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Rapid detection and typing of pathogenic nonpneumophila *Legionella* spp. isolates using a multiplex real-time PCR assay

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ABSTRACT

We developed a single tube multiplex real-time PCR assay that allows for the rapid detection and typing of 9 nonpneumophila *Legionella* spp. isolates that are clinically relevant. The multiplex assay is capable of simultaneously detecting and discriminating *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. Evaluation of the assay with nucleic acid from each of these species derived from both clinical and environmental isolates and typing strains demonstrated 100% sensitivity and 100% specificity when tested against 43 other *Legionella* spp. Typing of *L. anisa*, *L. parisiensis*, and *L. tucsonensis* sg 1 and 3 isolates was accomplished by developing a real-time PCR assay followed by high-resolution melt (HRM) analysis targeting the *ssrA* gene. Further typing of *L. bozemanii*, *L. longbeachae*, and *L. feeleii* isolates to the serogroup level was accomplished by developing a real-time PCR assay followed by HRM analysis targeting the *mip* gene. When used in conjunction with other currently available diagnostic tests, these assays may aid in rapidly identifying specific etiologies associated with *Legionella* outbreaks, clusters, sporadic cases, and potential environmental sources.

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1. Introduction

Legionellae are facultative intracellular gram-negative bacteria found ubiquitously in water and soil environments (Fields et al., 2002; Mercante and Winchell, 2015). Being an intracellular organism, Legionellae are capable of replicating in environmental protozoa as well as within alveolar macrophages and epithelial cells (Cianciotto et al., 1990; Fields, 1996; Newton et al., 2010). Legionellae are common contaminates of natural and man-made water systems, including cooling towers, air conditioning systems, fountains, and whirlpools, where conditions may be ideal for growth and propagation (Kozak et al., 2013; Steinert et al., 2002). 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 (Fields et al., 2002).

Over 60 *Legionella* spp. comprising 70 distinct serogroups have been identified to date (http://www.bacterio.cict.fr/l/legionella.html). Although the majority of cases of Legionnaires' disease are caused by *Legionella pneumophila*, nearly one-half of all *Legionella* spp. have been associated with human disease. It is possible that under the appropriate conditions, immunocompromised individuals can be infected with any *Legionella* spp. (Fields et al., 2002). Infections caused by *Legionella* spp. other than *L. pneumophila* are probably not diagnosed regularly due to limitations of current diagnostic methods which are biased toward the detection of *L. pneumophila*. *L. pneumophila* is the species most frequently isolated from water distribution systems, but *Legionella micdadei*, *Legionella bozemanii*, *Legionella dumoffii*, *Legionella anisa*, and *Legionella feeleii* are also isolated relatively frequently (Best et al., 1983; Bornstein et al., 1985; Joly et al., 1986; Lowry et al., 1991; Palutke et al., 1986; Parry et al., 1985). Worldwide, of the reported nonpneumophila infections, *L. micdadei* accounts for 60% of the cases; *L. bozemanii*, for 15%; *L. dumoffii*, for 10%; *Legionella longbeachae*, for 5%; and other species, for 10% (Amodeo et al., 2010; Benson et al., 1990; Fang et al., 1989; Herwaldt et al., 1984; Lee et al., 2009; Reingold et al., 1984; Yu et al., 2002). However, global incidence of *Legionella* infection surveillance data should be interpreted carefully as it is underrecognized in many countries because of the lack of diagnostics and surveillance systems.

