

## Detection of *Legionella pneumophila* by Real-Time PCR for the *mip* Gene

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**A real-time PCR assay for the *mip* gene of *Legionella pneumophila* was tested with 27 isolates of *L. pneumophila*, 20 isolates of 14 other *Legionella* species, and 103 non-*Legionella* bacteria. Eight culture-positive and 40 culture-negative clinical specimens were tested. This assay was 100% sensitive and 100% specific for *L. pneumophila*.**

*Legionella pneumophila* is the most common pathogenic species of the 42 recognized *Legionella* species (3, 4, 29). Significant mortality rates among the elderly and patients with severe underlying disease may occur as a result of infection with this pathogen (5). Diagnostic delay may also result in increased mortality (15). Therefore, rapid tests, such as direct fluorescent-antibody stains and urinary antigen assays, have been developed (10, 18). Although useful, these assays have sensitivities less than 100% (9, 10, 11, 14). Nucleic acid amplification assays have been shown to be useful for the detection of *Legionella* (1, 8, 14, 17, 19, 20, 24, 27, 28). The genes that encode the 5S and 16S ribosomal subunits have been shown to contain signature sequences that are useful for the identification of *L. pneumophila* (8, 14, 15, 17, 24, 28) and a variety of other organisms. More recently, target sequences on these genes have been used in conjunction with real-time PCR for the detection of the *Legionella* genus, as well as the species *L. pneumophila* (14, 27).

The macrophage infectivity potentiator gene, which encodes a 24-kDa protein virulence factor that facilitates the entry of legionellae into amoebae and macrophages, has sufficient sequence variability between the *Legionella* species to also afford the specific detection of *L. pneumophila* by PCR (2, 6, 7, 12, 13, 14, 16, 21, 22, 23, 25). Although two groups have described real-time PCR assays for the detection of *L. pneumophila* via detection of the *mip* gene in water samples, to date only one group has evaluated a real-time PCR for this genetic target for the detection of *L. pneumophila* in clinical specimens (1, 14, 28). Therefore, we have attempted to confirm the utility of this gene as a target for the detection of *L. pneumophila* by real-time PCR, using a set of primers and hybridization probes that were different from those previously described (14).

We describe a sensitive and specific hybridization probe-based real-time PCR assay for the detection of *L. pneumophila* through the detection of the *mip* gene (GenBank accession number AF095230). This assay was used in conjunction with

the LightCycler System (Roche Molecular Biochemicals, Indianapolis, Ind.) with fluorescent resonance energy transfer technology. Primers and fluorescently labeled hybridization probes were designed by Brian Caplin, formerly of Idaho Technologies, Salt Lake City, Utah. The sequences of the primers were as follows: forward primer (LpmipFp), 5'-GCAATGTCAACAGCAA 3'; reverse primer (LpmipRp), 5'-CATAGCGTCTTGCATG 3'. The 3' end of the first hybridization probe (LpmipHP-1) was labeled with fluorescein (*fam*); the sequence of this probe was 5'-CAACTTATCCTTGTCTGTAGCT-[*fam*]-3'. The 5' end of the second hybridization probe (LpmipHP-2) was labeled with 640-*N*-hydroxysuccinimide ester (Red 640), and the 3' end of the probe was phosphorylated (*p*) to prevent probe extension during PCR; the sequence of this probe was 5'-[Red 640]-TGATGTGGCATCGGTTG-*p*-3'. The amplicon was 159 nucleotides in length. A BLAST search of the GenBank database demonstrates a high predicted specificity, with the only cross-reacting bacteria being *Legionella worsleiensis* (GenBank accession number LWU60164) and *Legionella fairfieldensis* (GenBank accession number LFU60163).

The LightCycler FastStart DNA Master Hybridization Probe Kit (Roche) was used with a final volume of 20  $\mu$ l, which consisted of 18  $\mu$ l of master mix and 2  $\mu$ l of nucleic acid extract. The master mix was prepared with the following final concentrations per capillary tube: 0.5  $\mu$ M (both) primers, 0.2  $\mu$ M LpmipHP-1, 0.4  $\mu$ M LpmipHP-2, and 3.0 mM MgCl<sub>2</sub>. The experimental LightCycler protocol consisted of 10 min at 95°C for *Taq* polymerase activation, 45 cycles of PCR amplification (95°C for 0 s, 54°C for 10 s, and 72°C for 6 s), melting (40 to 95°C at 0.1°C/s), and a cooling step (40°C for 2 min). To determine the analytical sensitivity of this assay, bacterial DNA for testing was prepared by using the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's instructions. The DNA concentration was determined for pooled DNA extracts from the same isolate of *L. pneumophila* by measuring the optical density at 260 nm with the GeneQuant spectrophotometer (Pharmacia, Piscataway, N.J.). The DNA concentration was 2.1 ( $\pm$  0.2) mg/ml. Serial dilutions of the extract were tested, and the described assay was able to detect 10 fg of DNA, which is equivalent to approximately 10 organisms.

