Detection of *Legionella* Species in Respiratory Specimens Using PCR with Sequencing Confirmation

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*Legionella* spp. are a common cause of community-acquired respiratory tract infections and an occasional cause of nosocomial pneumonia. A PCR method for the detection of *legionella* in respiratory samples was evaluated and was compared to culture. The procedure can be performed in 6 to 8 h with a commercially available DNA extraction kit (Qiagen, Valencia, Calif.) and by PCR with gel detection. PCR is performed with primers previously determined to amplify a 386-bp product within the 16S rRNA gene of *Legionella pneumophila*. We can specifically detect the clinically significant *Legionella* species including *L. pneumophila*, *L. micdadei*, *L. longbeachae*, *L. bozemanii*, *L. feeleii*, and *L. dumoffii*. The assay detects 10 fg (approximately two organisms) of legionella DNA in each PCR. Of 212 clinical specimens examined by culture, 100% of the culture-positive samples (31 of 31) were positive by this assay. By gel detection of amplification products, 12 of 181 culture-negative samples were positive for *Legionella* species by PCR, resulting in 95% specificity. Four of the 12 samples with discrepant results (culture negative, PCR positive) were confirmed to be positive for *Legionella* species by sequencing of the amplicons. The *legionella*-specific PCR assay that is described demonstrates high sensitivity and high specificity for routine detection of *legionella* in respiratory samples.

*Legionella* spp. are gram-negative bacteria responsible for epidemic and sporadic cases of pneumonia after inhalation of contaminated water droplets from a variety of water sources (7). Currently, there are 42 species that comprise 64 sero-groups of *legionella* (3), most of which have caused disease (6, 15).

An estimated 3 to 8% of all community-acquired pneumonias are caused by *Legionella* spp. (3). The case-fatality rate for patients with legionellosis is 5 to 30%, with the elderly and immunocompromised patients at greater risk of death (4). Although nosocomial cases are not uncommon, with sources of infection often being hospital showers and respiratory therapy equipment, most cases occur sporadically. Generally, the clinical presentation of legionellosis includes systemic problems that involve kidney, liver, gastrointestinal, and central nervous system dysfunction (5).

Currently, culture is regarded as the “gold standard” for detection of *legionella*. However, due to the fastidious nature of the organism and problems inherent with specimen collection and transport, culture may have a sensitivity as low as 50 to 60% (4). While culture for *legionella* will detect many different species, special medium is required and the organisms take 3 to 5 days to form visible colonies. Also, testing for legionella is often requested after the patient has been treated with antibiotics, which decreases the chance of isolation by culture. Serologic testing is useful only for the retrospective diagnosis of infection, and as many as 25% of patients with legionellosis may fail to demonstrate diagnostic titers (8). Urinary antigen tests are convenient but only detect antigens of *Legionella pneumophila* serogroup 1, which cause more than 70% of cases of legionellosis in the United States (4). A properly designed PCR assay could improve the speed, accuracy, and sensitivity of diagnosis.

A PCR assay was developed in which the target for the test is the 16S rRNA gene, which exists in multiple copies per genome, thus improving the sensitivity of detection. Several medically relevant *Legionella* species including *L. pneumophila*, *L. micdadei*, *L. bozemanii*, *L. longbeachae*, *L. feeleii*, and *L. dumoffii* can be detected without the use of culture. This study compares PCR to culture with respiratory samples from patients suspected of having legionella infection.

**MATERIALS AND METHODS**

**Clinical specimens.** The PCR procedure described here is designed for use with DNA isolated from respiratory specimens. One hundred eighty-six respiratory samples from patients suspected of having pneumonia caused by *Legionella* spp. were submitted to the Associated Regional and University Pathologists Diagnostic Infectious Diseases Laboratory for culture with or without direct fluorescent-antibody assay (DFA). The number and type of each specimen submitted were as follows: 31 sputum specimens, 66 bronchial washing specimens, 74 bronchoalveolar lavage (BAL) specimens, 8 pleural fluid specimens, and 7 lung tissue specimens. Portions of 26 of the respiratory samples (13 BAL, 7 sputum, 4 bronchial washing, and 2 pleural fluid specimens) were spiked so that they contained a final concentration of approximately 10 to 100 organisms of *L. pneumophila* (ATCC 33512) per ml.

