Direct Detection and Differentiation of *Legionella* spp. and *Legionella pneumophila* in Clinical Specimens by Dual-Color Real-Time PCR and Melting Curve Analysis

Udo Reischl,^{1*} Hans-Jörg Linde,¹ Norbert Lehn,¹ Olfert Landt,² Kevin Barratt,³
and Nele Wellingshausen⁴

Institute of Medical Microbiology and Hygiene, University of Regensburg, D-93053 Regensburg,¹ TIB Molbiol GmbH, Tempelhofer Weg 11-12, D-10829 Berlin,² and Institute of Medical Microbiology and Hygiene, University of Ulm, D-89081 Ulm,⁴ Germany, and Health Waikato Laboratory, Hamilton, New Zealand³

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A dual-color LightCycler PCR assay targeting the 16S rDNA gene of *Legionella* spp. was established. By using two pairs of hybridization probes, *Legionella* spp. and *Legionella pneumophila* could be detected and differentiated simultaneously. With 26 culture-positive and 42 culture-negative respiratory specimens from patients with atypical pneumonia, 100% sensitivity and specificity was observed for *L. pneumophila*.

Legionellae are ubiquitous in environmental water sources and may cause sporadic as well as epidemic cases of atypical pneumonia after inhalation or aspiration of contaminated water droplets. Presently, 42 species with 64 serogroups are known (2), many of which cause disease in humans (13, 16, 20). *Legionella pneumophila* is the most common pathogenic species, accounting for up to 90% of legionellosis cases (20). Mortality of patients with pneumonia may exceed 30% for elderly and immunocompromised patients (3), and prognosis of patients depends in part on rapid identification of the causative agent (8).

Diagnostic culture is considered the gold standard for the laboratory detection of *Legionella* infections, but legionellae are slow-growing and fastidious bacteria and successful culture requires selective media and prolonged incubation periods. Furthermore, diagnostic culture is often requested after initiating antibiotic therapy, which lowers the recovery rate substantially. Direct fluorescent antibody assays (DFA) on respiratory secretions or urinary antigen detection tests can be performed rapidly but have limitations. DFA has low sensitivity, and cross-reactions with other bacteria may result in false-positive results. Urinary antigen testing has up to 85% sensitivity but detects only a limited range of pathogenic *Legionella* species (5, 10).

Serological methods are also widely used in clinical laboratories, but they are not helpful for rapid diagnosis of acute legionellosis, since seroconversion of the infected patients is delayed (about 50% of patients develop specific antibodies more than 2 weeks after onset of clinical symptoms) or is even absent in some cases (6).

For these reasons, nucleic acid amplification techniques are attractive tools for detection of *Legionella* species in clinical as well as in environmental samples. A variety of diagnostic PCR

protocols have been reported that target specific regions within 16S ribosomal DNA (rDNA) (4, 7, 9, 17, 19), 5S rDNA (7), or the macrophage infectivity potentiator (*mip*) gene (1, 7, 11, 12, 14, 15, 18, 19).

Here we have described the development of a sensitive and specific hybridization probe-based dual-color real-time PCR assay for the simultaneous detection and differentiation of *Legionella* spp. and *L. pneumophila* in clinical specimens. In order to meet the requirements of true rapid diagnostics, the LightCycler device (Roche Diagnostics, Mannheim, Germany) was used for rapid thermal cycling and dual-color monitoring of specific PCR products. Amplification was based on a previously published *Legionella*-specific primer pair (4, 9), flanking a segment within the bacterial multicopy 16S rRNA gene. For the detection of *Legionella* spp. a pair of LightCycler Red640-labeled hybridization probes (Leg-HP-1 and Leg-HP-2) was selected complementary to a region conserved among all *Legionella* spp. For the detection of *L. pneumophila*, a pair of LightCycler Red705-labeled hybridization probes (Lpn-HP-1 and Lpn-HP-2) was selected complementary to an *L. pneumophila*-specific region within the amplicon. Sequence alignments of sensor hybridization probe Lpn-HP-1 revealed a perfect match to *L. pneumophila* 16S rDNA but at least two nucleotide mismatches between its sequence and 16S rDNA sequences of all other bacterial species. Oligonucleotide primers and fluorescence-labeled hybridization probes were obtained from TIB Molbiol (Berlin, Germany). Nucleotide sequences and positions are listed in Table 1.