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TABLE 1. Isolates of *Legionella* species tested

Source	Organism(s)	No. of isolates tested	LightCycler results
University of Regensburg	<i>L. pneumophila</i> serogroups 1–14 (one isolate of each serogroup)	14	All positive
	<i>L. bozemaniae</i> and <i>L. longbeachae</i>	2	Both negative
	<i>L. micdadei</i> , <i>L. gormanii</i> , <i>L. dumoffii</i> , <i>L. jordanis</i> , <i>L. hackeliae</i> , <i>L. oakridgensis</i> (ATCC 33761), <i>L. israelensis</i> (ATCC 43119), <i>L. wadsworthii</i> (ATCC 33877), <i>L. tucsonensis</i> (ATCC 49180), <i>L. feeleii</i> (ATCC 35849), <i>L. sainthelensi</i> (ATCC 35248), and <i>L. birminghamensis</i> (ATCC 43702)	1 each	All negative
Cleveland Clinic Foundation	<i>L. pneumophila</i> (five of serogroup 1, one of serogroup 6, and seven that were not serotyped)	13	All positive
	<i>L. bozemaniae</i>	1	Negative
	<i>L. jordanis</i>	1	Negative
	<i>L. micdadei</i>	2	Both negative

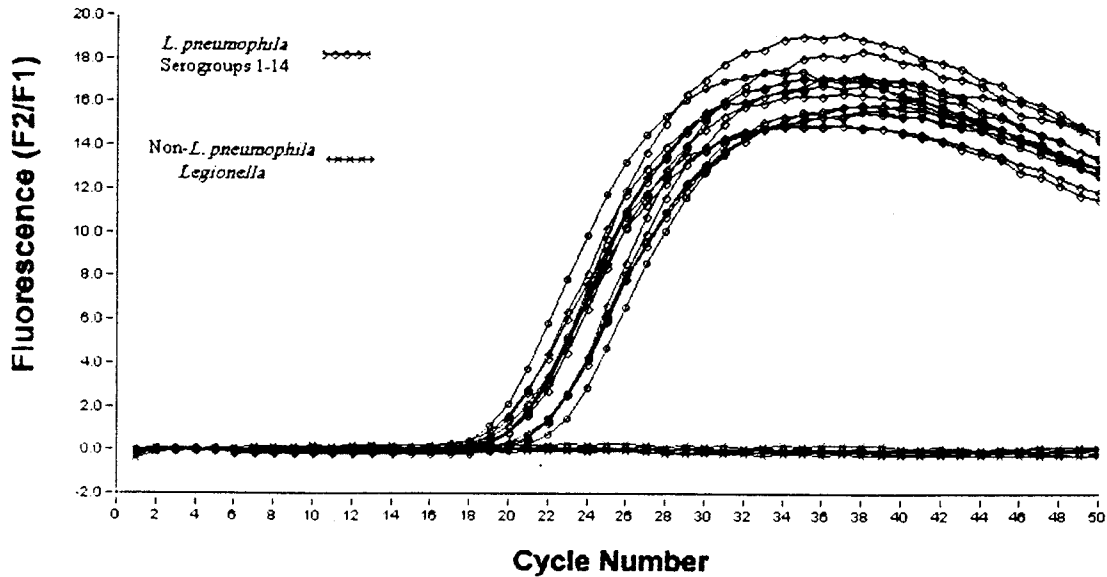
Bacterial isolate DNA extracts used for assay validation were obtained from the Institute of Medical Microbiology and Hygiene, University of Regensburg, Regensburg, Germany, and the Cleveland Clinic Foundation, Cleveland, Ohio. The Institute of Medical Microbiology and Hygiene provided DNA extracts from 30 of the *Legionella* isolates, which included one representative isolate of each of the *L. pneumophila* serogroups 1 to 14 and 16 isolates of 14 *Legionella* species other than *L. pneumophila* (Table 1). These DNA extracts were prepared from cultured organisms by using the MagNA Pure LC DNA Isolation Kit I (Roche) according to the manufacturer's instructions. The Cleveland Clinic Foundation provided DNA extracts from 17 *Legionella* isolates (13 *L. pneumophila* isolates and 4 isolates of *Legionella* species other than *L. pneumophila*) and 103 non-*Legionella* bacteria representing 76 species (Tables 1 and 2). The DNA from the *Legionella* isolates was obtained by using the QIAamp DNA Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. The nucleic acid from the non-*Legionella* bacteria was obtained from cultured organisms by boiling a loopful of organisms in a simple lysis buffer, which has been previously described (26). This assay was positive for all of the *L. pneumophila* isolates and negative for all of the *Legionella* species other than *L. pneumophila* and for the non-*Legionella* bacteria tested (Fig. 1). The sensitivity and specificity of this

assay for confirmation of cultured isolates tested were 100 and 100%, respectively. We consider that the true specificity of the assay may be slightly less than 100%, since *L. worsleiensis* and *L. fairfieldensis* would be expected to produce a positive result with this assay based on the results of the BLAST search. These organisms, however, were not available for testing.