**Culture.** Each specimen was aliquoted into two samples, one was frozen at −70°C, and the other was applied to buffered charcoal yeast extract (BCYE) agar and BCYE agar with polymyxin B, anisomycin, and vancomycin (Remel, Lenexa, Kans.). For culture analysis, sputum specimens and specimens obtained by bronchoscopy were plated undiluted, pleural fluid specimens were concentrated by centrifugation, and lung tissue was homogenized in a Stomacher 80 Lab Blender (CMI/Fisher, Houston, Tex.) with approximately 2.0 ml of Trypticase soy broth. Specimens were processed immediately upon receipt. The plates were incubated at 35°C with 70 to 80% relative humidity. All cultures were examined daily and were held for 5 days before final reporting of results. Visible colonies were subcultured onto a BCYE agar plate and a blood agar plate with 5% sheep blood (Remel). Bacteria that grew on BCYE agar but not on a blood agar plate were considered possible *Legionella* spp. and were tested by DFA (Genetic Systems Corp, Redmond, Wash.) for *L. pneumophila* serogroups 1 to 6. If the DFA with the isolate was negative, the organism was sent to the Utah State Health Laboratory for further identification.

**Extraction of DNA from respiratory samples.** (i) **Sample preparation.** Samples over 1 ml in volume were centrifuged in a Beckman model TJ-6 centrifuge at 2,000 × g for 5 min, the supernatant was discarded, and the pellet volume was brought up to 1 ml with phosphate-buffered saline or sterile water before DNA extraction. Thick samples were diluted with phosphate-buffered saline and vortexed until they were homogeneous, and 1 ml was aliquoted for analysis.
mixture was held at this temperature until gel analysis. After a 20-min hold at 95°C, 38 cycles consisting of 94°C for 45 s, 57°C for 45 s, and 72°C for 60 min, and the final extension at 72°C for 20 min, and the mixture was held at this temperature until purification.

(ii) PCR conditions. Five microliters of extracted template DNA was used in a 50-μl reaction mixture that included 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3)), 3 mM MgCl₂, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP, 1 μM primer JFP, 1 μM primer JRP, 2.8 U of AmpliTaq Gold per μl, and 1 U of uracil DNA glucosylase (UDG) (Life Technologies, Gaithersburg, Md.) μl of bovine serum albumin per ml, 20% sucrose, 1 mM cresol red, 20 mM MgCl₂, and the mixture was electrophoresed on 1.5% agarose gels in TBE buffer (5 mM Tris, 5 mM boric acid, 0.1 mM EDTA) with ethidium bromide (0.5 mg/ml) at 80 V for 60 min. The gels were viewed under UV light. The migration distance was compared to that of BioMark Low (BioVentures, Inc., Murfreesboro, Tenn.) to determine the approximate number of base pairs of the amplification product.

Confirmation of positive results by sequencing. (i) Primers. The primers used for the sequencing analysis of DNA samples with discrepant results (culture negative, PCR positive) were Leg F2 (‘-GGACGAGCATTGGAAGA’ and Leg R2 (‘-CAACCGCGTCAACTTAT’).

(ii) PCR conditions. Five microliters of template DNA was used in a 50-μl reaction mixture that included 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3)), 2.5 mM MgCl₂, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP, 1 μM primer Leg F2, 1 μM primer Leg R2, 2.8 U of AmpliTaq Gold per μl, and 1 U of UDG per μl. Cycling conditions began with an initial incubation at 95°C for 10 min to allow UDG degradation of uracil residues to prevent carryover amplicon contamination. After a 20-min hold at 95°C, 38 cycles consisting of 94°C for 45 s, 57°C for 45 s, and 72°C for 45 s were followed by a final extension at 72°C for 20 min, and the mixture was held at this temperature until gel analysis.

