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Simultaneous detection of *Legionella* species and *L. anisa*, *L. bozemanii*, *L. longbeachae* and *L. micdadei* using conserved primers and multiple probes in a multiplex real-time PCR assay☆

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ABSTRACT

Legionnaires' disease is a severe respiratory disease that is estimated to cause between 8,000 and 18,000 hospitalizations each year, though the exact burden is unknown due to under-utilization of diagnostic testing. Although *Legionella pneumophila* is the most common species detected in clinical cases (80-90%), other species have also been reported to cause disease. However, little is known about Legionnaires' disease caused by these non-*pneumophila* species. We designed a multiplex real-time PCR assay for detection of all *Legionella* spp. and simultaneous specific identification of four clinically-relevant *Legionella* species, *L. anisa, L. bozemanii, L. longbeachae,* and *L. micdadei,* using 5′-hydrolysis probe real-time PCR. The analytical sensitivity for detection of nucleic acid from each target species was ≤50 fg per reaction. We demonstrated the utility of this assay in spiked human sputum specimens. This assay could serve as a tool for understanding the scope and impact of non-*pneumophila Legionella* species in human disease.

1. Introduction

Legionellae are Gram-negative bacteria ubiquitous in fresh water and soil environments (Fields, 1996; Fields et al., 2002). Their ability to inhabit and thrive in man-made water systems, such as air conditioning units, cooling towers, hot tubs, and potable water systems creates a potential hazard to human health (Fields, 1996; Fields et al., 2002; Mercante and Winchell, 2015). At least half of the ~56 known species of *Legionella* have been shown to cause disease in humans based on detection in clinical specimens, but all species are thought to have pathogenic potential (Fields et al., 2002; Muder and Yu, 2002). Inhalation of aerosolized droplets containing *Legionella* may result in the development of a severe form of pneumonia called Legionnaires' disease (LD) or a milder, non-pneumonic form known as Pontiac fever (Fields et al., 2002). According to estimates from the U.S. Centers for Disease Control and Prevention, *Legionella* infections account for 8,000 to 18,000 hospitalizations each year (Fields et al., 2002; Marston et al., 1997). *Legionella* has been implicated as the etiology in 3–14% of community-acquired pneumonia (CAP) cases that require admission into the intensive care unit (File et al., 1998; Stout and Yu, 1997; Waterer et al., 2001). Determination of the true burden of disease is impacted by limited use of diagnostic assays paired with the dearth of readily available standardized diagnostic tests for non-pneumophila species.

L. pneumophila is the most commonly isolated organism from clinical cases of LD in the United States, accounting for up to 90% of cases (Benin et al., 2002; Yu et al., 2002). However, other serogroups and numerous other species of *Legionella* have been implicated in clinical cases (Benin et al., 2002; Muder and Yu, 2002; Yu et al., 2002). *L. longbeachae*, *L. micdadei*, and *L. bozemanii* together account for the majority of non-*pneumophila* LD cases, although the distribution may vary by geography and patient population (Benin et al., 2002; McNally et al., 2000; Mercante and Winchell, 2015; Muder and Yu, 2002; Yu et al., 2002). Though rarely isolated as the primary pathogen from clinical cases of pneumonia, *L. anisa* is frequently found along with *L. pneumophila* in hospital water systems and could serve as a surrogate indicator for increased outbreak risk (van der Mee-Marquet et al., 2006).

Bacterial culture directly from primary specimens remains the reference standard for detection of *Legionella* spp.; however, this method can take several weeks and is not feasible for identification of acute infection (Fields et al., 2002; Lee et al., 1993; Mercante and Winchell, 2015; Muder and Yu, 2002). Although many serology-based tests are still widely used, these suffer from limited specificity, lack of standardization, and subjective nature of interpretation, and thus have not been validated for diagnostic use (Mercante and Winchell, 2015). Furthermore, these assays are typically limited to detection of only

Abbreviations: LD, Legionnaires' disease; Lp1, *Legionella pneumophila* serogroup 1; CAP, Community-acquired pneumonia; LOD, Limit of detection.