Clinical specimens were obtained from the clinical microbiology laboratory at the Cleveland Clinic Foundation. Eight clinical specimens were identified that were culture positive for *Legionella* (7 *L. pneumophila* isolates and 1 *Legionella bozemaniae* isolate). These were stored at  $-20^{\circ}\text{C}$ . Twenty bronchoalveolar lavage (BAL) and 20 sputum specimens that were culture negative for *L. pneumophila* were also tested. All the clinical specimens were tested with the MONOFLUO *Legionella pneumophila* IFA (immunofluorescent-antibody) Test Kit (direct fluorescent-antibody assay [DFA]; Bio-Rad Laboratories, Redmond, Wash.), which was performed according to the manufacturer's guidelines, and cultured for *Legionella* by using buffered charcoal-yeast extract agar (BCYE agar; BD Diagnostic Systems, Sparks, Md.) with 10 days of incubation. DNA extracts from the clinical specimens were prepared by using the Qiagen DNA Mini Kit (Qiagen) according to the manufacturer's guidelines. Extracts were suspended in a final volume of 100  $\mu\text{l}$ . The seven specimens that were culture positive for *L. pneumophila* were PCR positive with the described assay, while

TABLE 2. Isolates of non-*Legionella* bacteria tested

Type	Organism(s)	No. of isolates tested	LightCycler results
Gram negative	<i>Salmonella</i> spp., <i>Shigella</i> spp., <i>Yersinia</i> sp., <i>Citrobacter</i> sp., <i>Escherichia coli</i> , <i>Proteus</i> spp., <i>Klebsiella</i> spp., <i>Serratia</i> sp., <i>Pseudomonas</i> sp., <i>Bacteroides</i> sp., <i>Moraxella</i> sp., <i>Haemophilus</i> sp., <i>Acinetobacter</i> sp., and <i>Enterobacter</i> spp.	3 each	All negative
	<i>Providencia</i> sp., <i>Bartonella</i> spp., and <i>Burkholderia cepacia</i>	2 each	All negative
	<i>Neisseria</i> spp.	9	All negative
	<i>Vibrio cholerae</i> , <i>Eikenella corrodens</i> , <i>Afipia felis</i> , <i>Campylobacter jejuni</i> , <i>Rhizobium</i> sp., and <i>Mesorhizobium</i> sp.	1 each	All negative
Gram positive	<i>Staphylococcus aureus</i> (including ATCC 25923)	5	All negative
	<i>Staphylococcus epidermidis</i>	3	All negative
	<i>Staphylococcus saprophyticus</i> , <i>Micrococcus</i> sp., <i>Stomatococcus</i> sp., and <i>Lactobacillus</i> sp.	2 each	All negative
	<i>Enterococcus</i> sp., viridans group streptococcus, <i>Streptococcus pneumoniae</i> , group A <i>Streptococcus</i> , group B <i>Streptococcus</i> , <i>Aerococcus</i> sp., <i>Bacillus</i> spp., and <i>Corynebacterium</i> spp.	3 each	All negative

FIG. 1. Real-time PCR results for *Legionella* isolates.

the specimen that contained *L. bozemanii* and the 40 culture-negative BAL and sputum specimens were negative (Fig. 2 and Table 3). Only five of the seven specimens that were culture and PCR positive for *L. pneumophila* were positive by DFA for *L. pneumophila*. In this limited study of clinical specimens, the results of real-time PCR were equivalent to those of culture (seven of seven positive) for the detection of *L. pneumophila* and better than those of the DFA (five of seven positive).

PCR has been shown to be a useful diagnostic tool for the clinical microbiologist for infections caused by a variety of human pathogens, including *L. pneumophila*. Real-time PCR is often favored over traditional PCR, since the results are avail-

able in a closed system, which diminishes the likelihood of laboratory contamination, and is more timely since detection and amplification occur simultaneously. Two real-time PCR assays have been described for the detection of the genus *Legionella* and the species *L. pneumophila*. One of these assays detects the *Legionella* genus and differentiates *L. pneumophila* by using two sets of hybridization probes that target signature sequences in the 16S ribosomal subunit gene (27). The other assay detects the *Legionella* genus by using a PCR with the 5S ribosomal rRNA gene and detects *L. pneumophila* by targeting the *mip* gene (14). We have confirmed the utility of the *mip* gene as a target for the detection of *L. pneumophila* by real-

