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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.

Keywords

Real-time PCR; *Legionella* spp.; Detection; Typing; HRM Analysis

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;

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Conflict of interest

The authors declare that they have no conflicts of interest.

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 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. longbeachae*, 11 *L. feeleii*, 43 *L. anisa*, 2 *L. parisiensis*, 2 *L. tucsonensis* sg 1 and sg 3, and 20 *L. sainthelensis* sg 1 and sg 2 clinical and environmental isolates (Table 2) previously identified by serological methods and/or *mip* sequencing following previously described methods (Katz, 1985; Ratcliff et al., 1998). Analytical sensitivity was established by testing 10-fold DNA dilutions from 100 pg/μL down to 1 fg/μL from each of the 9 *Legionella* spp. detected by the assay. Each dilution

was tested in 10 replicates, and limits of detection (LODs) were established for each assay, defined as the lowest dilution in which ≥50% of replicates had positive crossing threshold (Ct) values. The Ct values were plotted against nucleic acid concentration to determine slope and assay efficiencies (%). This was calculated by determining the percentage of difference either above or below the perfect slope of −3.3 and efficiency of 100%. Isolates were selected from the CDC reference diagnostics library and grown on buffered charcoal yeast extract agar plates at 35 °C with 2.5% CO_2 for 48–72 h. Total nucleic acid was extracted using the Roche MagNA Pure Compact and/or the MagNA Pure LC instruments (Roche Applied Science, Indianapolis, IN, USA) and normalized to 1 ng/μL using the NanoDrop® ND-1000 V3.5.2 Spectrophotometer (NanoDrop products, Wilmington, DE, USA).

3. Results

3.1. Multiplex real-time PCR analysis

Real-time PCR amplification curves for detection of 9 pathogenic nonpneumophila *Legionella* spp. typing strain isolates are shown in Fig. 1. Specific amplification of *L. micdadei* (Fig. 1A), *L. dumoffii* (Fig. 1B), and *L. feeleii* (Fig. 1C) typing strains is displayed in the green (FAM), yellow (HEX), and orange (TX-RED 615) channels, respectively. Fig. 1D displays amplification curves for *L. longbeachae* and *L. sainthelensis* sg 1 and sg 2 type strains in the red (Quasar670) channel. Ct values of 17–20 are observed for *L. longbeachae* isolates, whereas *L. sainthelensis* sg 1 and *L. sainthelensis* sg 2 isolates display Ct values of 32–35 and 24–27, respectively. Fig. 1E displays amplification curves for *L. bozemanii* (Ct 17–20), *L. anisa, L. pansiensis*, and *L. tucsonensis* sg 1 and sg 3 (Ct 24–27) in the crimson (Quasar705) channel. The multiplex assay achieved 100% analytical specificity when tested against a panel composed of 300 *Legionella* spp. type strains and clinical/environmental isolates (data not shown) (Table 2). An LOD of 50 fg per reaction was established for the *L. micdadei, L. dumoffii, L. feeleii, L. longbeachae*, and *L. bozemanii* targets. An LOD of 500 fg/reaction was established for the *L. anisa, L. pansiensis*, and *L. tucsonensis* sg 1 and sg 3 targets and 500 pg and 5 pg for *L. sainthelensis* sg 1 and *L. sainthelensis* sg 2 targets, respectively. The LODs were equivalent for the singleplex and multiplex *Legionella* spp.–specific assays (data not shown). Assay efficiencies ranged from 80% to 110% depending on the target (data not shown).

3.2. Real-time PCR and HRM analysis

Speciation of *L. anisa, L. parisiensis*, and *L. tucsonensis* sg 1 and sg 3 isolates is shown in Fig. 2. HRM analysis allows for detection of each species based on the melting pattern of the 101-bp amplicon targeting the *ssrA* gene. Specific nucleotide differences at 4 different positions within the *ssrA* amplicon dictate the specific melting pattern of each species. *L. anisa* isolates displayed nucleotide bases T, T, C, and A; *L. tucsonensis* sg 1 and 3 displayed A, A, A, and A; and *L. parisiensis* displayed A, T, and C and a base pair deletion at positions 50, 67, 68, and 77 within the amplicon, respectively. A total of 43 *L. anisa*, 2 *L. parisiensis*, 1 *L. tucsonensis* sg 1, and 1 *L. tucsonensis* sg 3 isolates were tested with 100% specificity. Fig. 3A displays HRM analysis of the 112-bp amplicon targeting the *mip* gene of 26 *L. bozemanii* isolates previously serotyped. The curve melting pattern shows clear discrimination between 1 sg 1 and 13 sg 2 isolates which targets a 2-bp