The current gold standard for typing of isolated *Legionella* spp. is based on PCR amplification followed by sequencing of the macrophage infectivity potentiator gene (*mip*) (Ratcliff et al., 1998). Although reliable, this method is time consuming and requires specialized training. In this study, we developed a rapid, single-tube real-time multiplex PCR assay capable of identifying 9 nonpneumophila *Legionella* spp.: *L. micdadei*, *L. bozemanii*, *L. dumoffii*, *L. longbeachae*, *L. feeleii*, *L. anisa*, *Legionella parisiensis*, *Legionella tucsonensis* sg 1 and 3, and *Legionella sainthelensis* sg 1 and 2 isolates. The assay was designed to identify 5 of the most prevalent nonpneumophila species involved in human disease (*L. micdadei*, *L. bozemanii*, *L. dumoffii*, *L. longbeachae*, and *L. feeleii*). However, we were able to exploit primer/probe cross-reactivity to

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identify an additional 4 nonpneumophila species (*L. anisa*, *L. parisiensis*, *L. tucsonensis* sg 1 and 3, and *L. sainthelensis* sg 1 and 2) that, although rare, have also been identified as cause of human disease. Additional follow-up high-resolution melt (HRM) assays were developed to discriminate nucleotide polymorphisms within specific targets to differentiate *L. anisa*, *L. parisiensis*, and *L. tucsonensis* sg 1 and sg 3 isolates and serogroups for *L. bozemanii*, *L. longbeachae*, and *L. feeleii* isolates. Identification of specific serogroups could be valuable when trying to pinpoint the source of infection during outbreak investigations.

2. Materials and methods

2.1. Primer and probe design

PrimerQuest® Software (Integrated DNA Technologies, Coralville, IA, USA) was used to design multiple TaqMan® primer-probe sets targeting *gyrB* (*L. bozemanii*), *legS2* (*L. dumoffii*), *figA* (*L. feeleii*), *ligB* (*L. longbeachae*), and the *migB* genes (*L. micdadei*). Primer sets for real-time PCR-HRM typing assays were designed to target the *mip* gene (*L. bozemanii*, *L. feeleii*, and *L. longbeachae*) and the *ssrA* gene (*L. anisa*, *L. parisiensis*, and *L. tucsonensis* sg 1 and 3). Primer and probe sequences, GenBank accession number, final concentrations, and the distinct fluorophores for each probe used in the multiplex and HRM assays are listed in Table 1. Primers and probe sets were initially tested and optimized in singleplex format (data not shown).

2.2. Multiplex real-time PCR assay

The multiplex reaction contained 12.5 μL of Quanta PerfeCTa™ Multiplex qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), 0.5 μL of each primer and probe (Table 1), 5 ng of template, and nuclease-free water to a final volume of 25 μL. The assay was performed on the Rotor-Gene Q (Rotor-Gene 6000) (Qiagen, Valencia, CA, USA) instrument with the following thermocycling conditions: 95 °C for 5 minutes followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute (data acquisition in all 5 channels). Channel gain settings were set at green (Fields, 1996), yellow (Cianciotto et al., 1990), orange (Newton et al., 2010), red (Mercante and Winchell, 2015), and crimson (Cianciotto et al., 1990). All samples were tested in duplicate. Primers and probe sets were initially tested and optimized in singleplex format (data not shown).

2.3. Real-time PCR-HRM assay

The *ssrA* and *mip* real-time PCR-HRM assays were prepared using the Universal SYBR GreenER qPCR kit (Life Technologies, Grand Island, NY, USA), containing the following components per reaction: 12.5 μL of 2× master mix, 0.5 μL of each primer (Table 1), 1 μL 10 mmol/L dNTP mix, 0.25 μL of Platimun Taq polymerase, 5 ng of template, and nuclease-free water to a total final volume of 25 μL. All HRM assays were performed on the Rotor-Gene Q instrument with the following thermocycling conditions: 95 °C for 5 minutes followed by 35 cycles of 95 °C for 15 seconds and 60 °C for 1 minute (data acquisition in green channel). Following amplification, HRM was performed between 74 °C and 84 °C (*ssrA*) and 68 °C and 82 °C (*mip*) at a rate of 0.03 °C per step. HRM normalizing regions for each assay were as follows: *ssrA* 77 °C–78 °C/81 °C–82 °C, *L. bozemanii* (*mip*) 70.5 °C–71.5 °C/79 °C–80 °C, *L. feeleii* (*mip*) 71.5 °C–72.5 °C/78.5 °C–79.5 °C., and *L. longbeachae* (*mip*) 73.5 °C–74.5 °C/78.5 °C–79.5 °C. All samples were tested in duplicate. Direct fluorescent antibody (DFA) and *mip* sequencing procedures to confirm HRM results were performed as previously described (Katz, 1985; Ratcliff et al., 1998).

