JCM
Journals.ASM.org

Clinical Application of a Multiplex Real-Time PCR Assay for Simultaneous Detection of *Legionella* Species, *Legionella pneumophila*, and *Legionella pneumophila* Serogroup 1

Alvaro J. Benitez, Jonas M. Winchell

Respiratory Diseases Branch, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

We developed a single-tube multiplex real-time PCR assay capable of simultaneously detecting and discriminating *Legionella* spp., *Legionella pneumophila*, and *Legionella pneumophila* serogroup 1 in primary specimens. Evaluation of 21 clinical specimens and 115 clinical isolates demonstrated this assay to be a rapid, high-throughput diagnostic test with 100% specificity that may aid during legionellosis outbreaks and epidemiologic investigations.

Legionellae are facultative intracellular Gram-negative bacteria found in soil and water environments, where they parasitize and proliferate within free-living protozoa (1). Legionellae possess the ability to replicate in environmental protozoa and within mammalian alveolar macrophages and epithelial cells (2–4). Legionellae are common contaminates of artificial water systems, including air-conditioning systems, cooling towers, and jacuzzis, where conditions may be optimal for growth and proliferation (5, 6). Once aerosolized, the bacteria can enter the human respiratory tract and cause disease manifesting as Legionnaires' disease, a severe form of pneumonia, or Pontiac fever, a self-limiting flu-like illness (1).

More than 50 species comprising 70 distinct serogroups have been identified to date (http://www.bacterio.cict.fr/l/legionella.html). Although more than 90% of isolates associated with Legionnaires' disease are *Legionella pneumophila*, with 84% being *L. pneumophila* serogroup 1 (sg1), nearly one-half of *Legionella* species have been associated with human disease (1, 7, 8). Given this epidemiological background, an ideal diagnostic assay would rapidly detect the presence of *Legionella* and simultaneously identify the species and, if applicable, the serogroup. Although bacterial culture remains the gold standard for the diagnosis of Legionnaires' disease, it requires specialized media and expertise and is time-consuming, requiring several days to obtain definitive and reliable results. The urine antigen test is a common and convenient test used for diagnosis but detects only *L. pneumophila* sg1. Serology-based tests, although widely used, suffer from specificity limitations (1). Currently, molecular diagnosis is based largely on detection of the 16S rRNA gene for identification of the *Legionella* genus and the *mip* gene, used for identification to the species level, along with sequence based-typing (SBT) for *L. pneumophila* characterization (9–11). These tests require post-PCR analysis and/or sequencing to distinguish *L. pneumophila* sg1 from other serogroups and/or non-*pneumophila* species of *Legionella*, thus making them less ideal (12, 13). The lack of available diagnostic tests for species other than *L. pneumophila* may lead to underreporting and/or unrecognized cases of legionellosis. Since the worldwide burden of disease caused by non-sg1 *L. pneumophila* and other species is largely unknown but is suspected to be significant (14, 15), an improved detection method would ideally integrate a more inclusive approach for identification and characterization.

This article reports the development and evaluation of a single-tube multiplex real-time PCR assay that allows simultaneous detection and differentiation of *Legionella* spp., *Legionella pneumophila*, and *Legionella pneumophila* sg1 for clinical isolates and in primary specimens. The assay targets the *ssrA* (*Legionella* species) gene (16), the *mip* (*L. pneumophila*) gene, and the recently identified *wzm* gene (17), specific for *L. pneumophila* sg1.

Multiple TaqMan primer-probe sets were designed targeting the *ssrA* gene (GenBank accession no. AE017354 [bp 172917 to 173015]), the *mip* gene (GenBank accession no. AJ810179 [bp 76 to 190]), and the *wzm* gene (GenBank accession no. AM778127 [complement bp 6098 to 6167]) either manually (*ssrA*) or using the software program Primer Express 3.0 (Applied Biosystems, Foster City, CA). The RNase P assay designed to detect human DNA was described previously (18). Primers and probe sets were initially tested and optimized in singleplex format (data not shown). Primer and probe sequences, their final concentrations, and the distinct fluorophores for each probe used in the multiplex assay are listed in Table S1 in the supplemental material. Purified total nucleic acid from *L. pneumophila* sg1 strain Philadelphia was used as a positive control. The multiplex real-time PCR mixture was prepared in a total volume of 25 μl containing the following, per reaction: 12.5 μl of PerfeCTa Multiplex qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD), the appropriate volume of each primer and probe (see Table S1), 5 μl of template, and nuclease-free water (Promega), to achieve a 25-μl final volume. The assay was carried out using the ABI 7500 real-time PCR system (Applied Biosystems) under the following conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Received 19 September 2012 **Returned for modification** 19 October 2012
Accepted 2 November 2012
Published ahead of print 7 November 2012
Address correspondence to Jonas M. Winchell, jwinchell@cdc.gov.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.02510-12.

