

Identification and Differentiation of *Legionella pneumophila* and *Legionella* spp. with Real-Time PCR Targeting the 16S rRNA Gene and Species Identification by *mip* Sequencing

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Fluorescent resonance energy transfer probes targeting the 16S rRNA gene were constructed for a sensitive and specific real-time PCR for identification and differentiation of *Legionella pneumophila* from other *Legionella* spp. For identification of non-*L. pneumophila* spp. by direct amplicon sequencing, two conventional PCR assays targeting the *mip* gene were established.

There are currently 50 species (<http://www.dsmz.de/bactnom/bactname.htm>) comprising about 70 distinct serogroups in the genus *Legionella*. *Legionella pneumophila* serogroup 1 accounts for the majority of infections of humans, but association with human disease has been reported for >20 of the species in the genus *Legionella* (4). It is, however, likely that most legionellae can cause human disease under appropriate conditions due to their capability for cellular invasion and intracellular growth (3, 4).

Diagnostic delay may result in increased mortality for patients with legionellosis (6). Culture is considered the “gold standard” for detection of legionellae, but due to the slow-growing and fastidious nature of legionellae, other strategies to ensure a rapid diagnosis of legionellosis have become imperative. Several PCR assays targeting *Legionella* sp. and *L. pneumophila* genes have been reported, including assays targeting the 16S rRNA gene (2, 5, 8, 9, 11, 12), the 5S rRNA gene (5), the 23S–5S spacer region (7), and the macrophage infectivity potentiator gene *mip* (1, 5, 10, 13). The aim of this study was to develop molecular tools enabling (i) rapid detection of *Legionella* spp. in clinical and environmental specimens, (ii) fast differentiation between *L. pneumophila* and other *Legionella* spp., and (iii) suitable amplicons for species identification by DNA sequencing.

The following *Legionella* reference strains were used: *L. birminghamensis* serogroup 1 (CCUG 31233 A), *L. bozemanai* serogroup 2 (CCUG 16416), *L. cincinnatiensis* serogroup 1 (CCUG 31230 A), *L. dumoffii* (CCUG 47789), *L. hackeliae* serogroup 1 (CCUG 31232 A), *L. lansingensis* serogroup 1 (CCUG 31227), *L. longbeachae* serogroup 1 (CCUG 28612), *L. longbeachae* serogroup 2 (CCUG 46623), *L. micdadei* serogroup 1 (CCUG 31229 A), three strains of *L. pneumophila* serogroup 1 (CCUG 33058, CCUG 13395, and CCUG 9568), *L. pneumophila* serogroup 2 (CCUG 13396), *L. pneumophila* serogroup 6 (CCUG 13400), *L. pneumophila* serogroup 14 (CCUG 44898), *L. sainthelensi* (CCUG 29672), *L. tucsonensis*

(CCUG 31119), and *L. wadsworthii* (CCUG 16415). Legionellae were grown at 37°C on buffered charcoal-yeast extract (BCYE) agar for 48 to 72 h in humidified air. One colony was emulsified in 0.2 ml sterile water and heated for 15 min at 95°C. Bacterial DNA was purified using a DNeasy tissue kit (QIAGEN, Hilden, Germany).

A real-time PCR assay with hybridization probes targeting the bacterial multicopy 16S rRNA gene of *Legionella* spp. was established, using a primer pair described previously (8). By aligning published *Legionella* 16S rRNA gene sequences, the probes for fluorescent resonance energy transfer (FRET) technology were constructed to be 100% homologous to *L. pneumophila* and to have various numbers of mismatches to other *Legionella* spp. (Table 1). The PCR mixture consisted of 2 µl 10× LightCycler FastStart DNA Master Hybridization Probes mix (Roche Diagnostics, Basel, Switzerland), 3 mM MgCl₂, 0.5 µM each primer, 0.2 µM each hybridization probe, 0.2 U of uracil-*N*-glycosylase (MedProbe, Oslo, Norway), and 2 µl of template DNA in a final volume of 20 µl. The PCR was monitored on a LightCycler device (Roche Diagnostics), starting with an initial denaturation step for 10 min at 95°C to activate the *Taq* DNA polymerase and proceeding with 50 cycles of amplification (5 s at 95°C, 15 s at 58°C, and 15 s at 72°C), followed by a melting curve analysis (40°C to 85°C with a heating rate of 0.1°C/s). A second PCR assay aiming at identification of *Legionella* species by DNA sequencing targeted the *mip* gene of *Legionella* spp. Based on alignment of *mip* sequences in the GenBank database, two primer sets were selected (Table 1). The PCR mixture consisted of 200 ng of each primer, 50 µM each deoxynucleoside triphosphate, 1× PCR buffer (Applied Biosystems, Foster City, CA), 1.5 mM MgCl₂ (Applied Biosystems), 0.25 U of AmpliTaqGold DNA polymerase (Applied Biosystems), 2 µl template DNA, and sterile water to a final volume of 50 µl. Amplifying conditions were as follows: an initial denaturation step for 15 min at 94°C to activate the *Taq* polymerase; 35 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min; and finally a prolonged extension step for 7 min at 72°C. The amplicons were visualized by a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) or gel electrophoresis. The amplicons were purified with a QIAquick PCR purification kit (QIAGEN) and sequenced