difference at positions 22 and 81 within the amplicon, G and C (sg1) and A and T (sg2), respectively. An additional distinct melting pattern was observed in 12/26 isolates (sg 1 variant) previously typed by DFA as sg 1, and nucleotides A and C were identified after *mip* sequencing analysis at positions 22 and 81 within the amplicon (data not shown). Typing of *L. longbeachae* serogroups was accomplished by targeting a 2-bp difference within a 180-bp region in the *mip* gene. *L. longbeachae* sg 1 displayed nucleotide bases C and C at positions 139 and 145 within the amplicon, whereas sg 2 isolates displayed nucleotide bases A and T, respectively. Fig. 3B displays HRM analysis of 77 *L. longbeachae* isolates [sg 1 (73), sg 2 (Cianciotto et al., 1990), and sg 2 variant (Fields et al., 2002)]. Results were confirmed by DFA and *mip* sequencing analysis (data not shown). HRM analysis of a 100-bp amplicon within the *mip* gene region of 11 *L. feeleii* isolates is displayed in Fig. 3C. A single base pair nucleotide difference was targeted, and clear discrimination between 1 *L. feeleii* sg 1 isolate with nucleotide G at position 24 and 10 sg 2 isolates with nucleotide T at position 24 within the amplicon was observed. Results were confirmed by *mip* sequencing analysis (data not shown).

4. Discussion

Although *L. pneumophila* causes the majority of cases of Legionnaires' disease, the number of cases caused by other *Legionella* spp. is not well established, as the infections are difficult to diagnose due to limitations of the current diagnostic methods, which are biased toward the detection of *L. pneumophila* (Svarrer and Uldum, 2012). A study of community-acquired Legionnaires' disease identified culture-confirmed cases where *L. pneumophila* was responsible for the greatest percentage of cases (91.5%), followed by *L. longbeachae* (3.9%) and *L. bozemanii* (2.4%). The remainder of cases were due to *L. micdadei, L. feeleii, L. dumoffii, Legionella wadsworthii*, and *L. anisa* (Yu et al., 2002). Due to the lack of molecular detection diagnostics for nonpneumophila *Legionella*, we developed a multiplex real-time PCR assay for detection of 9 clinically relevant nonpneumophila *Legionella* spp. isolates, as well as real-time HRM assays to rapidly subtype *L. longbeachae, L bozemanii*, and *L. feeleii* isolates to the serogroup level. Detection of *L. longbeachae* is of particular importance outside of the United States. In Australia and New Zealand, *L. longbeachae* has been reported to be more prevalent in clinical cases than in other parts of the world (Murdoch, 2003; Whiley and Bentham, 2011). In addition to its detection capabilities, the assay allows for a time and cost-efficient alternative to *mip* sequencing, which can often take 8+ hours to complete and requires specialized equipment and training.

The current multiplex assay uses hydrolysis probe-based chemistry and targets distinct regions within the *L. dumoffii, L. feeleii*, and *L. micdadei* genomes, allowing for specific identification of these species. Primers and probes were designed to be specific for both *L. longbeachae* and *L. bozemanii* targets. Mismatches of primers and/or probes to target sequences of other *Legionella* spp. were exploited to enable additional identification. The lower assay efficiency due to primer and/or probe mismatches, along with the use of normalized nucleic acid, allows for successful discrimination of the cross-reactive species within each assay based on a differential Ct value and fluorescence levels of the amplification curve. Lower efficiency in oligo binding in the *L. longbeachae* assay allows for detection of *L. sainthelensis* sg 1 and sg 2 isolates, while the *L. bozemanii* assay can

also detect *L. anisa, L. parisiensis*, and *L. tucsonensis* sg 1 and sg 3 isolates. Although rarely isolated from clinical cases, *L. anisa, L. parisiensis*, and *L. tucsonensis* have been identified in the past as direct causes of human disease, including pneumonia and Pontiac fever (Fallon and Stack, 1990; Fenstersheib et al., 1990; Jones et al., 2003; Lo Presti et al., 1997; Thacker et al., 1989). In addition, *L. anisa* is the most frequently isolated species from hospital water systems, in conjunction with *L. pneumophila* (van der Mee-Marquet et al., 2006).