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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L. pneumophila, and, therefore, are inadequate for identification of infection with non-*pneumophila* species (Fields et al., 2002; Mercante and Winchell, 2015; Muder and Yu, 2002). In comparison, PCR has been shown to be a rapid and reliable method for detection of *Legionella* in lower respiratory specimens and thus has emerged as a preferred diagnostic strategy (Diederen, 2008; Murdoch, 2003). One recent study demonstrated that systematic screening of respiratory specimens from patients with a clinical diagnosis of pneumonia improved case detection, particularly of milder cases (Murdoch et al., 2013). Still, PCR is not widely and systematically implemented for diagnostic testing, and detection of nucleic acid in a lower respiratory specimen has not yet been recognized as sufficient laboratory evidence for confirmation of a legionellosis case in the United States or Europe (Mercante and Winchell, 2015).

The urinary antigen enzyme immunoassay (EIA) (Binax Legionella Urinary Antigen EIA kit, Alere, Waltham, MA) is the primary method used for diagnosis of *Legionella* infections in the United States and the European Union, and a positive result using this method is widely considered confirmatory laboratory evidence for diagnosis of legionellosis (Benin et al., 2002; Den Boer and Yzerman, 2004; Dominguez et al., 1998; Lepine et al., 1998; Mercante and Winchell, 2015). A critical limitation of this method is that only the most prevalent species and serogroup of *Legionella*, *L. pneumophila* serogroup 1 (Lp1), is detected using this assay, thus precluding diagnosis of non-Lp1 and non-*pneumophila* LD cases. Currently, PCR and sequencing of the *mip* and 16S genes are the primary molecular methods available for identification of non-*pneumophila* species (Cloud et al., 2000; Ratcliff et al., 1998; Svarrer and Uldum, 2012), but these methods are typically only performed at specialized reference laboratories and do not yield results in a sufficiently rapid manner to inform patient clinical management.

In the current study, we describe a novel, rapid, single-tube multiplex real-time PCR assay for detection of all *Legionella* species and simultaneous specific identification of clinically relevant non-*pneumophila* species, including *L. bozemanii*, *L. longbeachae*, *L. anisa* and *L. micdadei*. We demonstrate the utility of this assay for detection of the four targeted *Legionella* species in mock human sputum specimens. This assay represents an extension of PCR methods for rapid detection of targeted non-*pneumophila Legionella* species and could serve as a tool for understanding the scope and impact of these species in human disease.

2. Materials and methods

2.1. Bacterial strains/isolates and nucleic acid extraction

Representative isolates of 50 available *Legionella* species (Supplementary Table 1) and isolates from clinical specimens (n = 29) or environmental samples (n = 34) were obtained from collections at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA. *Legionella* were grown on buffered charcoal yeast extract agar, and nucleic acid was extracted using the MagNA Pure Compact instrument (Roche Applied Science, Indianapolis, IN) with total nucleic acid isolation kit I according to manufacturer's instructions. All extracted nucleic acid templates were normalized to 1 ng/μL.

2.2. Primer and probe design

Primers were designed manually or using Primer Express v3.0.1 (Thermo Fisher Scientific, Waltham, MA) based on alignment of the 23S-5S intergenic spacer region for all *Legionella* species provided by Grattard et al. (Grattard et al., 2006). Primers were designed to anneal specifically to a highly conserved region within the genome of all *Legionella* species, and five unique 5′ hydrolysis probes were designed within this ~200 basepair region for detection of any *Legionella* species and specific identification of *L. anisa*, *L. bozemanii*, *L. longbeachae*, and *L. micdadei*. Sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and primers and probes were chosen for compatible melting temperatures, ideal G-C content, and minimal cross and self-complementarity. The final selected sequences and modifications are shown in Table 1. All oligonucleotides were manufactured by Integrated DNA Technologies (Coralville, IA) with HPLC purification. All assays were initially tested for oligonucleotide dimerization and cross-reactivity by testing water as template (no template control (NTC), n = 68).