doi:10.1128/JCM.02510-12

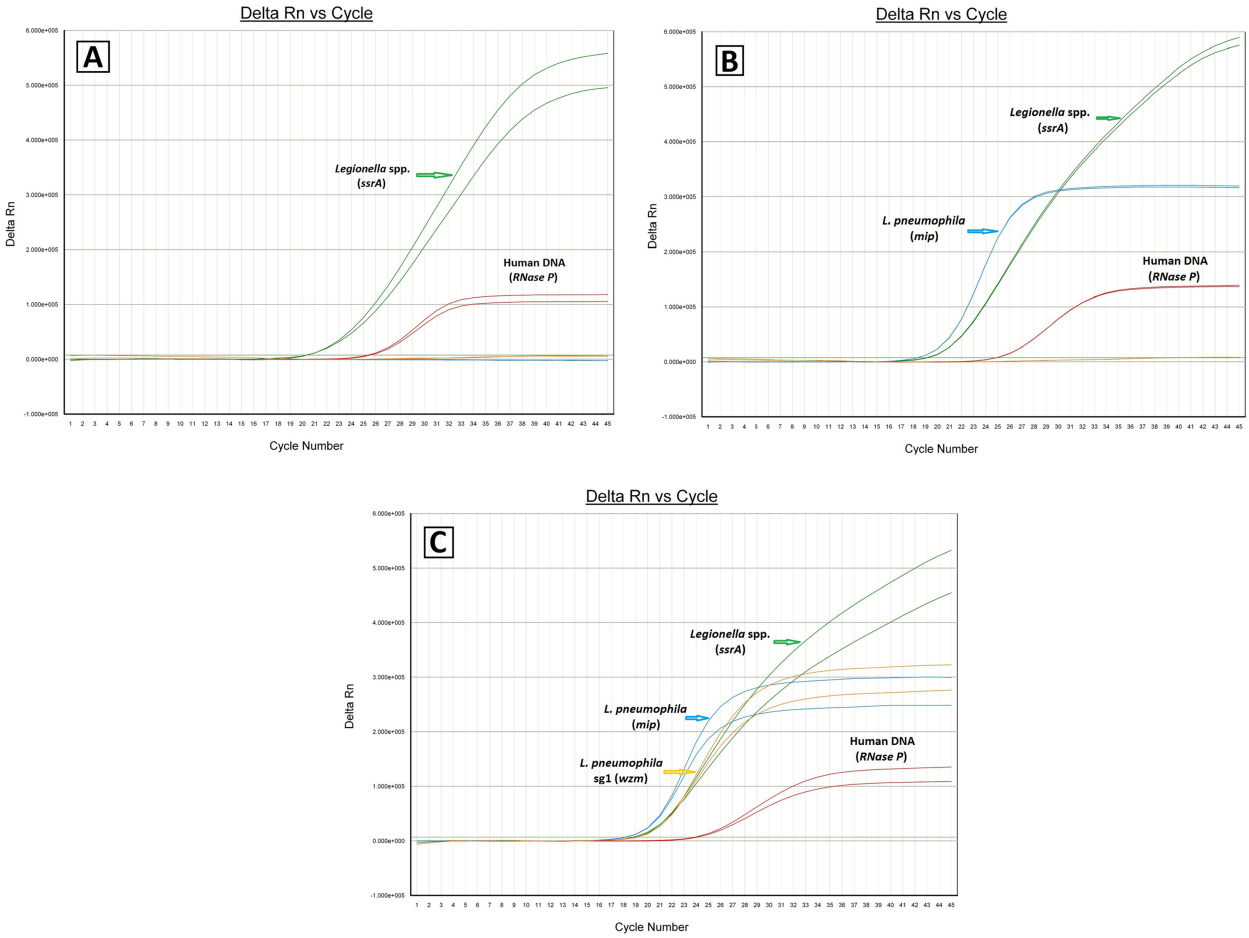

FIG 1 Multiplex assay for detection of *Legionella* spp., *Legionella pneumophila*, *Legionella pneumophila* sg1, and human DNA. (A) *L. anisa* DNA/human DNA; (B) *L. pneumophila* sg2 DNA/human DNA; (C) *L. pneumophila* sg1 DNA/human DNA. Samples were run in duplicate. All channels are shown to display specificity. Delta Rn, baseline-corrected fluorescence.

TABLE 1 Multiplex real-time analysis of *Legionella*-positive and -negative clinical specimens

Sample no.	Source[c]	Culture result	DFA	Multiplex real-time PCR C_T value			
				Legionella spp. (*ssrA*)	*L. pneumophila* (*mip*)	*L. pneumophila* sg1 (*wzm*)	Human DNA (RNase P)
1	Sputum	+	sg1	27.63	26.84	27.74	25.96
2	Lung	+	sg1	17.15	16.85	17.11	17.66
3	Lung	+	sg1	15.14	14.97	15.19	16.56
4	Br. swab	+	sg1	25.53	24.47	24.53	23.91
5	Lung	+	sg1	26.89	26.18	25.95	20.00
6	Lung	+	sg1	28.55	28.07	26.98	17.66
7	Lung	+	sg1	25.19	24.37	24.17	19.59
8	Lung	+	sg1	25.93	25.30	24.72	19.05
9	Lung	+	sg1	29.36	28.74	28.27	20.50
10	Lung	+	sg1	26.04	25.18	25.15	17.50
11	Lung	+	sg1	26.54	25.97	25.81	21.24
