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/bactnomenclature.html) 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), and 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 *Legionella* spp. with Real-Time PCR Targeting the 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 MgCl\(_2\), 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 MgCl\(_2\) (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.
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

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

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′→3′)</th>
<th>Gene</th>
<th>GenBank accession no. of reference sequence</th>
<th>Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leg primer 1</td>
<td>AGGGTTGATAGGTTAAGAGC</td>
<td>16S rRNA</td>
<td>M59157</td>
<td>451–470</td>
<td>8</td>
</tr>
<tr>
<td>Leg primer 2</td>
<td>CCAACAGCTATGGACATCG</td>
<td>16S rRNA</td>
<td>M59157</td>
<td>836–817</td>
<td>8</td>
</tr>
<tr>
<td>Leg probe 1</td>
<td>GAGTCAACCTATATGACGTCCACCC[FL]</td>
<td>TAACTTAATCTAGCATCA</td>
<td>653–626</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Leg probe 2</td>
<td>[Red 640]GGTAAAGGCGAGAATTTCCAGA</td>
<td>16S rRNA</td>
<td>M59157</td>
<td>624–590</td>
<td>This study</td>
</tr>
<tr>
<td>mip FI</td>
<td>GGTGCCTGCAGCTGYCATRR</td>
<td>mip</td>
<td>S62141</td>
<td>700–719</td>
<td>This study</td>
</tr>
<tr>
<td>mip RI</td>
<td>GCATTAAATGYARWGGCTTCAGT</td>
<td>mip</td>
<td>S62141</td>
<td>1280–1259</td>
<td>This study</td>
</tr>
<tr>
<td>mip FII</td>
<td>GGGGATTTGGTGAAGATGA</td>
<td>mip</td>
<td>U91607 [S62141]</td>
<td>467–487 [675–695]</td>
<td>This study</td>
</tr>
<tr>
<td>mip RII</td>
<td>ACCAGACGGCATATAATTTAGA</td>
<td>mip</td>
<td>U91607 [S62141]</td>
<td>1050–1030 [1288–1268]</td>
<td>This study</td>
</tr>
</tbody>
</table>

* 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.

![FIG. 1. Melting curve analysis with *L. pneumophila*-specific hybridization probes.](http://aem.asm.org)
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 lurchbacked melting curve was observed for all *L. pneumophila* strains and also for *L. cincinnatiensis*. The explanation for this phenomenon probably is related to the probe design, i.e., the melting temperatures (*Tm*) 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 *Tm* determination, emphasis is placed on the peak with the higher *Tm* 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 *Tm* 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. birminghamensis*, *L. longbeachae*, *L. bozemanae*, and *L. cincinnatiensis* have sufficient 16S rRNA similarity to the *L. pneumophila* probe. The melting temperatures of these non-*L. pneumophila* species range from 60°C to 66°C, and thus all these species are differentiated from *L. pneumophila* by the melting curve. The *L. hackeliae* species is not detected by the probe described here, however, possibly because it lacks the necessary polymorphic sites in the probe region.
FIG. 2. Alignment of the mip PCR products of the non-L. pneumophila reference strains. Asterisks indicate identity with the L. micdadei strain (GenBank accession no. S62141). A dash indicates a gap or no base analyzed.
Ratcliff et al. (10) have previously described 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 sets 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.

REFERENCES