2.3. Mastermix and run conditions

The ideal annealing temperature was determined by performing a gradient PCR followed by a 1% ethidium bromide gel analysis. Various primer and probe concentrations were tested to identify the optimal ratio of oligonucleotides in the reaction mix. Each 25 μL multiplex reaction contained 12.5 μL of PerfeCta® MultiPlex qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD), 150 nm each of the forward and reverse primer, 50 nm each of the *L. bozemanii* (ROX) and *L. anisa* (HEX) probes, 25 nm each of the *L. micdadei* (Cy5) and *L. longbeachae* (Quas705) probes, and 100 nm of the pan-*Legionella* (FAM) probe; 5 μL of normalized template was used in each reaction. All reactions were run using the Rotor-Gene Q instrument (Qiagen, Venlo, Netherlands) with the following cycling conditions: 5 minute denaturation at 95 °C followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds with data acquisition in all five channels during the last step in each cycle.

2.4. Analytical sensitivity and specificity

The limit of detection (LOD) and assay efficiency were determined for each target, and values were compared between reactions in which the mastermix included only the primers and the single probe specific for the target being tested (singleplex) and reactions in which all five probes were included (multiplex). The LOD was determined for each assay format by testing a series of six ten-fold dilutions of nucleic acid from each targeted species (100 pg to 1 fg per reaction). The LOD was identified as the lowest dilution at which amplification was observed in at least 50% of 10 replicates. Graphs were created using the Rotor-Gene Q analysis software where log (DNA concentration) is on the x-axis and Crossing threshold (Ct) value is on the y-axis, and reaction efficiencies were calculated based on the slope of the standard curve.

Pan-*Legionella* primers and probe were tested against 50 available *Legionella* species, including multiple serogroups of each species, if applicable (n = 67, Supplementary Table 1). A panel of viral and bacterial targets commonly found in lower respiratory tract specimens or environmental samples were tested with the multiplex assay at a concentration of 5 ng per reaction, including: *Candida albicans, Chlamydophila pneumoniae, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Moraxella catarrhalis, Neisseria meningitidis, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Chlamydia psittaci, Lactobacillus plantarum, Neisseria elongata, Ureaplasma urealyticum,* human metapneumovirus, human parainfluenza virus 1–4, *Bordetella pertussis, Mycoplasma pneumoniae,* respiratory syncytial virus (RSV), human enterovirus, and rubella

Table 1
Primer and probe sequences.

Primer/Probe Name	Sequence (5′→3′)
Pan-*Legionella* F primer	GTACTAATTGGCTGATTGTCTTG
Pan-*Legionella* R primer	TTCACTTCTGAGTTCGAGATGG
Pan-*Legionella* Probe	FAM-CGCTATRGTCGCCAGGAAA-MGBNFQ
L. micdadei Probe	Cy5-AGCTGATTGGTTAATAGCCCAATCGG-BHQ_2
L. anisa Probe	HEX-CTCAACCTACGCAGAACTACTTGAGG-BHQ_1
L. bozemanii Probe	ROX-TACGCCCATTCATCATGCAAACCAGTTT-BHQ_2
L. longbeachae Probe	Quasar705-CTGAGTATCATGCCAATAATGCGCGC-BHQ_3

MGBNFQ, Minor Groove Binder non-fluorescent quencher.
BHQ, Black Hole Quencher.

virus. Human genomic DNA (Promega Corporation, Madison, WI; 5 ng per reaction) was also tested.

2.5. Mock clinical specimen testing

Pooled human sputa (BioreclamationIVT, Hicksville, NY) were homogenized, incubated with 8 mM DTT (Thermo Fisher Scientific, Waltham, MA) at room temperature for 30 minutes, and extracted as described in Section 2.1. The pooled sputa were then screened for the presence of *Legionella* species and other respiratory pathogens using the TaqMan Array Card (TAC) (Thermo Fisher Scientific, Waltham, MA) as previously described (Diaz et al., 2013). Culture stocks of *Legionella* were quantified by measuring optical density and comparing to a standard curve. Quantified culture stocks of *Legionella* species were spiked into 400 μL aliquots of the pooled human sputum or water in order to simulate a clinical specimen containing 60, 40, 20, 10 or 1 CFU. Mock specimens were homogenized, pre-treated with dithiothreitol (DTT), and extracted as described in Section 2.1 eluting 100 μL from 400 μL. Mixed specimens were generated by spiking 40 CFU/mL of *L. pneumophila* sg1 along with 20 CFU/mL of *L. micdadei*, *L. longbeachae*, *L. anisa*, or *L. bozemanii* in order to assess the ability to detect the less common species in the presence of excess *L. pneumophila*.