12	Lung	+	sg1	26.72	27.07	26.52	19.83
13	BAL	+	sg1	33.17	32.13	31.97	24.70
14	Sputum	+	sg1	27.88	27.21	26.66	16.75
15	Lung	+	sg1	28.18	27.24	26.95	20.48
16	OP swab	−	NT[b]	ND[c]	ND	ND	24.11
17	Urine	−	NT	ND	ND	ND	23.07
18	OP swab	−	NT	ND	ND	ND	25.17
19	NP swab	−	NT	ND	ND	ND	24.56
20	Sputum	−	NT	ND	ND	ND	22.26
21	Sputum	−	NT	ND	ND	ND	23.98

[a] Br. swab, OP swab, and NP swab, bronchial, oropharyngeal, and nasopharyngeal swabs, respectively; BAL, bronchoalveolar lavage specimen.
[b] NT, not tested.
[c] ND, not detected.

TABLE 2 Multiplex real-time analysis of *Legionella* clinical isolates

Species (n^a)	Multiplex real-time PCR C_T value					
	Legionella spp. (*ssrA*)		*L. pneumophila* (*mip*)		*L. pneumophila* sg1 (*wzm*)	
	Avg (SD)	Range	Avg (SD)	Range	Avg (SD)	Range
Legionella species, non-*pneumophila* (13)	21.84 (0.85)	20.64–23.76	ND[b]		ND	
L. pneumophila sg2 to sg14 (42)	20.59 (0.68)	18.11–21.70	19.19 (0.63)	17.01–20.36	ND	
L. pneumophila sg1 (60)	19.07 (0.63)	18.04–20.25	17.97 (0.60)	17.04–19.13	19.11 (0.59)	17.95–20.10

[a] *n*, no. of isolates.
[b] ND, not detected.

