbon (GAC) filtered water, and the network of a public drinking water system in east central Indiana using a pilot three-tiered approach. Water samples were enriched for *Legionella* spp. by cultivation on selective media, followed by direct fluorescent antibody (DFA) staining for *L. pneumophila* serogroups 1–14, and duplex real-time polymerase chain reaction (PCR). PCR targets included a 16S rDNA segment and a macrophage infectivity potentiator (*mip*) gene fragment of *L. pneumophila*. Sensitivity of culture methods and PCR was determined by percent recovery and by using serial dilutions of *L. pneumophila* DNA, respectively. Sensitivity of the real-time PCR assay was 230 genome equivalents (GE) of *L. pneumophila* per liter of GAC filtered or distribution waters, and 230 GE per 100 ml source water. All source water samples were positive for *L. pneumophila* by DFA *versus* 80% by real-time PCR. Forty percent of GAC water samples were positive by DFA *versus* 20% by real-time PCR. Potable water samples yielded the lowest percentage with 12.5% positive for both DFA and real-time PCR.

Keywords: *Legionella pneumophila*, biofilm, fluorescent microscopy, real-time PCR

Legionella pneumophila is Gram-negative opportunistic pathogen, ubiquitous in freshwater systems, capable of causing severe pneumonia, and is the most reported cause of clinical legionellosis cases (Fields 2002; Reischl et al. 2002; Templeton et al. 2003; Fields 2007). In potable water systems, *L. pneumophila* survives in the microbial communities of biofilms (Atlas 1999; Molofsky & Swanson 2004; Sheehan et al. 2005; Lau & Ashbolt 2009) with human infection occurring through inhalation of contaminated water particles (Reischl et al. 2002; Templeton et al. 2003), including hot water systems (Hruba 2009). While in water systems, *L. pneumophila* may persist within biofilms because of increased opposition to biocidal and chlorination treatments (Costerton et al. 1995; Stickler 1999; Harb et al. 2000). The replication of *L. pneumophila* within amoeba is well documented, but proliferation of *L. pneumophila* may occur in mixed bacterial communities in water as well, outside of a host cell environment (Kuiper et al. 2004). Indeed, the amount of bacteria present in distribution system biofilms may be near 10^7 cells cm^2 (Olson & Nagy 1984; LeChevallier et al. 1987). However, many of the bacteria present in biofilms have been uncharacterized, and their contribution to *L. pneumophila* survival is not fully understood.

Like many bacterial pathogens, detection and recovery of *Legionella* spp. from water may involve culture-based methods and/or molecular approaches, depending on the laboratory resources available (Tronel & Hartemann 2009). Traditional methods of *L. pneumophila* detection utilize culture-based assays that require lengthy incubation times and may lead to false negatives or require cellular resuscitation due to bacteria entering a viable-but-nonculturable (VBNC) state (Atlas 1999; Reischl et al. 2002; Ohno et al. 2003; Oezcakir

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2007). Other means of detection include direct fluorescent antibody methods (DFA) and nucleic acid detection methods such as polymerase chain reaction (PCR) that target ribosomal RNA (rRNA) genes and/or the *mip* gene (Behets et al. 2007; Dusserre et al. 2008; Nazarian et al. 2008; Morio et al. 2008), which encodes the macrophage infectivity potentiator, a virulence factor mediating intracellular infection of human macrophages as well as free-living protozoa (Cianciotto & Fields 1992). The DNA intercalating dye SYBR Green can be used in real-time PCR to differentiate multiple PCR products with melting temperatures (T_m) differing less than 2°C (Ririe et al. 1997; Velasek & Repa 2005). The objectives of this study were to assess the prevalence and distribution of *L. pneumophila* in public drinking water sources in and around the Delaware County, Indiana area using selective culture methods, fluorescence microscopy, and species-specific real-time PCR methods.

The White River in Muncie (Delaware County), Indiana is source water for the surrounding area. This river basin has a drainage area of 625 km^2 with the majority of the surrounding land used for agriculture. The average river flow is 4.92×10^5 m^3/day. The Indiana-American Water Company treatment plant is a conventional treatment facility that involves pre-treatment with powdered activated carbon for micro-pollutant removal and chlorine for oxidation and disinfection. The settling step utilizes ferric chloride and a cationic polymer. The filtration step consists of 61 cm of granular activated carbon (GAC) followed by 15–20 cm of sand. Post-treatment includes the addition of chlorine and ammonia to form chloramines, fluoride, and orthophosphate for corrosion inhibition prior to distribution. The treatment plant produces 42,000 m^3 of potable water per day. The distribution system covers an area approximately 70 km^2 with roughly 43% of the distribution network consisting of pipes 15 cm in diameter or smaller.