3. Results

3.1. Analytical sensitivity and specificity

All *Legionella* strains tested (n = 67, Supplementary Table 1) were detected with the pan-species probe, and each representative isolate of *L. anisa* (n = 1), *L. bozemanii* (n = 2), *L. longbeachae* (n = 2), and *L. micdadei* (n = 1) was detected in the appropriate channel corresponding to the species-specific probe reporter dye. No cross-reactivity was detected between the five probes (data not shown). No amplification was observed in any channel for other bacteria (n = 17) or viruses (n = 6) tested (data not shown). The LOD was 10 fg per reaction in both singleplex and multiplex reaction formats for *L. bozemanii* (Fig. 1A) and *L. micdadei* (Fig. 1B). The LOD for *L. longbeachae* (Fig. 1C) and *L. anisa* (Fig. 1D) was 50 fg in the singleplex reaction format and 10 fg in the multiplex reaction. The LOD of each targeted species and *L. pneumophila* using the pan-*Legionella* probe was 10 fg per reaction (Fig. 2).

3.2. Comparison of singleplex and multiplex assay efficiencies

Efficiency of each assay in the multiplex reaction was ≥94%, with *L. longbeachae* having the highest efficiency (99%), followed *by L. micdadei* (97%), *L. anisa* (96%), and *L. bozemanii* (94%) (Fig. 1). The reaction efficiencies for *L. anisa* and *L. bozemanii* were higher in singleplex than multiplex format whereas the *L. micdadei* and *L. longbeachae* assays had slightly lower efficiencies in singleplex compared to multiplex (Fig. 1). The efficiency of the pan-*Legionella* assay was ≥92% for each of the five species tested (*L. pneumophila*, *L. anisa*, *L. bozemanii*, *L. longbeachae*, and *L. micdadei*, Fig. 2).

3.3. Clinical and environmental isolate testing

Isolates from clinical specimens (n = 29) and environmental samples (n = 34) previously identified as *L. micdadei* (n = 10), *L. longbeachae* (n = 12), *L. anisa* (n = 33), or *L. bozemanii* (n = 8) were