(iii) Amplicon purification. PCR products were purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and were held at −20°C until sequencing.

(iv) Sequencing. Sequencing was performed by use of BigDye chemistry with the ABI Prism 377 Sequencer (PE Applied Biosystems). Sequence analysis was performed by a BLAST search of the GenBank database.

Dilution studies. The detection limit of the current PCR assay was determined by using limiting dilutions of DNA. DNAs from several separate Legionella species were quantified spectrophotometrically and were brought to 2 pg/μl with water. Tenfold dilutions were made, and 5 μl of each dilution was analyzed in a 50-μl PCR. Detection limits were determined for six separate species of legionellae consisting of L. pneumophila ATCC 33152, L. micdadei ATCC 33218, L. bozemanii ATCC 33217, L. longbeachae ATCC 33462, L. feelei ATCC 33849, and L. dumoffii ATCC 33279.

To determine the number of viable organisms in a sample, a 0.5 McFarland suspension of organisms was made, followed by preparation of a 10⁻⁴ dilution and a 10⁻⁵ dilution in water, from which 10 μl of each dilution was plated onto BCYE agar plates for colony counts. Two fivefold dilutions were further obtained, and 10 μl of each solution was used for DNA extraction.

**RESULTS**

Detection of a 386-bp amplification product is a positive result by the present PCR assay for legionellae. The amount of DNA consistently detected in each PCR is 10 fg by use of limiting dilutions for each of L. pneumophila, L. micdadei, L. bozemanii, L. longbeachae, L. feelei, and L. dumoffii. Occasional, 1 fg of DNA was detected, suggesting that the exact detection limit is probably between 1 and 10 fg of DNA. L. pneumophila has a genome consisting of approximately 3.9 × 10⁶ bp (about 4.3 fg of DNA) (2), indicating that 2.3 organisms can be detected by this assay. Since 5 of the 100 μl of DNA extracted from a sample was used in each PCR, 5 to 46 organisms per specimen corresponds to a detection limit of 1 to 10 fg of DNA per PCR.

The detection range of 5 to 46 organisms per sample was further confirmed by determining the number of organisms detected when performing PCR with the same dilutions that had been plated for CFU determination. It remains difficult to find the ultimate endpoint; however, after four separate dilution series with different strains of legionellae, we found a lower endpoint of 13.4 organisms for L. bozemanii. We showed the limit of detection to be as high as 55 organisms (L. pneumophila). Dilutions of L. micdadei and L. longbeachae resulted in the detection of 30.8 and 23.2 organisms, respectively.

Twenty separate species of organisms commonly occurring in the respiratory tract were analyzed by the current PCR assay. No cross-reactivity was found with any of these organisms, which included Candida albicans, Streptococcus pneumoniae, Arcanobacterium haemolyticum, Staphylococcus aureus, Actinobacillus actinomycetemcomitans, Capnocytophaga granulosa, Eikenella corrodens, Pseudomonas aeruginosa, Moraxella catarrhalis, Pasteurella multocida, Haemophilus influenzae, Escherichia coli, Klebsiella pneumoniae, Neisseria sicca, Neisseria meningitidis, Bordetella bronchiseptica, Acinetobacter baumannii, Acinetobacter junii, Chlamydia pneumoniae, and Mycoplasma pneumoniae. Limiting dilutions of A. baumannii ATCC 19606 and A. junii ATCC 17908 were further analyzed and were not found to amplify a 386-bp product.