2.4. Analytical specificity, sensitivity, isolates, and nucleic acid extraction

Analytical specificity was verified using a comprehensive panel of 52 *Legionella* spp. type strains representing most species and serogroups along with 21 *L. micdadei*, 28 *L. bozemanii*, 21 *L. dumoffii*, 80 *L.*

Table 1
Primers and probes for multiplex real-time and HRM detection/typing of nonpneumophila *Legionella* spp.

Primer/probe	Sequence (5′→3′)	Species target	Gene target	GenBank accession no.	Primer/probe final concentration
bozemanii-F	TCCGCTGCTGAAGTGATTATG	*L. bozemanii*, *L. anisa*,			125 nmol/L
bozemanii-R	CATGCAAACCACCCGATACT	*L. parisiensis*,	*gyrB*	HQ717438	125 nmol/L
bozemanii-P3	Q705-AAATTTACCACCGGCGTGAAGCAC-BHQ3	*L. tucsonensis* sg 1 and 2			25 nmol/L
dumoffii-F	CAGGAAAGCGCGACATCTAT				250 nmol/L
dumoffii-R	ATCCAGCTCGTTCGCAATAA	*L. dumoffii*	*legS2*	EU107519	250 nmol/L
dumoffii-P	HEX-TGGAAACCCTCAATGGTCCGTTCT-BHQ1				50 nmol/L
feeleii-F	AACCGGTTTATCGGTCTTT				125 nmol/L
feeleii-R	ATCAACCAGCTTGTCTCG	*L. feeleii*	*figA*	AY753535	125 nmol/L
feeleii-P2	TEX615-AGCTTGAGAATTTGATGGATTATCACTCGC-BHQ2				25 nmol/L
LLB-F1	CTGCAGAAGTTGCTGATTGTG	*L. longbeachae*,			125 nmol/L
LLB-R1	GACGTGGCGAATGACTTATCT	*L. sainthelensis* sg 1 and 2	*ligB*	AY512558	125 nmol/L
LLB-P1	Q670-TGTCGCCAAGAAGTTGTATCTCATGCT-BHQ3				25 nmol/L
micdadei-F	TGACAAGTGAGAGCAAGAGTT				125 nmol/L
micdadei-R	GTATCTATTCCGACAGCGATAGG	*L. micdadei*	*migB*	AY512559	125 nmol/L
micdadei-P	FAM-ACAGAAGGAGAACCTTCCGGTGTG-BHQ1				25 nmol/L
LLB-HRM-F	TGGCTAAGCGTAGTGCTG	*L. longbeachae*	*mip*	X83036	250 nmol/L
LLB-HRM-R	CAACAGTTACAGTATCTGATTTAC				250 nmol/L
bozemanii-HRM-F1	CCAAGCGGCTTGCAATATAA	*L. bozemanii*	*mip*	U91609	250 nmol/L
bozemanii-HRM-R1	CAAATACAGTACCATCAATTAAAGTACC				250 nmol/L
feeleii-HRM-F1	GACCTAATGGCAAAACGAAATG	*L. feeleii*	*mip*	U92205	250 nmol/L
feeleii-HRM-R	CGCCTTCTTTGGCCTTATTC				250 nmol/L
tmRNA-HRM-F	GGCGACCTGGCTTC	*L. anisa*, *L. parisiensis*,	*ssrA*	HG525464	250 nmol/L
tmRNA-HRM-R	GGTCATCGTTTGCATTTATATTTA	*L. tucsonensis* sg 1 and 2			250 nmol/L