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TABLE 1. Oligonucleotide primers and hybridization probes used in this study

Oligonucleotide	Sequence (5'-3') ^a	Gene	GenBank accession no. of reference sequence	Position	Reference
Leg primer 1	AGGGTTGATAGGTTAAGAGC	16S rRNA	M59157	451-470	8
Leg primer 2	CCAACAGCTAGTTGACATCG	16S rRNA	M59157	836-817	8
Leg probe 1	GAGTCAACCAGTATTATCTGACCGTCCC-[FL]	16S rRNA	M59157	653-626	This study
Leg probe 2	[Red 640]GGTTAAGCCCAGGAATTCACAGA TAACTTAATCA-[Ph]	16S rRNA	M59157	624-590	This study
<i>mip</i> FI	GGTCGCTGCAGCTGYCATRR	<i>mip</i>	S62141	700-719	This study
<i>mip</i> RI	GCATTAATTGYARWGCTTCAGT	<i>mip</i>	S62141	1280-1259	This study
<i>mip</i> FII	GGGGATTSTTTATGAAGATGA	<i>mip</i>	U91607 [S62141]	467-487 [675-695] ^c	This study
<i>mip</i> RII	ACCAGCAGGCATTAATTGTAA	<i>mip</i>	U91607 [S62141]	1050-1030 [1288-1268] ^{b,c}	This study

^a [FL], fluorescein; [Red 640], LightCycler-Red 640-*N*-hydroxy-succinimide ester; [Ph], 3'-phosphate.

^b Four mismatches in this region compared to the *mip* RII primer.

^c For primer set FII/RII, the corresponding positions in S62141 are given in brackets.

on a CEQ 8800 genetic analysis system (Beckman Coulter, Fullerton, CA), using a CEQ DTCS Quick Start kit (Beckman Coulter), 10 to 50 fmol purified PCR product, and 3.2 pmol primer. Primers used in the sequencing reaction were identical with the PCR primers. Sequence analysis was performed by using the Sequencher program (Gene Codes Corporation, Ann Arbor, MI). For all analyses, data obtained with the forward and reverse primers were combined and aligned manually. The consensus sequence was compared with sequences in the GenBank database for identification using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) or with sequences in the *Legionella mip* gene sequence database provided by the European Working Group for Legionella Infections (<http://www.ewgli.org/>).

The real-time PCR detected all 6 *L. pneumophila* strains and 11 of the 12 non-*L. pneumophila* reference strains. The sensitivity was estimated to be 1.4 fg per PCR of *L. pneumophila* DNA template, corresponding to less than 1 genome equivalent (data not shown). To investigate the specificity of the 16S real-time PCR, DNAs extracted from a number of commonly encountered microorganisms were analyzed. The species investigated included *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Mycobacterium tuberculosis*, *Escherichia coli*, *Propionibacterium acnes*, *Bordetella pertussis*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Listeria monocytogenes*, and *Moraxella catarrhalis*. Neither during amplification nor during the