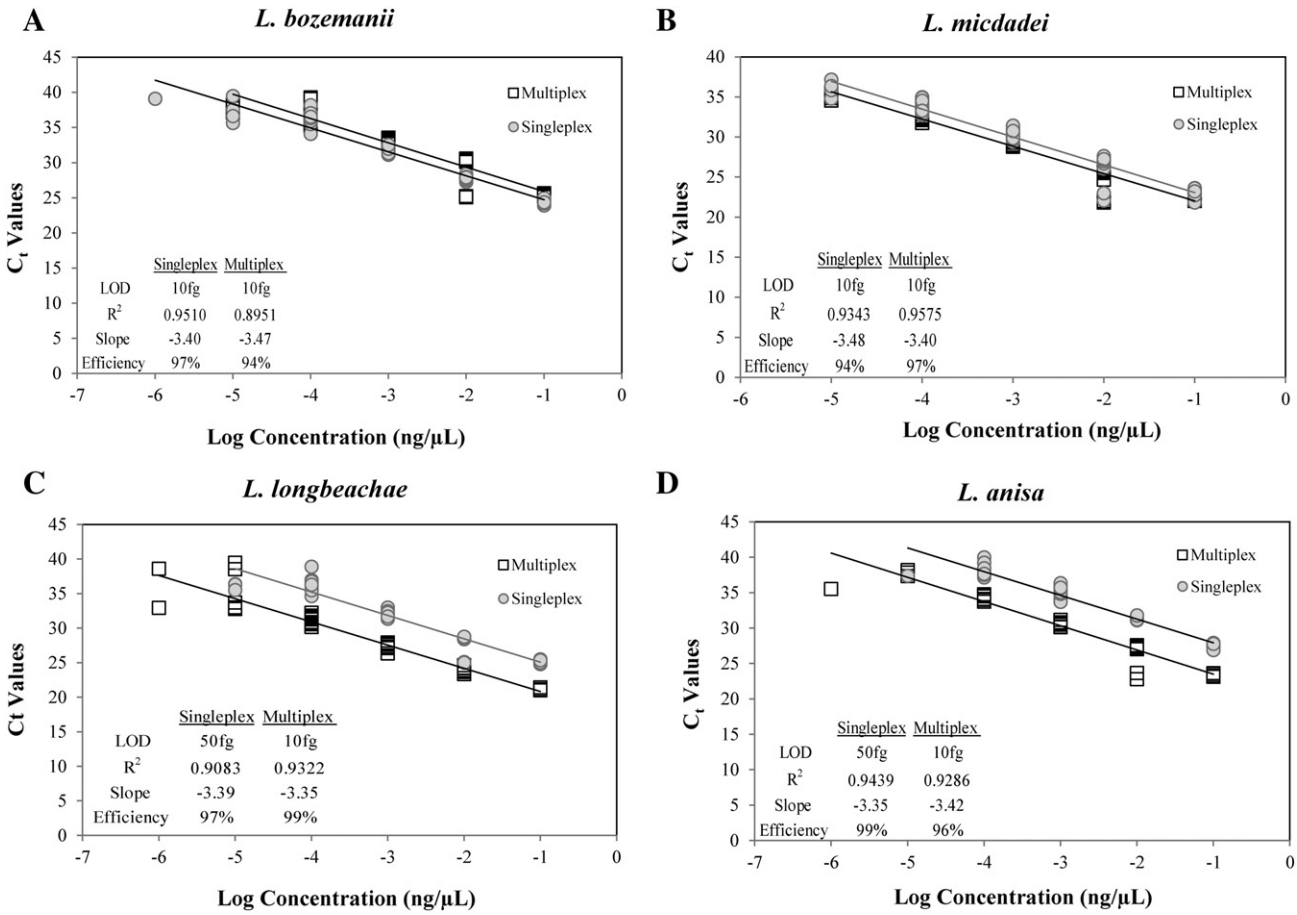
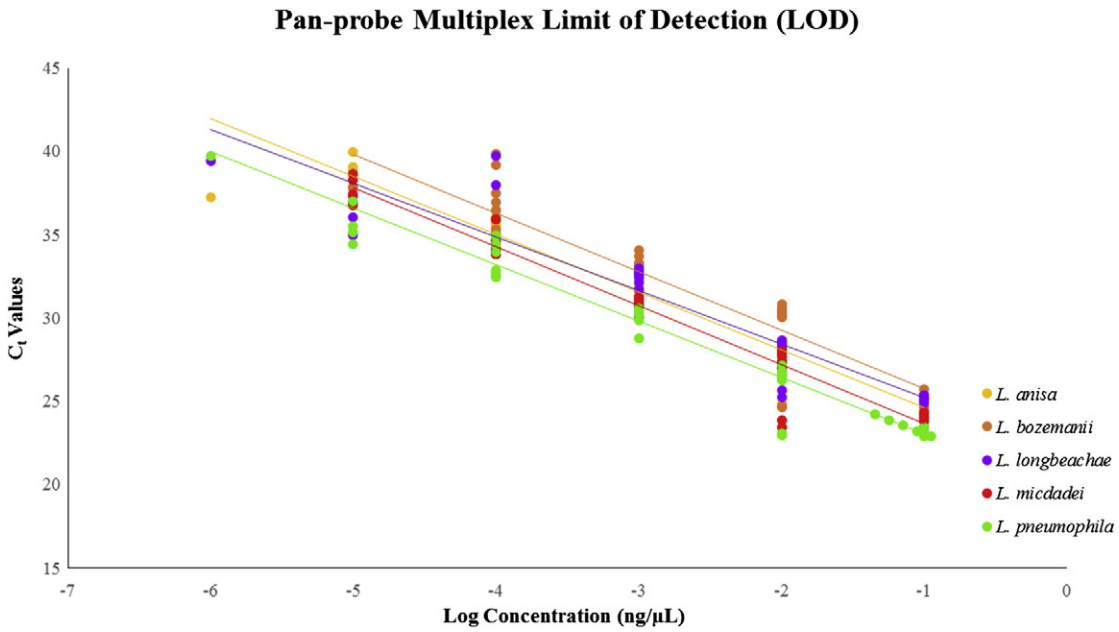


Fig. 1. Amplification efficiency of *L. bozemanii* (A), *L. micdadei* (B), *L. longbeachae* (C), and *L. anisa* (D) in singleplex (grey circles) and multiplex (white squares) reaction formats. A line of best fit is shown for both singleplex (grey) and multiplex (black) results. Data shown are ten replicate reactions at each concentration.