METHODS

Legionella pneumophila subspecies *pneumophila* serogroup 1 was obtained from American Type Culture Collection (ATCC 33152; Manassas, Virginia USA) and plated on buffered charcoal yeast extract (BCYE) agar plates containing polymyxin B, anisomycin, and vancomycin (Hardy Diagnostics; Santa Maria, California USA). BCYE agar plates were incubated in an extinguished candle jar at 37 °C for 72 h. Colonies of *L. pneumophila* ATCC 33152 were transferred to sterile saline to reach a 0.5 McFarland turbidity standard. Serial dilutions were performed to a concentration of 1.5×10^2 cells/100 ml then filtered with a 0.45 µm membrane filter (Millipore, Billerica, Massachusetts USA) and placed on BCYE agar. After 72 h, percent recovery was determined by dividing the number of colonies in quadruplicate platings by total number of *L. pneumophila* cells in the sample. A 95% confidence interval (CI) was used to determine recovery efficiency. Other experiments analyzed replicate values using a one-tailed *t*-test and standard deviation (SD).

Source and GAC water samples, collected during a span from August 2003 to March 2005, were aseptically collected in sterile 1 L Nalgene containers (Nalge Nunc International Corp., Rochester, New York USA) and processed immediately. Source and GAC water samples were collected on site at the Indiana-American Water treatment plant in Muncie, Indiana. Distribution water samples were collected from the end of the distribution system and stored at 4 °C until enrichment was completed the same day. Distribution water samples were poor in quality containing residual disinfectants. Total Chlorine was measured using the DPD colorimetric method (Eaton et al. 1998). A study by Leoni & Legnani (2001) found that combining membrane filtration with a heat treatment at 50 °C for 30 min yielded higher incidence of *Legionella* spp. than acid treatment or no treatment. The authors used a method which consisted of membrane filtration (0.2 µm) with 1 L of potable water followed by aseptically cutting the membrane filter and vigorously shaking the filter homogenate for 15 min in 10 ml of phosphate buffer solution (PBS; pH 7.2) and plated on BCYE. This study uses the same method with the following modifications. One hundred ml of source water, 1 L of GAC filter water, and 1 L of distribution water samples were heated at 50 °C for 30 min prior to membrane filtration, and distribution water containers included 0.1% sodium thiosulfate to neutralize residual chlorine (Walker et al. 2000). In the present study, 0.45 µm membrane filters were used instead of 0.2 µm filters, which differs from the Leoni and Legnani method.

The sample was filtered through 0.45 μm membrane filters, the filter was placed on BCYE agar, and incubated in a candle jar at 37 °C for 72 h.

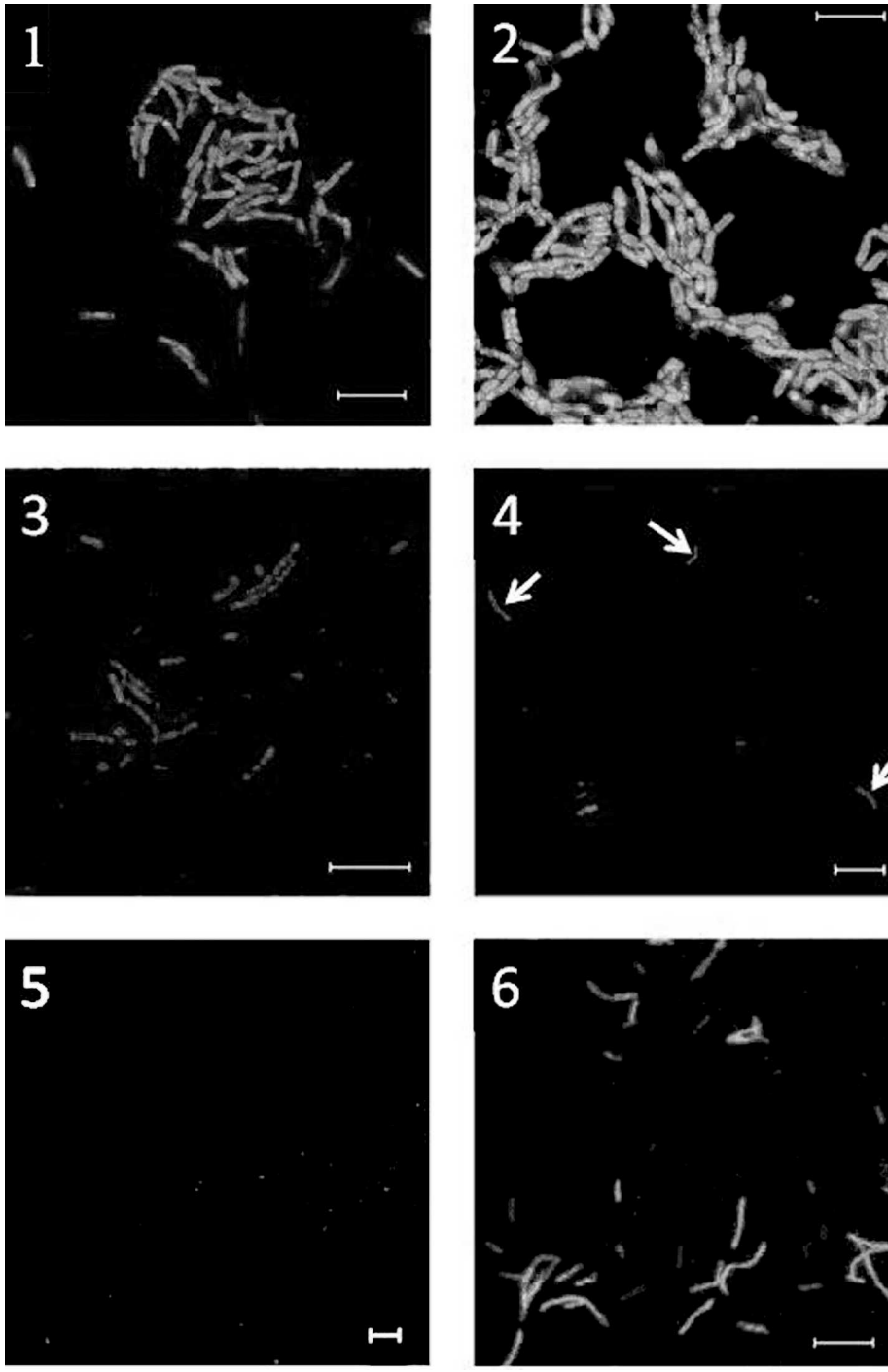
Monoclonal fluorescein isothiocyanate (FITC) labeled antibodies for *L. pneumophila* serogroups 1–14 (m-Tech Monoclonal Technologies; Alpharetta, Georgia USA) were used in DFA assay according to manufacturer's specifications. For staining, the membrane filter was cut and placed in 0.85% NaCl, vortexed, and shaken at 250 rcf for 15 min. Then, samples were vortexed again, and 100 μl was placed on a microscope slide for DFA. Images were obtained by confocal microscopy (Laser Scanning Confocal Microscope 5 PASCAL, Carl Zeiss International; Minneapolis, Minnesota USA). Two water meters were removed from the distribution after approximately 10 years of use and were swabbed for biofilm sampling. Following swabbing, the cotton tip was removed from the wooden shaft of the swab and placed in a 1.5 ml Eppendorf tube containing 1.0 ml of sterile 0.85% NaCl. This cotton tip contained the biofilm sample and was vortexed at maximum speed for 1 min. Then, the sample was shaken at 250 rcf for 15 min. After 15 min, the sample was vortexed at maximum speed for 10 sec and the cotton tip was removed. The remaining volume was placed in 100 ml of 0.85% NaCl and processed by membrane filtration and treated as mentioned above by DFA.