Of 212 specimens examined by both culture and PCR for detection of Legionella spp., 31 samples positive by culture were also positive by PCR. Sequencing was performed with DNA amplicons from 12 samples that were PCR positive and culture negative. The results for 4 of 181 culture-negative samples remained discrepant after an attempt to confirm the results by sequencing. A GenBank BLAST search (1) indicated that all samples with discrepant results did, in fact, contain Legionella spp. and that the remaining eight PCR-positive samples were perhaps contaminated. Addition of a sequencing step to confirm all PCR-positive results for culture-negative samples improved the specificity from 93 to 98%.

We designed sequencing primers whose sequences were upstream and downstream of the original PCR amplicon so that the legionella primer sequences (primers JFP and JRP) were included in our analysis (Table 1). Comparison of the sequences of the JFP and JRP primer regions among different Legionella species showed very few, if any, base differences from the L. pneumophila sequence. For routine analysis, we have also validated that sequencing of the amplicon obtained in the original PCR with primers JFP and JRP provides the same results obtained with the amplicon generated by using the less specific outside primers.

A BLAST search of the 386-bp PCR product from the first eight samples with discrepant results showed that they contained sequences with homology to the 16S rRNA gene of Acinetobacter species or an unidentified Proteobacterium 16S rRNA gene and does not give any indication that they are Legionella species. The sequence in each of the remaining samples with discrepant results (samples 9 to 12 in Table 1) was homologous to those of Legionella species and had fewer base pair differences from L. pneumophila than did samples 1 to 8, which had discrepant results. The sequences of the entire 386-bp amplicon of samples 9 and 10, which had discrepant results, were identical to that of L. micdadei ATCC 33218.
while the sequence in sample 11, which had a discrepant result, was identical to that of _L. longbeachae_ ATCC 33462.

**DISCUSSION**

The legionella-specific PCR assay described in our study targets the 16S rRNA gene and is very sensitive, detecting 100% of all culture-positive respiratory samples. Among those culture-negative samples that were PCR positive, we were able to confirm the results for samples with true-positive results and distinguish them from samples with false-positive results with good confidence using sequence analysis. By sequencing confirmation, 98% specificity was accomplished compared to the results of culture.

Tang et al. (14) showed that identification of bacterial species by sequence analysis can successfully be performed with the MicroSeq system (PE Applied Biosystems Division, Foster City, Calif.), which includes a sequence database library to be used for identification of several bacteria to the species level. We cannot use the MicroSeq system primers to amplify DNA obtained from respiratory samples because the primers are not legionella specific and there may be several different types of bacteria in a respiratory sample. In our study, there appears to be enough variability within the 386-bp amplicon generated (approximate bases 451 to 837 of the 16S rRNA gene) to determine the species of several strains of legionellae.

Less stringent conditions were applied to the assay for detection of most, if not all, of the 64 or more species and serogroups of legionellae. Jonas et al. (9) used identical primers and similar conditions compared with those used for PCR in the current study in conjunction with a probe-capture technique to delineate samples with false-positive results. While we favored their choice of primers for detection of multiple species, we found their detection procedure to be undesirable since their probe did not detect _L. micdadei_, which is a common disease-causing strain. Our studies indicate that sequencing of all PCR-positive samples for confirmation of results provides a higher degree of specificity without a loss of sensitivity, as may occur with probe-capture detection systems.

Genetic targets other than the 16S rRNA gene, such as the intergenic 16S-23S ribosomal spacer region (11, 13) and the _mip_ gene (12), have been used to determine the species of pure isolates of legionellae. For the detection of legionella DNA extracted directly from respiratory samples, our goal was to use genus-specific primers so that other non-Legionella organisms would not be detected. In the present study, the primers that target the 16S rRNA gene appear to be very specific for _Legionella_ spp., while they amplify a variety of different species which cause legionellosis.