Analytical specificity of the multiplex assay was 100% (data not shown), as verified using a comprehensive panel of 215 clinical and environmental isolates representing 52 *Legionella* species and 44 non-*Legionella* strains (see Table S2 in the supplemental material). Analytical sensitivity was established by testing *L. pneumophila* sg1 nucleic acid diluted from 1 ng/μl, followed by 10-fold dilutions down to 1 fg/μl. Each dilution was tested in 10 replicates, and limits of detection (LOD) were established for each assay, defined as the lowest dilution in which ≥50% of replicates had positive crossing threshold (C_T) values. An LOD of 25 fg was established for each assay, except for the RNase P assay, which had an LOD of 25 pg. Figure 1 presents real-time PCR analysis curves of samples spiked with 500 pg of *Legionella anisa* (Fig. 1A), *L. pneumophila* sg2 (Fig. 1B), *L. pneumophila* sg1 (Fig. 1C), and human nucleic acid. Samples were run in duplicate with all channels shown to display assay specificity and the lack of cross-channel signal bleed.

Clinical specimens previously collected by the CDC *Legionella* Lab from individual cases and/or domestic outbreaks and identified as positive ($n = 15$) or negative ($n = 6$) for *Legionella* by culture and direct immunofluorescence assay (DFA) were tested with the current assay. Culture-positive clinical specimens were limited to *L. pneumophila* sg1 positives due to limited availability of other *Legionella*-positive specimens. Total nucleic acid from each clinical specimen or culture was extracted with the MagNA Pure compact instrument (Roche Applied Bioscience, Indianapolis, IN) using the total nucleic acid isolation kit, following the manufacturer's instructions. Clinical sensitivity was established by testing nucleic acid extracts in triplicate using the multiplex assay, which included an RNase P internal control to ensure proper nucleic acid extraction and integrity. Table 1 shows the average C_T values obtained from each clinical specimen extract and demonstrates 100% clinical sensitivity. All negative specimens demonstrated no reactivity with any of the *Legionella*-specific markers but gave positive RNase P signals (Table 1). Total nucleic acid extracted from clinical isolate cultures ($n = 115$) previously characterized in our lab by *mip* sequencing and/or DFA was normalized to 1 ng/μl and tested using the multiplex assay for specificity (Table 2). These data demonstrate assay specificity and sensitivity and provide an overall improvement in the diagnostic capability for detecting and characterizing causes of legionellosis by demonstrating a sensitive, high-throughput, and rapid testing procedure.

To our knowledge, our study is the first report of a single-tube, 4-plex real-time PCR assay capable of simultaneous detection and differentiation of *Legionella* spp., *Legionella pneumophila*, and *Legionella pneumophila* sg1, along with an internal control (RNase P) in clinical specimens. The multiplex assay is considerably faster (4 h versus ≥8 days) and easier to interpret than culture. In addition, it allows direct quantification of *Legionella*. Although this study was limited to *Legionella pneumophila* sg1-positive clinical specimens, the assay affords the opportunity to expand the diagnostic capabilities to detect species other than *L. pneumophila* sg1, thereby yielding a better understanding of the causative agent of legionellosis. The assay does not require an isolate, thereby providing an advantage in cases where antibiotics may have been administered that may render the *Legionella* nonviable but allow for the potential to detect any remaining nucleic acid present. Although further testing would be required, this assay could potentially be used for surveillance purposes or to screen environmental samples to assist in identifying a source during an outbreak. Use of this test along with currently established diagnostic tests for legionellosis may lead to a more effective public health response and expedite appropriate treatment of patients, especially during outbreaks.