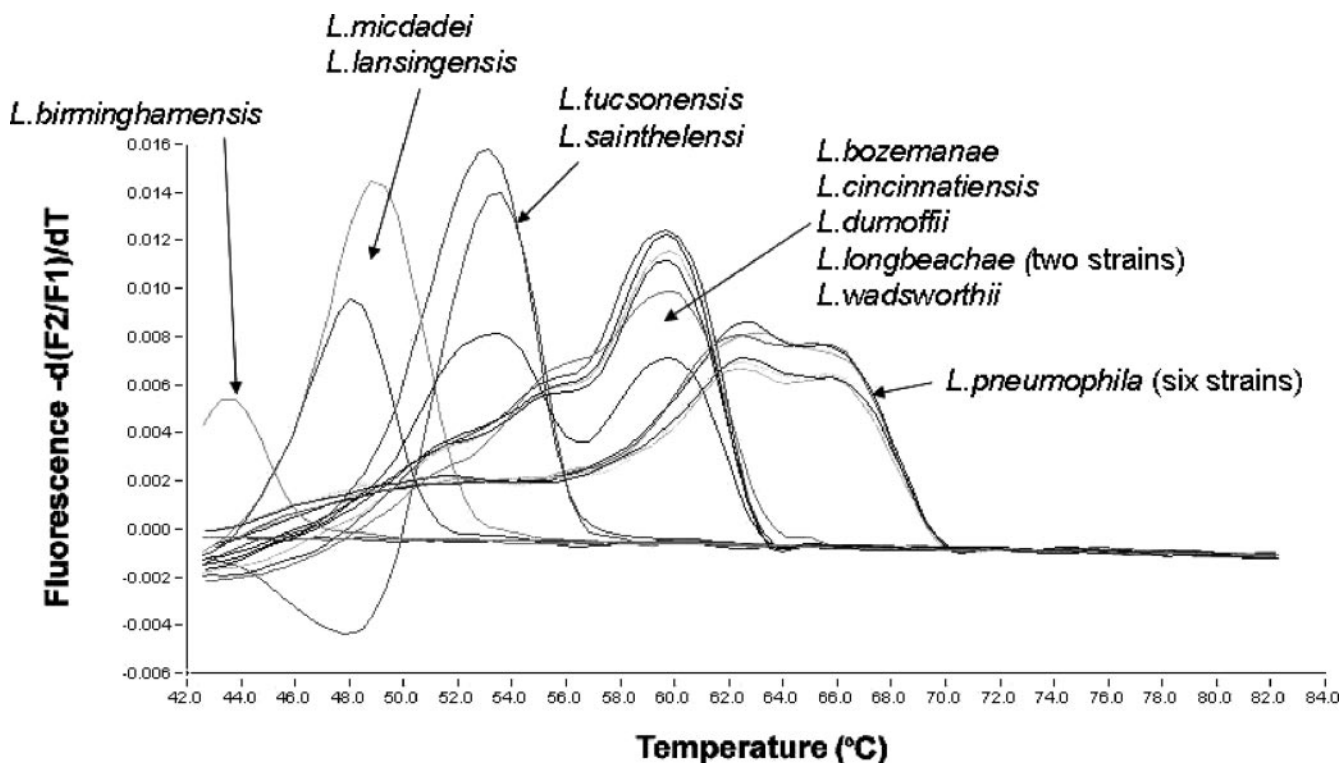


FIG. 1. Melting curve analysis with *L. pneumophila*-specific hybridization probes.

TABLE 2. Polymorphism in the probe region among the various *Legionella* reference strains employed in this study in comparison with results of melting curve analysis

Species	CCUG accession no.	Nucleotide sequence in the probe region ^a	Melting temp (°C)	No. of nt mismatches in the probe region
<i>L. pneumophila</i>	33058	TGATTAAGTTATCTGTGAATTCCTGGGCTTACC TGGGACGGTCAGATAATAC TGGTTGACTC	66	Perfect match
<i>L. pneumophila</i>	44898	*****	66	Perfect match
<i>L. pneumophila</i>	13400	*****	66	Perfect match
<i>L. pneumophila</i>	9568	*****	66	Perfect match
<i>L. pneumophila</i>	13396	*****	66	Perfect match
<i>L. pneumophila</i>	13395	*****	66	Perfect match
<i>L. bozemaniae</i>	16416	*****C*****CA*****G*****	60	4
<i>L. wadsworthii</i>	16415	*****C**C*****CA*****	60	4
<i>L. cinchonensis</i>	31230A	*****C*****CA*****G*****	60	4
<i>L. dumoffi</i>	47789	*****C*****CA*****G*****	60	4
<i>L. longbeachae</i>	46623	*****C*****CA*****G*****	60	4
<i>L. tucsonensis</i>	28612	*****C*****CA*****G*****	60	4
<i>L. sainthelensii</i>	31119	*****A*****CA*****G*****	53	6
<i>L. mitcladii</i>	29672	*A*****CA*****G*****A*****	53	6
<i>L. 31229A</i>	31229A	*TTTA*****C*****A**GT*****TGA*****T	12	
<i>L. 31227</i>	31227	*T**A*****G*****C*****G*****C**G*****T**A*****T	49	
<i>L. 31233A</i>	31233A	*T**A*****A*****C*****T*****G*****T**A*****A	44	
<i>L. hackeliae</i>	31232A	*T**A*****GT*****ATTG**CC**G*****T**A*****G	No data	15

^a Asterisks indicate identity with *L. pneumophila* CCUG 33058.

melting curve analysis did these specimens produce any FRET signal (data not shown).