Although the sequences of the false-positive samples were very homologous to those of _Acinetobacter_ spp., we could not get a positive PCR result with DNA extracted from _A. baumanii_ or _A. junii_. There remains the possibility that the sequence with a false-positive result is not that of an _Acinetobacter_ sp., although the BLAST search showed high scores (767 bits; 100% [387 of 387] identities for the _A. junii_ 16S rRNA gene).

It is also interesting that samples 9, 10, and 11, which had discrepant results (they were not detected by culture), were non- _L. pneumophila_ strains of legionellae. Without sufficient data with which to draw conclusions, a suspicion of the loss of sensitivity of culture for detection of legionellae that are not _L. pneumophila_ arises. It may be possible that less common strains are not as easily detected in the microbiology laboratory by culture and DFA. There is a broad range (50 to 99%) of reported sensitivities of culture in the literature (4, 6). This discrepancy is most likely due to differences in the types and qualities of the specimens used in each study. Collection of specimens in saline (BAL specimens, bronchial washes) lowers the sensitivity of culture because saline inhibits the growth of legionellae (6). Sputum samples may include contamination with enough oropharyngeal organisms to overgrow and mask _Legionella_ spp. Another contribution to a lowered sensitivity of culture is the use of samples collected from patients currently being treated with antibiotics. A PCR test is likely to overcome some of these issues.

The accurate diagnosis of legionella pneumonia has important implications for treatment of the infection. Many first-line antibiotics commonly used to treat typical bacterial pneumonias (i.e., beta-lactams) are ineffective against _Legionella_ species (10). This is at least partially due to the fact that legionella bacteria are intracellular pathogens. There is a great need for a rapid diagnosis of legionella infection. While DFA is rapid, the test detects only _L. pneumophila_ serogroups 1 to 6 and fails to detect other _Legionella_ species and serogroups. Testing for...

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**Table 1. Alignment of partial 16S rRNA sequences from the 12 samples with discrepant results as well as the indicated Legionella species**

<table>
<thead>
<tr>
<th>Species or PCR sample no.</th>
<th>Forward primer region (bases 451 to 470) sequence</th>
<th>Variable region (bases 609 to 649) sequence</th>
<th>Reverse primer region (bases 818 to 837) sequence</th>
<th>No. of bases different from <em>L. pneumophila</em> (bases 451 to 837)</th>
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<td><em>L. pneumophila</em></td>
<td>AGGTTGATAGTGTTAGAACG</td>
<td>CATTCTGGCGTTACGTCGACGTCTAGATAATCTGGT</td>
<td>CGATGCTCAAAGCTGCGTGTTGG</td>
<td>0</td>
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<tr>
<td><em>L. micdadei</em></td>
<td>-G-</td>
<td>-C-</td>
<td>-G-</td>
<td>-TGA</td>
</tr>
<tr>
<td><em>L. longbeachae</em></td>
<td>-G-</td>
<td>-C-</td>
<td>-G-</td>
<td>-TGA</td>
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</table>

* a Samples 1 to 12 had discrepant results (culture negative, PCR positive).

* b Dots indicate identity with _L. pneumophila_; hyphens indicate deletions.

* c Sequencing revealed a Y at position 742.

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**Table 2. Genetic targets other than the 16S rRNA gene, such as the intergenic 16S-23S ribosomal spacer region (11, 13) and the _mip_ gene (12), have been used to determine the species of pure isolates of legionellae. For the detection of legionella DNA**
legionella antigen in urine is a good test but also fails to detect multiple serotypes and species. Culture of respiratory samples can detect multiple species, but it may take up to 5 days for results. Our findings show that a legionella-specific PCR of respiratory samples that targets a 386-bp portion of the 16S rRNA gene can be performed in 6 to 8 h with high sensitivity and high specificity of results. The specificity is further improved by confirming positive results by sequencing analysis. If the PCR-positive samples confirmed to be positive by sequencing are accepted as true positives on the basis of a comparison with sequences in the GenBank database, then no specimens have false-positive results (100% specificity) by the combined PCR-sequencing assay.

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REFERENCES