	L. anisa	*L. bozemanii*	*L. longbeachae*	*L. micdadei*	*L. pneumophila*
LOD	10fg	10fg	10fg	10fg	10fg
R^2	0.9721	0.9368	0.9447	0.9788	0.9733
Slope	-3.47	-3.52	-3.22	-3.54	-3.93
Efficiency	94%	92%	105%	92%	97%

Fig. 2. Limit of detection and efficiency of pan-*Legionella* probe detection in multiplex for *L. anisa* (yellow), *L. bozemanii* (orange), *L. longbeachae* (purple), *L. micdadei* (red) and *L. pneumophila* (green). Data shown are ten replicate reactions at each concentration.

tested using the multiplex assay (Table 2). All isolates showed amplification of the pan-*Legionella* target region in the green channel, and each targeted species displayed amplification only in the channel corresponding to the species-specific hydrolysis probe reporter dye. Results of the multiplex PCR assay matched the species previously identified by sequencing the *mip* gene for all isolates.

3.4. Mock clinical specimen testing

Because primary clinical specimens for the targeted *Legionella* spp. were lacking, mock specimens were generated by spiking varying concentrations of *Legionella* into pooled human sputa. Unspiked sputum did not contain *Legionella* spp. or any other organisms included in the testing panel used here (data not shown). The specimen with the lowest pathogen load (1 CFU) was detected for all four species, and the LOD of each species was similar for nucleic acid extracted from spiked sputum or water (Supplementary Table 2). The Ct values for detection of each of the four targeted *Legionella* species were comparable in the presence or absence of excess *L. pneumophila* (data not shown).

4. Discussion

Diagnosis of legionellosis caused by non-*pneumophila* species is limited by the lack of available diagnostic methods for testing of clinical specimens. We developed a multiplex real-time PCR assay for detection of four clinically-relevant non-*pneumophila* species in a rapid and reliable manner. This assay enables detection of non-*pneumophila* *Legionella* species without post-PCR processing or sequencing through the use of one set of conserved primers along with five uniquely-labeled probes. Typically, multiplex real-time PCR assays require three oligonucleotides (two primers and one probe) for each target in the reaction. By targeting the 23S-5S intergenic spacer region, which has both conserved and variable regions, we were able to amplify a single target region in any *Legionella* spp. and detect fluorescent signal from each uniquely-labeled probe when bound to its species-specific target. This approach minimizes the number of oligonucleotides in the reaction mix, reducing the potential for cross-reactivity. This assay could be modified to allow detection of other *Legionella* species of interest by designing a species-specific hydrolysis probe within the target region and re-evaluating the multiplex assay performance. This would allow for customization of the assay to interrogate specimens for the most common non-*pneumophila* species, which may vary substantially between different geographic regions or specific populations.

This assay was designed to complement or augment existing screening recommendations, which include culture paired with the urinary antigen test for Lp1 (Fields et al., 2002; Mercante and Winchell, 2015). Increasing confidence in the reliability of PCR for diagnosis of LD is likely to result in a shift toward nucleic acid detection methods for *Legionella*. To this end, we previously described a multiplex PCR assay to detect all *Legionella* species, *L. pneumophila,* and *L. pneumophila* serogroup1 (Benitez and Winchell, 2013). The current assay was designed to be used as a follow-up test for any isolate or specimen in which non-*pneumophila* *Legionella* may be identified. More recently, we reported a multiplex real-time PCR high-resolution melt (PCR-HRM) assay to be used for detection and typing of non-*pneumophila* *Legionella* spp., including *L. micdadei*, *L. bozemanii*, *L. dumoffii*, *L. longbeachae*, *L. feeleii*, *L. anisa*, *L. parisiensis*, *L. tucsonensis* serogroup (sg) 1 and 3, and *L. sainthelensis* sg 1 and 2 isolates (Benitez and Winchell, 2016). While

Table 2
Detection of clinical and environmental *Legionella* isolates (n=63) with the multiplex assay in the current study.