DNA was extracted from samples, following 72 h incubation on BCYE plates, using InstaGene Matrix (BioRad; Hercules, California USA). The membrane filter was cut and placed in 0.85% NaCl, vortexed, and shaken at 250 rcf for 15 min. Then, samples were vortexed again and 1.5 ml was removed for DNA extraction. DNA extraction followed manufacturer's specifications. DNA concentration was determined by absorbance at 260 nm.

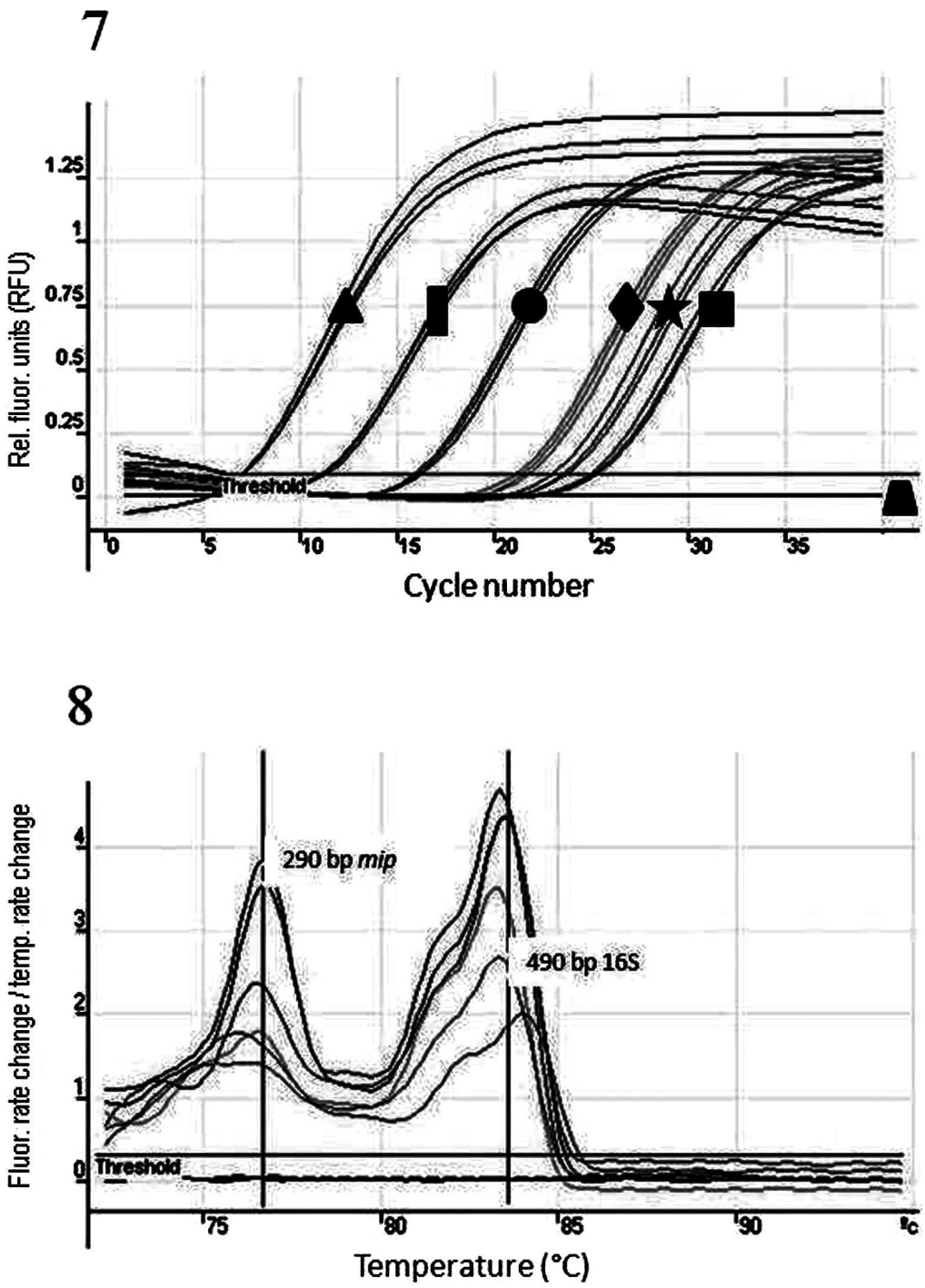
Oligonucleotide primers (Integrated DNA Technologies; Coralville, Iowa, USA) were designed using *L. pneumophila* subspecies *pneumophila* serogroup 1 genome (accession number AE017354). LegF1 (5′-CCT ACC AAG GCG ACG ATC GGT AGC T-3′) and LegR2 (5′-GTG TCA GTA TTA GGC CAG GTA GCC G-3′) were designed to amplify a 490 base-pair (bp) product within the 16S rDNA gene. MipF1 (5′-GAC GCT ATG AGT GGC GCT CA-3′) and MipR2 (5′-ACG GTA CCA TCA ATC AGA CGA CCA G-3′) were designed to amplify a 290 bp product within the macrophage infectivity potentiator (*mip*) gene. Duplex amplicon sizes were of sufficient relative length to be discerned by melting curve analyses. Real-time PCR consisted of iTaq SYBR Green Supermix with ROX (BioRad; Hercules, California USA). Reaction volumes were 25 μl containing 12.5 μl Supermix, 3.0 mM $MgCl_2$, 0.04 μM 16S primers, 0.08 μM *mip* primers, 2% bovine serum albumin (BSA, Sigma-Aldrich; St. Louis, Missouri USA), and 2% dimethyl sulfoxide (DMSO, Sigma-Aldrich). Real-time PCR reactions were done in triplicate with 100 ng of total genomic DNA (gDNA) from environmental samples assayed. Thermocycling parameters were as followed: 95 °C for 3 min followed by 40 cycles of 95 °C for 20 sec, 60 °C for 75 sec (decreasing 1 °C for first six cycles until annealing temp of 55 °C was reached), and 72 °C for 75 sec with acquisition of data following extension step using Rotor-Gene 3000 (Corbett Research; Mortlake, NSW, Australia). A touchdown approach was implemented for real-time multiplex PCR to decrease nonspecific amplification in environmental samples, which may contain large amounts of nontarget DNA. Melting curve analysis consisted of 45 sec at 72 °C followed by increments of 0.5 °C to 95 °C with a hold of 5 sec at each increment. Serial dilutions of *L. pneumophila* gDNA from 100 ng to 1 pg amounts were used to determine reaction sensitivity. Assay specificity was tested by titrating purified *L. pneumophila* gDNA into 10-fold excess of nontarget *Escherichia coli* K12 gDNA as template and subjecting to the same PCR reaction conditions described above.

RESULTS

Recovery efficiency was 70.2% ± 8.3 (95% CI, range 62–79.3%, $n = 4$) for spiked *Legionella* water samples, corresponding to an approximate recovery sensitivity of 105 CFU/100ml ± 12 CFU. Figures 1–6 show representative data from DFA detection using confocal microscopy. All source water samples ($n = 5$) were positive by DFA detection *vs.* 40% of GAC filter water samples ($n = 5$) and 12.5% of potable water samples ($n = 8$). The temperature, total Cl_2, and pH for this sample were 6.11 °C, 0.71 mg/l, and 7.8 with the mean values for all potable water samples being 6.81 °C ± 1.18,



Figures 1–6.—DFA detection of *Legionella pneumophila* in water samples. 1. Source water; 2. Biofilm from water meter; 3. GAC filter water sample 1; 4. Potable water sample 1; 5. Negative control *Pseudomonas aeruginosa*; 6. *Legionella pneumophila* ATCC 33512. Scale bar (10μm) added with LCM software while arrows and lettering inserted using Adobe Photoshop CS2.