REFERENCES

1. **Fields BS, Benson RF, Besser RE.** 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin. Microbiol. Rev. **15**:506–526.
2. **Cianciotto NP, Bangsborg JM, Eisenstein BI, Engleberg NC.** 1990. Identification of *mip*-like genes in the genus *Legionella*. Infect. Immun. **58**:2912–2918.
3. **Fields BS.** 1996. The molecular ecology of legionellae. Trends Microbiol. **4**:286–290.
4. **Newton HJ, Ang DK, van Driel IR, Hartland EL.** 2010. Molecular pathogenesis of infections caused by *Legionella pneumophila*. Clin. Microbiol. Rev. **23**:274–298.
5. **Fields BS.** 2008. *Legionella* in the environment, p 85–91. *In* Hoffmann P, Friedman H, Bendinelli M (ed), *Legionella pneumophila*: pathogenesis and immunity. Springer, New York, NY.
6. **Steinert M, Hentschel U, Hacker J.** 2002. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol. Rev. **26**:149–162.
7. **Kozak NA, Benson RF, Brown E, Alexander NT, Taylor TH, Jr, Shelton BG, Fields BS.** 2009. Distribution of lag-1 alleles and sequence-based types among *Legionella pneumophila* serogroup 1 clinical and environmental isolates in the United States. J. Clin. Microbiol. **47**:2525–2535.
8. **Muder RR, Yu VL.** 2002. Infection due to *Legionella* species other than *L. pneumophila*. Clin. Infect. Dis. **35**:990–998.
9. **Ratcliff RM, Lanser JA, Manning PA, Heuzenroeder MW.** 1998. Sequence-based classification scheme for the genus *Legionella* targeting the *mip* gene. J. Clin. Microbiol. **36**:1560–1567.
10. **Ratzow S, Gaia V, Helbig JH, Fry NK, Luck PC.** 2007. Addition of *neuA*, the gene encoding *N*-acylneuraminate cytidylyl transferase, increases the discriminatory ability of the consensus sequence-based scheme for typing *Legionella pneumophila* serogroup 1 strains. J. Clin. Microbiol. **45**:1965–1968.
11. **Templeton KE, Scheltinga SA, Sillekens P, Crielaard JW, van Dam AP, Goossens H, Claas EC.** 2003. Development and clinical evaluation of an internally controlled, single-tube multiplex real-time PCR assay for detection of *Legionella pneumophila* and other Legionella species. J. Clin. Microbiol. **41**:4016–4021.
12. **Maurin M, Hammer L, Gestin B, Timsit JF, Rogeaux O, Delavena F,**

Tous J, Epaulard O, Brion JP, Croize J. 2010. Quantitative real-time PCR tests for diagnostic and prognostic purposes in cases of legionellosis. Clin. Microbiol. Infect. **16**:379–384.
13. **Yang G, Benson RF, Pelish T, Brown E, Winchell JM, Fields BS.** 2010. Dual detection of *Legionella pneumophila* and *Legionella* species by real-time PCR targeting the 23S-5S rRNA gene spacer region. Clin. Microbiol. Infect. **16**(3):255–261.
14. **Gobin I, Newton PR, Hartland EL, Newton HJ.** 2009. Infections caused by non-pneumophila species of *Legionella.* Rev. Med. Microbiol. **20**:1–11.
15. **Yu VL, Plouffe JF, Pastoris Castellani M, Stout JE, Schousboe M, Widmer A, Summersgill JT, File TM, Jr, Heath CM, Paterson DL, Chereshsky A.** 2002. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. J. Infect. Dis. **186**:127–128.
16. **Thurman KA, Warner AK, Cowart KC, Benitez AJ, Winchell JM.** 2011. Detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* spp. in clinical specimens using a single-tube multiplex real-time PCR assay. Diagn. Microbiol. Infect. Dis. **70**:1–9.
17. **Merault N, Rusniok C, Jarraud S, Gomez-Valero L, Cazalet C, Marin M, Brachet E, Aegerter P, Gaillard JL, Etienne J, Herrmann JL, Lawrence C, Buchrieser C.** 2011. Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples. Appl. Environ. Microbiol. **77**:1708–1717.
18. **Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, Tong S, Cook BT, Holloway BP, McCaustland KA, Rota PA, Bankamp B, Lowe LE, Ksiazek TG, Bellini WJ, Anderson LJ.** 2004. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. Emerg. Infect. Dis. **10**:311–316.

JCM
Journals.ASM.org

ERRATUM

Clinical Application of a Multiplex Real-Time PCR Assay for Simultaneous Detection of *Legionella* Species, *Legionella pneumophila*, and *Legionella pneumophila* Serogroup 1

Alvaro J. Benitez, Jonas M. Winchell

Respiratory Diseases Branch, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Volume 51, no. 1, p. 348–351, 2013. Table S1 in the supplemental material: The PanLeg-P1 sequence was missing the first 5′-end base pair (A). Revised supplemental material is posted at http://jcm.asm.org/content/51/1/348/suppl/DCSupplemental.

doi:10.1128/JCM.03391-13