Source	*Legionella* spp. (n=63)	*L. micdadei* (n=10)	*L. longbeachae* (n=12)	*L. anisa* (n=33)	*L. bozemanii* (n=8)
	Ct value	Ct value	Ct value	Ct value	Ct value
BAL	19.01	17.39	-	-	-
bronchial wash	20.19	18.01	-	-	-
sputum	20.11	18.22	-	-	-
BAL	20.7	18.67	-	-	-
lesion	21.44	18.85	-	-	-
bronchial wash	20.52	18.5	-	-	-
bronchial wash	19.97	17.84	-	-	-
environmental	19.45	17.44	-	-	-
environmental	20.48	17.95	-	-	-
environmental	19.5	17.36	-	-	-
human, unspecified	19.48	-	18.54	-	-
bronchial wash	18.73	-	18.06	-	-
BAL	20.47	-	19.79	-	-
human, unspecified	21.86	-	21.05	-	-
human, unspecified	20.96	-	20.43	-	-
sputum	20.8	-	20.08	-	-
bronchial wash	20.28	-	19.65	-	-
bronchial wash	19.21	-	18.33	-	-
bronchial wash	19.63	-	18.94	-	-
BAL	22.26	-	21.33	-	-
bronchial wash	21.25	-	20.47	-	-
bronchial wash	19.35	-	18.82	-	-
environmental	17.57	-	-	20.32	-
environmental	18.77	-	-	21.30	-
human, unspecified	20.00	-	-	22.01	-
environmental	18.32	-	-	20.58	-
environmental	18.42	-	-	21.10	-
environmental	18.73	-	-	21.14	-
environmental	18.23	-	-	20.84	-
environmental	19.13	-	-	21.20	-
bronchial wash	24.07	-	-	26.95	-
environmental	20.02	-	-	22.37	-
environmental	17.18	-	-	19.65	-
environmental	16.34	-	-	19.11	-
environmental	17.37	-	-	20.75	-
environmental	19.47	-	-	22.13	-
environmental	19.69	-	-	21.61	-
environmental	16.89	-	-	19.83	-
environmental	20.35	-	-	21.91	-
environmental	19.91	-	-	21.71	-
environmental	19.46	-	-	21.69	-
environmental	19.21	-	-	21.50	-
environmental	18.95	-	-	21.01	-
environmental	20.18	-	-	22.69	-
environmental	23.56	-	-	24.60	-
environmental	18.32	-	-	20.88	-
environmental	19.09	-	-	19.93	-
environmental	21.86	-	-	23.79	-
environmental	21.76	-	-	25.10	-
environmental	19.88	-	-	22.62	-
environmental	20.99	-	-	24.82	-
environmental	16.94	-	-	18.84	-
environmental	18.55	-	-	21.12	-
environmental	19.46	-	-	22.66	-
environmental	21.21	-	-	24.31	-
bronchial wash	21.14	-	-	-	20.90
BAL	21.53	-	-	-	19.20
human, unspecified	21.92	-	-	-	18.81
BAL	19.79	-	-	-	20.51
BAL	20.74	-	-	-	17.78
BAL	24.18	-	-	-	19.52
BAL	21.26	-	-	-	19.81
BAL	20.93	-	-	-	18.65

Ct, Crossing threshold.
BAL, bronchoalveolar lavage.

the PCR-HRM assay allows for identification of a higher number of species, it requires more specialized equipment, operator training, and a longer run time compared to the multiplex hydrolysis probe assay described here. Each method may be more suitable for different types of laboratories in academic, clinical, and public health sectors depending on the demand for *Legionella* test offerings and issues related to compliance with regulations for patient testing, among other laboratory-specific considerations. Implementation of real-time PCR assays such as these could be used to create a new diagnostic algorithm that would facilitate identification of LD cases caused by both *L. pneumophila* and other less common, yet clinically significant, *Legionella* species.