An initial evaluation of the PCR assay was performed with 68 bronchoalveolar lavage (BAL) specimens from patients with atypical pneumonia which tested positive ($n = 26$) or negative ($n = 42$) for *L. pneumophila* in diagnostic culture. Three of the *Legionella*-negative BAL specimens were spiked with about 10^6 CFU of cultured *Legionella longbeachae* (ATCC 33462) (American Type Culture Collection, Manassas, Va.), *Legionella micdadei* (ATCC 33218), or *Legionella bozemanii* (ATCC 33217) organisms. For template DNA preparation, 500- μ l aliquots of the BAL specimens were centrifuged for 2 min at $8,000 \times g$, and the resulting pellet plus 100 μ l of the

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, Franz-Josef-Strauß-Allee 11, D-93053 Regensburg, Germany. Phone: 49-941-944-6450. Fax: 49-941-944-6402. E-mail: Udo.Reischl@klinik.uni-regensburg.de.

TABLE 1. Oligonucleotide primers and LightCycler hybridization probes used in the PCR assay^a

Oligonucleotide	Sequence ^b	Nucleotide position	Reference
Leg-1	AGGGTTGATAGGTTAAGAGC	451–470	9
Leg-2	CCAACAGCTAGTTGACATCG	836–817	9
Leg-HP-1	AGTGGCGAAGGCGGCTACCT-[FL]	721–740	19
Leg-HP-2	[Red 640]-TACTGACACTGAGGCACGAAAGCGT-[Ph]	748–772	19
Lpn-HP-1	CCAGTATTATCTGACCGTCCCA-[FL]	646–625	Present study
Lpn-HP-2	[Red705]-TAAGCCCAGGAATTTACAGATAACTT-[Ph]	621–595	Present study

^a The target gene in each case was 16S rDNA, and the GenBank accession number was M59157.
^b [FL], fluorescein; [Red 640], LightCycler-Red 640-*N*-hydroxy-succinimide ester; [Red 705], LightCycler-Red 705-phosphoramidite; [Ph], 3'-phosphate.

supernatant was processed with the High Pure PCR Template Preparation kit (Roche Diagnostics) according to the manufacturers instructions. Amplification mixtures consisted of 2 μ l of 10 $\times$ LightCycler-FastStart DNA Master Hybridization

Probes mix (Roche Diagnostics), which contains DNA polymerase, reaction buffer, deoxynucleoside triphosphates in a ready-to-use formulation, 3 mM MgCl₂, 0.5 μ M concentrations of each primer oligonucleotide, 0.2 μ M concentrations of each

FIG. 1. Evaluation of the dual-color *Legionella* PCR assay with clinical specimens. A representative set of 23 *L. pneumophila* culture-positive BAL specimens, 4 *Legionella* culture-negative BAL specimens, and 3 *Legionella* culture-negative BAL specimens spiked with cultured *L. longbeachae*, *L. micdadei*, or *L. bozemanii* organisms was analyzed. Ten nanograms of *L. pneumophila* serogroup 1 (ATCC 33152) template DNA was used as a positive control. (A) Fluorescence readout at 640 nm showing the LightCycler results with the *Legionella* spp.-specific set of hybridization probes Leg-HP-1 and Leg-HP-2. (B) Fluorescence readout at 705 nm showing the LightCycler results with the *L. pneumophila*-specific set of hybridization probes Lpn-HP-1 and Lpn-HP-2.

FIG. 2. Melting curve analysis performed on the *L. pneumophila*-specific set of hybridization probes (fluorescence readout at 705 nm). Melting curves corresponding to *L. micdadei*-, *L. longbeachae*-, *L. bozemanii*-, and *L. pneumophila*-specific amplicons are indicated by arrows.

hybridization probe oligonucleotide, and 2 μ l of template DNA in a final volume of 20 μ l. After an initial denaturation step for 10 min at 95°C to activate the FastStart Taq DNA polymerase, the thermocycle program included 50 cycles of three steps each, comprised of heating at 20°C/s to 95°C with a 5-s hold, cooling at 20°C/s to 55°C with a 10-s hold, and heating at 20°C/s to 72°C with a 20-s hold. Following amplification a LightCycler melting curve analysis was performed with a heating rate of 0.2°C/s starting at 40°C. Fluorescence values of each capillary were measured at 640 and 705 nm (dual-color option).