Figures 7, 8.—Sensitivity of real-time PCR to detect *Legionella pneumophila* in water samples. 7. Fluorescence curves from diluted template DNA. Normalized fluorescence throughout the PCR cycle is shown with exact *Ct* values listed in Table 2. Data from triplicate dilutions are shown. 8. Melting peaks of the 290 bp *mip* and the 490 bp 16S segments are shown. Y-axis values = rate of change in fluorescence/rate of change in temperature (°C) as a function of the rate of change in temperature. For both figures, each dilution was completed in triplicate with one representative sample shown. Melting temperatures were 76.4 °C ± 0.4 and 83.7 °C ± 0.4 for *mip* and 16S rDNA, respectively (±1 SD). Dilutions were done with 100 ng (▲), 10 ng (▮), 1 ng (●), 100 pg (◆), 10 pg (★), and 1 pg (■) of *L. pneumophila* DNA with no DNA (▲) as a contamination control.

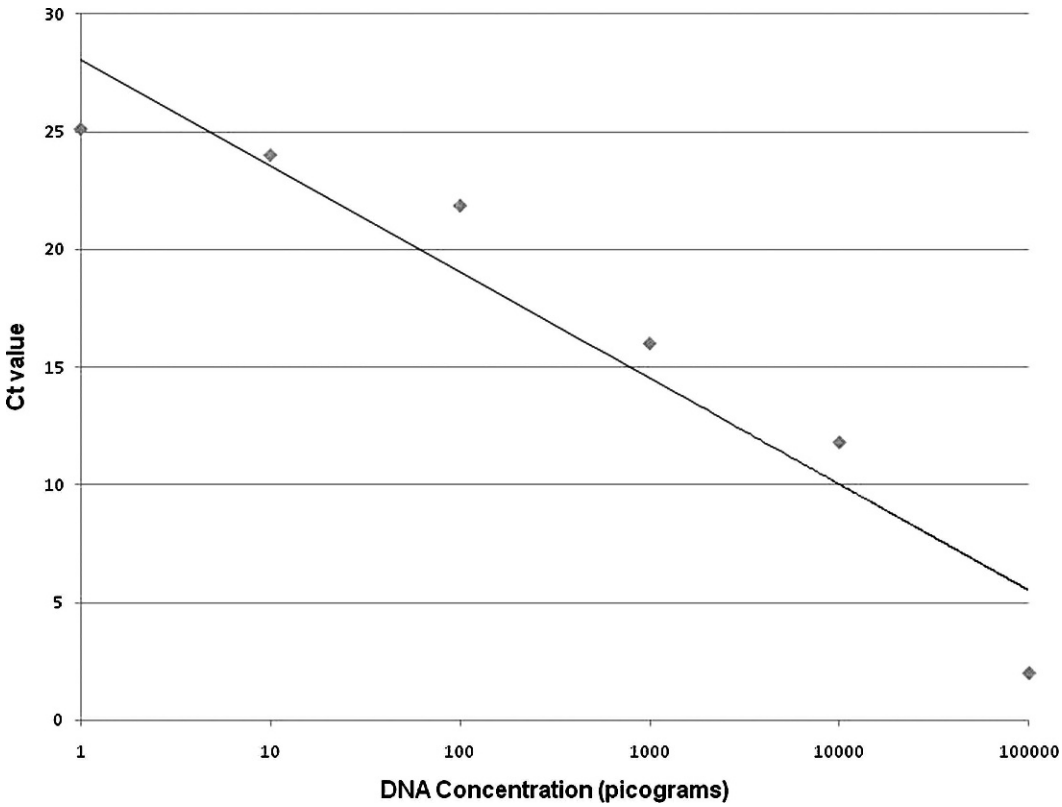


Figure 9.—Standard curve for real-time PCR of *L. pneumophila* template plotting cycle threshold Ct (or CT) values as a function of DNA concentration ($r^2 = 0.964$).

0.87 mg/l ± 0.38, and 7.69 ± 0.11, respectively. Biofilm samples from two residential water meters were tested by DFA with both samples being positive.

Eighty percent of source water samples tested positive by real-time PCR *vs.* 20% of GAC filter water samples and 12.5% of potable water samples. T_m values were 76.2 °C ± 0.23 and 83.88 °C ± 0.40 for *mip* and 16S rDNA products in environmental samples (±1 standard deviation, SD). Table 1 shows comparative T_m data from real-time PCR for positive environmental samples of both targets.

Serial dilutions of *L. pneumophila* were used to determine the sensitivity of the duplex real-time PCR assay (Figs. 7–9). *Legionella pneumophila* was detected in this study down to levels of 1pg gDNA. The *L. pneumophila* genome consists of 3.4×10^6 bp and is approximately 4.3 fg (Cloud et al. 2000). Therefore, 1 pg of gDNA corresponds to approximately 230 cells. The T_m of *mip* and 16S rDNA products were 76.4 °C ± 0.4 and 83.7 °C ± 0.4, respectively (±1 SD). Cycle threshold (*Ct*) values from this experiment are listed in Table 2. Amplification of *L. pneumophila* gDNA (starting template concentration of

Table 1.—Melting temperatures (T_m) of real-time PCR products from environmental samples. Data shown as mean of duplicate reactions, ±1 SD where shown.