Numerous recent studies suggest that *Legionella* species and serogroups other than Lp1 are responsible for a substantial portion of clinical cases, thus supporting the need for new diagnostic approaches capable of broader detection of *Legionella*, such as the assay described here. Among cases investigated by the U.S. CDC between 1980 and 1989 from which an isolate was recovered from a clinical specimen, approximately 10% were caused by species other than *L. pneumophila* (Marston et al., 1994). *L. micdadei* and *L. bozemanii* are frequently isolated during LD in immunocompromised patients (Doebbeling et al., 1989; Fang et al., 1989; Humphreys et al., 1992; Knirsch et al., 2000; McNally et al., 2000; Parry et al., 1985). *L. longbeachae* was the predominant *Legionella* species identified among patients with severe pneumonia in Thailand in 2004 (Phares et al., 2007) and is reported as a cause of LD as often as *L. pneumophila* in Australia (Group NARW, 2013; Yu et al., 2002). Incidence of LD due to *L. longbeachae* has also increased in countries where it was previously unreported, including Japan, Thailand, Scotland and the Netherlands (Den Boer and Yzerman, 2004; Koide et al., 2001; Paveenkittiporn et al., 2012; Pravinkumar et al., 2010), in some places becoming even more prevalent than *L. pneumophila* (Whiley and Bentham, 2011). In 2000 the U.S. CDC reported the first case of *L. longbeachae* transmission from potting soil occurring in the United States (Centers for Disease Control and Prevention (CDC), 2000), but the true burden of disease attributable to *L. longbeachae* in the United States is not known.

Augmentation of current diagnostic methods is needed in order to fully appreciate the contribution of various *Legionella* species to the global burden of LD. The replacement of culture-based methods with the urinary antigen test as the primary diagnostic method may actually mask the true incidence of LD cases caused by other serogroups of *L. pneumophila* and non-*pneumophila* species due to the limited specificity of this test for detection of Lp1 only. Benin and colleagues reported a decrease from 28% to 4% in the frequency of isolates other than Lp1 from 1980 to 1998, during which time urine antigen testing emerged as the primary diagnostic method (Benin et al., 2002). The importance of the urine antigen test cannot be overstated; however, in cases of suspected legionellosis in which this screening is negative, diagnostic testing should be expanded to include non-*pneumophila* species.

Identification of the species causing LD during an outbreak is crucial to the protection of the public, particularly in the cases of *Legionella* proliferation in hospital water systems. Public health officials require both environmental samples and clinical specimens in order to determine the species and strain causing an LD outbreak. Our assay was designed to include a probe for the detection of *L. anisa*, as it is the most frequently isolated species from hospital water systems, often found along with *L. pneumophila* (van der Mee-Marquet et al., 2006). In some reported outbreaks attributed to *L. pneumophila* based on detection in clinical specimens, *L. anisa* has been the only *Legionella* species detected in the potable water (van der Mee-Marquet et al., 2006). In these situations, it is hypothesized that *L. pneumophila* was the minority population and therefore was beyond the limit of detection of the testing methods. The abundance of *L. anisa* in water systems makes it a potentially useful surrogate indicator of the presence of *L. pneumophila*. Additional testing will be necessary to evaluate the utility of the current assay for detection of *L. anisa* and other *Legionella* species in environmental samples.

This study has a few limitations, most notably the lack of available primary clinical specimens to evaluate the new assay. We attempted to closely approximate such specimens by introducing varying amounts of *Legionella* into real human sputum. In addition, like all nucleic acid amplification tests, this assay cannot distinguish viable from non-viable *Legionella* present in a sample. While the positive predictive value for detection of *Legionella* in lower respiratory specimens by PCR is very high, the detection of non-viable organisms could complicate the interpretation of positive results obtained from environmental samples and impact recommended remediation efforts. Further evaluation is needed to fully define the performance characteristics of this assay for testing respiratory specimens from LD cases as well as environmental samples.

5. Conclusions

We developed a novel multiplex real-time PCR assay that allows detection of all *Legionella* species while simultaneously distinguishing four of the most commonly isolated non-*pneumophila* species. This assay fills a need for detection of clinically relevant non-*pneumophila* species of *Legionella* to complement existing methods for diagnosis of LD. Implementation of this technique could lead to improved detection of infections caused by non-*pneumophila* species of *Legionella*, contribute to more rapid outbreak recognition and response, and improve our understanding of the scope and impact of non-*pneumophila Legionella* species in human disease.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.diagmicrobio.2016.03.022.

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