All 29 *Legionella*-positive samples (26 *L. pneumophila* culture-positive samples and 3 samples spiked with other *Legionella* species) were correctly amplified and detected with the *Legionella* spp.-specific set of hybridization probes (Fig. 1A; fluorescence readout at 640 nm). Although all of the 29 *Legionella*-positive samples were also detected with the *L. pneumophila*-specific set of hybridization probes (Fig. 1B; fluorescence readout at 705 nm), *L. pneumophila*-specific amplicons could be easily distinguished by their characteristic melting temperature (T_m) of above 61°C in LightCycler melting curve analysis (Fig. 2). All 39 *Legionella*-negative samples tested negative with both sets of hybridization probes. None of the samples showed inhibition as judged by separate amplification of the human beta-globin gene by the LightCycler Control kit DNA (Roche Diagnostics). Compared with the results of diagnostic culture, the PCR assay results showed 100% specificity and sensitivity for *L. pneumophila* detection and species identification of the 68 BAL specimens investigated.

Assay specificity was further confirmed by testing genomic DNA of 46 *Legionella* species and serogroups (19), including all pathogenic species, all 15 serogroups of *L. pneumophila*, and *Legionella*-like amoebal pathogen 10 as well as with genomic DNA of 26 clinical isolates of gram-negative or gram-positive bacterial species other than *Legionella*, including *My-*

coplasma pneumoniae, *Chlamydia pneumoniae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Moraxella catarrhalis*. Positive PCR results, as evidenced by probe fluorescence, were observed only with *Legionella* spp., and all *L. pneumophila* isolates were distinguished by their characteristic T_m in melting curve analysis at 705 nm (data not shown).

To determine the lowest number of *L. pneumophila* cells detectable by the assay, serial dilutions of genomic DNA prepared from cultured *L. pneumophila* serogroup 1 (ATCC 33152) were tested. An analytical sensitivity of 10 fg of template DNA was determined for both pairs of hybridization probes, which corresponded to about 3 genome equivalents per PCR. Therefore, our assay concept should be sensitive enough for the direct detection of *Legionella* organisms in suitable patient specimens, such as BAL or tracheal secretions.

A cross-reaction of the *L. pneumophila*-specific set of hybridization probes with some non-*L. pneumophila* species was observed in the LightCycler amplification plots (Fig. 1B), thus requiring melting curve analysis for unambiguous identification of *L. pneumophila*. Such partial hybridization events of probes Lpn-HP-1 and Lpn-HP-2 with amplicons generated from non-*L. pneumophila* species can be abolished by applying more stringent annealing temperatures between 56 to 60°C or a touchdown protocol from 60 to 56°C, but this slightly reduces total assay sensitivity to about 10 *Legionella* genome equivalents per PCR.

In clinical practice, a variety of discordant results with DFA, urinary antigen, serology, and culture may be observed with patient specimens. For evaluation of our novel PCR assay, the selection of *L. pneumophila* culture-positive and culture-negative specimens was intentional in order to compare the results with the gold standard. Since results of DFA, urinary antigen, and serology were not available for all patients, the overall diagnostic sensitivity and specificity of the assay remain to be established in the course of a prospective study.

The sensitive detection of *Legionella* on the genus level as well as the differentiation between *L. pneumophila* and non-*L. pneumophila* species is important in the case of a clinically suspected legionellosis. Using only one pair of primers and two sets of elongation-blocked hybridization probes avoids sensitivity problems generally associated with duplex PCR and distinguishes this dual-color protocol from previously described real-time PCR assays (1, 7, 17, 19). Following template DNA preparation, PCR screening of up to 30 clinical specimens plus a positive and a negative control can be performed in a single LightCycler run, resulting in a total assay time of less than 2.5 h. LightCycler melting curve analysis allows the reliable differentiation of *L. pneumophila* from other *Legionella* species, but there is not enough sequence heterogeneity in the hybridization region of probes Lpn-HP-1 and Lpn-HP-2 to separate individual non-*L. pneumophila* species by T_m analysis. *L. bozemanii* and *L. longbeachae*, for example, share an identical T_m of about 50°C (Fig. 2). In the case of a positive LightCycler PCR result for the genus *Legionella* (hybridization probes Leg-HP-1 and Leg-HP-2; fluorescence readout at 640 nm) but a negative result for *L. pneumophila* (observed T_m with hybridization probes Lpn-HP-1 and Lpn-HP-2 below 61°C; fluorescence readout at 705 nm), the underlying *Legio-*

nella species can be identified within about 3 h by direct sequencing of the spin-column-purified PCR products.

With respect to microbiological practice, the performance characteristics of the described LightCycler assay, such as its speed, single-capillary format, dual-color hybridization probe detection, and species information obtained with melting curve analysis, make it a valuable adjunct to the spectrum of direct methods like DFA or urinary antigen detection and an attractive alternative to conventional PCR assays for detection of *Legionella* spp.

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