Water sample	T_m (°C)*	Cycle threshold (*Ct*)
Source water ($n = 5$)		
1	76.3 and 83.6	17.45 ± 0.06
2	76.5 and 83.3	21.26 ± 1.22
3	76.3 and 84.3	23.87 ± 0.30
4	76.0 and 84.4	26.03 ± 1.02
GAC filter water ($n = 5$)		
1	76.2 and 83.9	26.76 ± 0.12
Potable water ($n = 8$)		
1	76.0 and 83.9	28.67 ± 0.39

Table 2.—Cycle Threshold (*Ct*) Values of Real-time PCR-based detection of *Legionella pneumophila* in water. Data are a mean of triplicate dilutions with ±1 SD.

Amount of *L. pneumophila* gDNA	Cycle threshold (*Ct*)
100 ng	7.19 ± 0.07
10 ng	11.37 ± 0.13
1 ng	16.22 ± 0.19
100 pg	21.60 ± 0.31
10 pg	23.57 ± 0.39
1 pg	25.27 ± 0.21

100ng) was not adversely affected in reactions containing 10-fold excess of nontarget *E. coli* gDNA (data not shown).

DISCUSSION

Legionella pneumophila is considered a facultatively intracellular pathogen, surviving outside of a human host in environments such as water sources, perhaps within biofilms or by forming an association with amoeba. The typical route of infection in humans is through the respiratory tract. If aerosolized in contaminated water and inhaled, pathogenic *L. pneumophila* may become internalized but survive within macrophages, and have been known to stimulate the complement cascade. As an opportunistic pathogen, immunocompromised individuals are at the highest risk of acquiring an infection, termed Legionellosis, or Legionnaires' disease. Symptoms may include fever, chills, nonproductive cough, muscle aches, and in some cases, reduced kidney and liver function. Clinical diagnosis relies on culturing the bacteria from sputum, and/or the presence of *Legionella* antigens in urine or blood (Cianciotto & Fields 1992). However, these approaches are time-intensive and tend to have a high frequency of false negative results due to the difficulty in culturing *L. pneumophila* and/or the utility of the antibody-based tests in only detecting a single serotype of the species.

In order to reduce the possibility of inaccurate results, and to reduce the time necessary for confirming *L. pneumophila* in water or clinical specimens, molecular detection assays are needed. Ideally, these approaches, when applied to detect pathogenic bacteria, need to be specific for multiple targets simultaneously—a multiplex strategy. This is because when applied to natural samples, wild type bacterial strains harboring some but perhaps not all of the key determinants would still be detectable. In the assay described herein, specific virulence gene sequences are ideal targets because they allow for the differentiation of pathogenic *versus* nonpathogenic *L. pneumophila* strains.

A search of the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://kegg.com/) and the The Institute for Genomic Research (TIGR, http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi) revealed that *L. pneumophila* subspecies *pneumophila* contains three copies of 16S rRNA genes and one copy of *mip*. The former essentially allows for the presumptive determination of genus, while the latter confirms the presence of a pathogenic species. Therefore, our assay has four nucleoid targets for real-time PCR in a multiplex reaction detecting *L. pneumophila* gDNA. This study implemented a Basic Local Alignment Search Tool (BLAST). BLAST is essentially an algorithm to compare primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search of the bacterial database (http://www.ncbi.nlm.nih.gov/BLAST/) with the LegF1 primer resulted in many non-*Legionella* spp. 'hits,' or possible matches (all had a total score of 50.1 with E values of 1×10^{-5}). Most hits were 16S sequences from uncultured bacteria recently deposited in the database. Additionally, a BLAST search of the bacterial database with the LegR2 primer resulted in many *Legionella* spp. hits with the top three scores belonging to *L. pneumophila* Paris strain, *L. pneumophila* Lens strain, and *L. pneumophila* Philadelphia strain (total score of 150 each with E values of 1×10^{-5}).

A BLAST search of the bacterial database with the MipF1 primer resulted in nearly exclusive *Legionella* spp. hits (all had a total score of 40.1 with E values of 7×10^{-3}) and a BLAST search with the MipR2 primer was similar with hits nearly exclusive to the genus *Legionella* (all had a total score of 50.1 with E values of 1×10^{-5}). Collectively, these findings allow us to validate the precision of the primers used to detect *Legionella* spp. without 'false positive' signal from unrelated bacteria.

Additionally, the specificity of 16S and *mip* primers to recognize *L. pneumophila* gDNA in the presence of 10-fold excess *E. coli* gDNA reveals that primers are functionally able to

recognize targets in a reaction containing dominant amounts of non-specific DNA, further speaking to the promise of this assay for a low occurrence of false-positive results. However, it is possible that one or both primer sets may, in practice, recognize other *Legionella* spp. genomic targets, a possibility to be explored in more extensive work in the future using a broad range of species and strains within this genus.

This duplex assay detected *L. pneumophila* to levels on the order of 230 cells. The sensitivity of this SYBR Green real-time PCR is comparable to a previous study (Rantakokokko-Jalava & Jalava 2001) detecting *L. pneumophila* that reported a sensitivity of 200 CFU/ml with their uniplex SYBR Green real-time PCR. Another study reported a sensitivity limit of 1 pg of *L. pneumophila* gDNA in the first stage amplification of a semi-nested PCR assay (Miyamoto et al. 1997). In our study, detection sensitivity below 1 pg of *L. pneumophila* gDNA was not achieved because of the increase in nonspecific amplification when targets were in low amounts (data not shown) (Ririe et al. 1997).