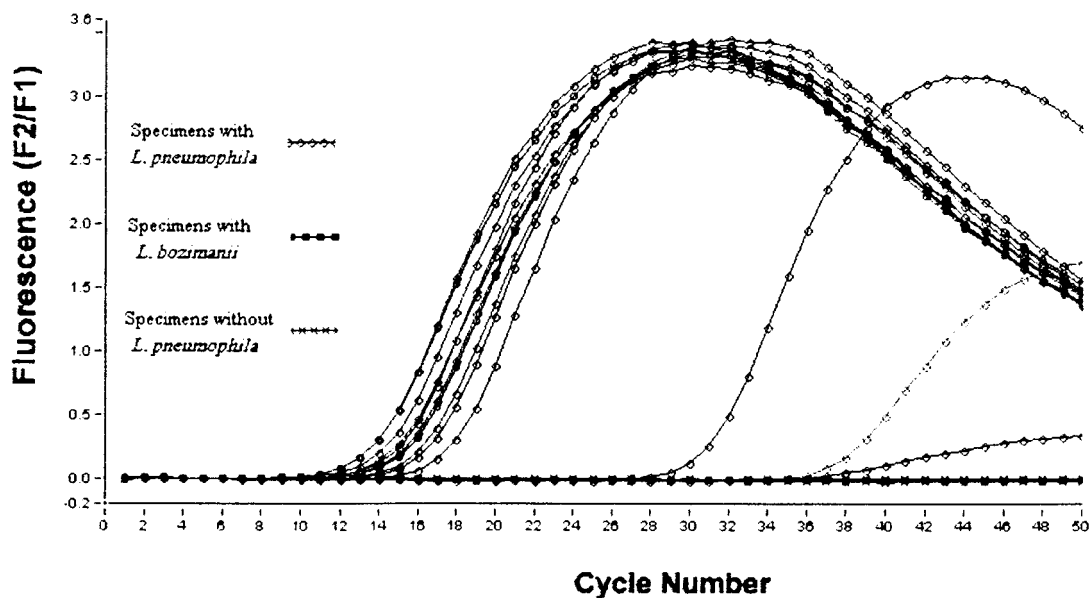
FIG. 2. Real-time PCR results for *L. pneumophila* in clinical specimens.

TABLE 3. Clinical specimens tested for *L. pneumophila* by real-time PCR detection of the *mip* gene

Patient no.	Specimen source <sup>a</sup>	Culture results <sup>b</sup>	DFA results <sup>c</sup>	LightCycler results
1	BAL fluid	<i>L. pneumophila</i> serogroup 1	+ (TP)	+ (TP)
2	BAL fluid	<i>L. pneumophila</i> serogroup 1	+ (TP)	+ (TP)
3	ET tube	<i>L. pneumophila</i> serogroup 1	+ (TP)	+ (TP)
4	LUL tissue	<i>L. pneumophila</i> serogroup 1	- (FN)	+ (TP)
5	BAL fluid	<i>L. pneumophila</i> (not serotyped)	+ (TP)	+ (TP)
6	BAL fluid	<i>L. pneumophila</i> serogroup 6	+ (TP)	+ (TP)
7	Sputum	<i>L. pneumophila</i> serogroup 1 <sup>d</sup>	- (FN)	+ (TP)
8	BAL fluid	<i>L. bozemanii</i> serogroup 1	- (TN)	- (TN)
9-28	Sputum	Negative	- (TN)	- (TN)
29-48	BAL fluid	Negative	- (TN)	- (TN)

<sup>a</sup> ET tube, endotracheal; LUL, left upper lobe of the lung.

<sup>b</sup> Results as reported by the Ohio State Department of Health and/or the Centers for Disease Control.

<sup>c</sup> TP, true positive; TN, true negative; FN, false negative.

<sup>d</sup> Subsequent specimens from the same patient not included in the analysis were negative by DFA and culture but remained positive by PCR.

time PCR by using an assay different than that previously reported. This assay could potentially also be used as a follow-up to a pan-*Legionella* assay to help determine whether the positive result was due to *L. pneumophila*, as previously described (14). Alternatively, we plan to use this assay as a replacement for DFA and as a confirmatory test for cultured isolates. We have opted to retain the BCYE culture medium, since occasionally we have recovered unexpected pathogens, such as *Nocardia* species and *Blastomyces dermatitidis*, from this medium. The retention of the BCYE medium will also serve to recover the non-*L. pneumophila* isolates, which overall are much less frequently encountered than the *L. pneumophila* isolates (3). In addition, retention of this medium affords the recovery of the *L. pneumophila* isolates for submission to the public health laboratories and for epidemiologic purposes.

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