By melting curve analysis, *L. pneumophila* could easily be differentiated from the other *Legionella* species (Fig. 1). A hunchbacked melting curve was observed for all *L. pneumophila* strains and also for *L. cinchonensis*. The explanation for this phenomenon probably is related to the probe design, i.e., the melting temperatures (T_m) for the hybridization probes are approximately equal, and thus neither will work like an anchor probe. Nevertheless, it is highly feasible to distinguish *L. pneumophila* from the other legionellae by its melting curve. For the strains mentioned, the appearance of the melting curve will vary somewhat from batch to batch of the probe. For T_m determination, emphasis is placed on the peak with the higher T_m of the two. As expected, the deviation of the melting point observed was approximately proportional to the number of mismatches in the probe region compared to *L. pneumophila* (Table 2). The minimum and maximum observed differences in T_m between *L. pneumophila* and the non-*L. pneumophila* strains were 6°C and 22°C, corresponding to 4 and 11 polymorphic sites in the probe region, respectively. One of the reference strains that gave no FRET signal during either amplification or melting curve analysis (i.e., *L. hackeliae*) was amplified by the primers when analyzed by gel electrophoresis. Here the number of mismatches in the probe region was 15. By comparing the various 16S rRNA genes of non-*L. pneumophila* species in the GenBank database with the hybridization probe region, approximately 70% have fewer than 12 polymorphic sites in the probe region and are thus likely to be detected by the 16S real-time assay described here. In addition, none of these strains have fewer than 4 polymorphic sites in the probe region (i.e., the number of polymorphic sites is sufficient to differentiate them from *L. pneumophila* by melting curve analysis). The high melting temperatures of the hybridization probes allow multiple polymorphic sites in the probe region. The melting temperature for the probes when hybridized to *L. pneumophila* was shown to be 66°C. The *L. pneumophila*-specific hybridization probes presented by Reischl et al. (11) are shorter and thus have a lower melting temperature (61°C). Our strategy, by using somewhat longer hybridization probes, allows for multiple mismatches along the entire probe sequence and thus enables the simultaneous detection and differentiation of *L. pneumophila* and a number of non-*L. pneumophila* spp. Since the hybridization probe assay will not detect every *Legionella* species, it is possible on negative samples to do a post-PCR analysis of the 386-bp PCR product generated by the primers. For that purpose we are using a capillary electrophoresis system on a disposable chip (Bioanalyzer; Agilent Technologies) using 1 µl from the LightCycler capillary tube, allowing results within 30 min. For the past 3 years, this real-time PCR assay has been successfully implemented in our routine laboratory with several positive findings in cases of legionellosis.

To identify non-*L. pneumophila* species of legionellae, direct sequencing of the amplicon produced by the 16S rRNA gene primers can be performed (2). We found similarities in the 16S rRNA gene amplicon among the 11 non-*L. pneumophila* species ranging from 93.3 to 99.7% (data not shown). Based on comparison with sequences in the GenBank database, only *L. dumoffi*, *L. birninghamensis*, *L. longbeachae*, *L. bozemaniae*, *L.*

micdadei, and *L. wadsworthii* gave the expected result for the BLAST search (i.e., for 5 of the 12 non-*L. pneumophila* reference strains, the best hit in the GenBank database was different from the reference species).

The *mip* gene has been reported to discriminate better among *Legionella* species, and identification through data comparison is available over the Internet (<http://www.ewgli.org/>). Ratcliff et al. (10) have previously described *mip* primers for this purpose, but these primers were to a very large extent degenerated. Based on alignment of *mip* sequences available in the GenBank database, we constructed two PCRs for detection and sequencing of non-*L. pneumophila* spp. Only 8 of 45 non-*L. pneumophila* *mip* sequences are not included in the assumed potential for identification by using these two primer sets. For these eight species, the *mip* sequences are not available for either or both of the primer binding sites, and thus the full potential for the modified primers cannot be assessed. Our main reason for constructing new primers is to avoid using too many degenerated base sites when the same primer sets are used for both PCR and sequencing. The conventional PCR assays targeting the *mip* gene detected all of the 12 non-*L. pneumophila* reference strains. By direct sequencing (Fig. 2), all of the non-*L. pneumophila* reference strains could be identified with high accuracy based on the GenBank data and the *Legionella mip* gene sequence database (<http://www.ewgli.org/>). These observations indicate that the *mip* gene sequence discriminates more reliably between *Legionella* species than does the 386-bp 16S rRNA gene sequence.

In conclusion, we have established a sensitive and specific real-time PCR assay capable of identifying *L. pneumophila* and simultaneously differentiating *L. pneumophila* from other, non-*L. pneumophila* species by melting curve analysis. Additionally, we present a procedure for identification of non-*L. pneumophila* spp. based on *mip* sequencing.

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