These data show that *L. pneumophila* are contaminating potable water in this distribution system. Indeed, data from DFA and real-time PCR generally correlate and exhibited a decreasing prevalence of *L. pneumophila* throughout the water treatment procedure. However, DFA and real-time PCR were not always identical. For instance, 100% of source water samples were positive by DFA compared to 80% by real-time PCR. Also, 40% of GAC water samples tested positive by DFA compared to 20% by real-time PCR. These variations are most likely due to PCR inhibitors present in water samples. Many water sources are known to contain PCR inhibitors which may become concentrated on the filters, subsequently transfer to the plated media, and carry over to the final DNA extraction. Such inhibitors, seen in many environmental samples, adversely affect PCR reaction efficiency (Leoni & Lagnani 2001; Levi et al. 2003). For instance, Miyamoto et al. (1997) discovered that 30.6% of cooling tower water samples contained PCR inhibitors, which may consist of divalent cations, minerals, or other debris that may antagonize the polymerase and decrease amplification efficiency (Wilson 1997). Another possible reason for the discrepancy between the DFA and real-time PCR results could be compromised DNA template quality, preventing detectable fluorescent signal from amplicons.

Morio et al. (2008) used real-time PCR for detection of *L. pneumophila* in six distinct water distribution systems and reported detection limits on the order of 100 genomic equivalents per liter with a different detection chemistry than reported in our study. Additionally, Morio et al. (2008) used a fluorescence resonance energy transfer (FRET)-based labeled probe system that allows for confirmation of amplicon identity during amplification rather than using SYBR Green-based melting curve analyses. This group reported that a portion of the samples exhibiting growth from *Legionella* on selective media did not give positive results by PCR and showed that this was likely due to inhibitors. Serial dilution of the extracted DNA is one means of circumventing carryover PCR inhibitors from natural sources as long as template quantity remains at adequate levels for amplification (McKillip et al. 2002), but certainly this depends on the specific type of antagonists present and the quality of template. In our study, dilution of *L. pneumophila* gDNA down beyond 1pg resulted in nonspecific amplification of false products, not an atypical outcome. This observation, along with the clean, successful amplification of *L. pneumophila* gDNA at higher template levels (even in the presence of excess *E. coli* gDNA) leads us to conclude that inhibition effects likely explain the real-time PCR not being in complete correspondence with DFA results.

Nazarian et al. (2008) also reported a slight disparity between culture recovery and PCR-based detection of *L. pneumophila* in both environmental and clinical specimens, and concluded that either approach alone is insufficient for accurate quantification of this bacterial pathogen in natural samples. Our study implemented a selective medium-based recovery of *Legionella* spp. and validation by species-specific duplex PCR (the *mip* primers which would confirm species as well as genus) as well as the use of immunofluorescence/confocal microscopic observation of bacteria. The latter two approaches allow for the detection of sublethally-injured, dead, or VBNC *L. pneumophila* that would not be enumerated on traditional selective media (Dusserre et al. 2008; Oezcakir 2007), leading to an underestimation of the true density of pathogens in the sample of interest, and false

negative assay determinations. Collectively, these data indicate that a combination of traditional culture-based methods and species-specific molecular approaches (such as PCR) provide a more accurate means of ascertaining presence of *L. pneumophila* in water samples.

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LITERATURE CITED

American Public Health Association (APHA). 1998. Standard methods for the examination of water and wastewater L.S. Clesceri, A.E. Greenberg & A.D. Eaton, (eds.), 20th ed. Washington, DC.

Atlas, R.M. 1999. *Legionella*: From environmental habitats to disease pathology, detection and control. Environmental Microbiology 1:283–293.

Behets, J., P. Declerck, Y. Delaedt, B. Creemers & F. Ollevier. 2007. Development and evaluation of a Taqman duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples. Journal of Microbiological Methods 68:137–144.

Cianciotto, N.P. & B.S. Fields. 1992. *Legionella pneumophila mip* potentiates intracellular infection of protozoa and human macrophages. Proceedings of the National Academy of Sciences USA 89:5188–5191.

Cloud, J.L., K.C. Carroll, P. Pixton, M. Erali & D.R. Hillyard. 2000. Detection of *Legionella* species in respiratory specimens using PCR with sequencing confirmation. Journal of Clinical Microbiology 38:1709–1712.

Costerton, J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber & H.M. Lapin-Scott. 1995. Microbial biofilms. Annual Reviews in Microbiology 49:711–745.

Dusserre, E., C. Genevre, S. Hallier-Soulier, F. Vandenesch, G. Festoc, J. Etienne, S. Jarraud & M. Molmeret. 2008. A PCR-based method for monitoring *Legionella pneumophila* in water samples detects viable but noncultivable Legionellae that can recover their cultivability. Applied and Environmental Microbiology 74:4817–4824.

Fields, B.S. 2007. *Legionella* in the environment. Pp. 85–94. *In* P. Hoffman, H. Friedman & M. Bendenelli (eds.). *Legionella pneumophila*: Pathogenesis and immunity. Springer, New York.

Fields, B.S., R.F. Benson & R.E. Besser. 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. Clinical Microbiology Reviews 15:506–526.

Harb, O.S., L. Gao & Y.A. Kwaik. 2000. From protozoa to mammalian cells: A new paradigm in the life cycle of intracellular bacterial pathogens. Environmental Microbiology 2:251–265.

Hruba, L. 2009. The colonization of hot water systems by *Legionella*. Annals of Agricultural and Environmental Medicine 16:115–119.

Kuiper, M.W., B.A. Wullings, A.D.L. Akkermans, R.R. Beumer & D. van der Kooij. 2004. Intracellular proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in aquatic biofilms grown on plasticized polyvinyl chloride. Applied and Environmental Microbiology 70:6826–6833.

Lau, H.Y. & N.J. Ashbolt. 2009. The role of biofilms and protozoa in *Legionella* pathogenesis: Implications for drinking water. Journal of Applied Microbiology 107:368–378.

LeChevallier, M.W., T.M. Babcock & R.G. Lee. 1987. Examination and characterization of distribution system biofilms. Applied and Environmental Microbiology 53:2714–2724.

Levi, K., J. Smedley & K.J. Towner. 2003. Evaluation of a real-time PCR hybridization assay for rapid detection of *Legionella pneumophila* in hospital and environmental water samples. Clinical Microbiology and Infection 9:754–758.

Leoni, E. & P.P. Legnani. 2001. Comparison of selective procedures for isolation and enumeration of *Legionella* species from hot water systems. Journal of Applied Microbiology 90:27–33.

McKillip, J.L., L. Jaykus & M.A. Drake. 2002. Influence of growth in a food medium on the detection of *Escherichia coli* O157:H7 by polymerase chain reaction. Journal of Food Protection 65:1775–1779.

Miyamoto, H., H. Yamomoto, K. Arima, J. Fujii, K. Maruta, K. Izu, T. Shiomori & S. Yoshida. 1997. Development of a new seminested PCR method for detection of *Legionella* species and its application to surveillance of legionellae in hospital cooling tower water. Applied and Environmental Microbiology 63:2489–2494.

Molofsky, A.B. & M.S. Swanson. 2004. Differentiate to thrive: Lessons from the *Legionella pneumophila* life cycle. Molecular Microbiology 53:29–40.

Morio, F., S. Corvec, N. Caroff, F. Le Gallou, H. Drugeon & A. Reynaud. 2008. Real-time PCR assay for the detection and quantification of *Legionella pneumophila* in environmental water samples: Utility for daily practice. International Journal of Hygiene and Environmental Health 211:403–411.

Nanzarian, E.J., D.J. Bopp, A. Saylors, R.J. Limberger & K.A. Musser. 2008. Design and implementation of a protocol for the detection of

Legionella in clinical and environmental samples. Diagnostic Microbiology and Infectious Disease 62:125–132.

Oezcakir, O. 2007. Viable but non-culturable form of bacteria. Mikrobiyoloji Bülteni 41:477–484.

Ohno, A., N. Kato, K. Yamada & K. Yamaguchi. 2003. Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water. Applied and Environmental Microbiology 69:2540–2547.

Olson, B.H. & L.A. Nagy. 1984. Microbiology of potable water. Advances in Applied Microbiology 30:73–132.

Rantakokko-Jalava, K. & J. Jalava. 2001. Development of conventional and real-time PCR assays for detection of legionella DNA in respiratory specimens. Journal of Clinical Microbiology 39:2904–2910.

Reischl, U., H. Linde, N. Lehn, O. Landt, K. Barratt & N. Wellinghausen. 2002. Direct detection and differentiation of *Legionella* spp. and *Legionella pneumophila* in clinical specimens by dual-color real-time PCR and melting curve analysis. Journal of Clinical Microbiology 40:3814–3817.

Ririe, K.M., R.P. Rasmussen & C.T. Wittwer. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical Biochemistry 245:154–160.

Sheehan, K.B., J.M. Henson & M.J. Ferris. 2005. *Legionella* species diversity in an acidic biofilm community in Yellowstone National Park. Applied and Environmental Microbiology 71:507–511.

Stickler, D. 1999. Biofilms. Current Opinions in Microbiology 2:270–275.

Templeton, K.E., S.A. Scheltinga, P. Sillekens, J.W. Crielaard, A.P. van Dam, H. Goosens & E.C.J. Claas. 2003. Development and clinical evaluation of an internally controlled, single-tube multiplex real-time PCR assay for detection of *Legionella pneumophila* and other *Legionella* species. Journal of Clinical Microbiology 41:4016–4021.

Tronel, H. & P. Hartemann. 2009. Overview of diagnostic and detection methods for legionellosis and *Legionella pneumophila*. Letters in Applied Microbiology 48:653–656.

Velasek, M.A. & J.J. Repa. 2005. The power of real-time PCR. Advances in Physiology Education 29:151–159.

Walker, J.T., D.J. Bradshaw, A.M. Bennett, M.R. Fulford, M.V. Martin & P.D. Marsh. 2000. Microbial biofilm formation and contamination of dental-unit water systems in general dental practice. Applied and Environmental Microbiology 66:3363–3367.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63:3741–3751.

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