Although widely used for subtyping of *Legionella* spp., DFA assay analysis is limited by cross-reactions among *Legionella* spp. and serogroups (Mercante and Winchell, 2015; Thacker et al., 1985). Since *L. anisa, L. parisiensis*, and *L. tucsonensis* sg 1 and sg 3 amplification curves fall within the same Ct value, a follow-up real-time PCR-HRM assay was developed in order to discriminate these species based on nucleotide differences within a 101-bp amplicon in the ssrA gene, as seen in Fig. 2. We were able to rapidly distinguish *L. longbeachae*–, *L. bozemanii*–, and *L. feeleii*–specific serogroups by designing an additional real-time PCR assay followed by HRM analysis targeting a specific region of the *mip* gene (Fig. 3). This increased resolution methodology has also been used to detect single-nucleotide polymorphisms in human genes (Gundry et al., 2003; Herrmann et al., 2006) and to subtype and identify antibiotic resistance in other bacteria (Bidet et al., 2012; Mitchell et al., 2009; Wolff et al., 2008).

Although limited to detection of isolates, this assay complements existing methods for detection and could serve as a tool for identification of clinically relevant nonpneumophila *Legionella* spp. in a clinical microbiology lab. The assay allows for rapid identification of 9 *Legionella* spp., along with subtyping of *L. longbeachae, L. bozemanii*, and *L. feeleii* isolates using HRM. The assay builds upon our recently developed multiplex assay (Benitez and Winchell, 2013), thus expanding the field of molecular diagnostics for *Legionella* detection. These assays can be used to complement bacteriological culture and antigen detection, allowing rapid and specific diagnosis, especially during outbreak investigations.

References

Amodeo MR, Murdoch DR, Pithie AD. Legionnaires' disease caused by *Legionella longbeachae* and *Legionella pneumophila*: comparison of clinical features, host-related risk factors, and outcomes. Clin Microbiol Infect 2010;16:1405–7. [PubMed: 19930271]

Benitez AJ, Winchell JM. Clinical application of a multiplex real-time PCR assay for simultaneous detection of Legionella species, Legionella pneumophila, and Legionella pneumophila serogroup 1. J Clin Microbiol 2013;51:348–51. [PubMed: 23135949]

Benson RF, Thacker WL, Fang FC, Kanter B, Mayberry WR, Brenner DJ. *Legionella sainthelensi* serogroup 2 isolated from patients with pneumonia. Res Microbiol 1990;141:453–63. [PubMed: 2399367]

Best M, Yu VL, Stout J, Goetz A, Muder RR, Taylor F. Legionellaceae in the hospital water-supply. Epidemiological link with disease and evaluation of a method for control of nosocomial Legionnaires' disease and Pittsburgh pneumonia. Lancet 1983;2:307–10. [PubMed: 6135832]

Bidet P, Liguori S, Plainvert C, Bonacorsi S, Courroux C, d'Humieres C, et al. Identification of group A streptococcal emm types commonly associated with invasive infections and antimicrobial resistance by the use of multiplex PCR and high-resolution melting analysis. Eur J Clin Microbiol Infect Dis 2012;31:2817–26. [PubMed: 22610663]

Bornstein N, Vieilly C, Marmet D, Surgot M, Fleurette J. Isolation of *Legionella anisa* from a hospital hot water system. Eur J Clin Microbiol 1985;4:327–30. [PubMed: 3894018]

Cianciotto NP, Bangsborg JM, Eisenstein BI, Engleberg NC. Identification of *mip*-like genes in the genus *Legionella*. Infect Immun 1990;58:2912–8. [PubMed: 2387627]

Fallon RJ, Stack BH. Legionnaires' disease due to *Legionella anisa*. J Infect 1990;20:227–9. [PubMed: 2341733]

Fang GD, Yu VL, Vickers RM. Disease due to the Legionellaceae (other than *Legionella pneumophila*). Historical, microbiological, clinical, and epidemiological review. Medicine 1989;68:116–32. [PubMed: 2646508]

Fenstersheib MD, Miller M, Diggins C, Liska S, Detwiler L, Werner SB, et al. Outbreak of Pontiac fever due to *Legionella anisa*. Lancet 1990;336:35–7. [PubMed: 1973219]

Fields BS. The molecular ecology of legionellae. Trends Microbiol 1996;4:286–90. [PubMed: 8829338]

Fields BS, Benson RF, Besser RE. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin Microbiol Rev 2002;15:506–26. [PubMed: 12097254]

Gundry CN, G.V. J, H.R. G, J.P. R, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 2003;49:396. [PubMed: 12600951]

Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. Clin Chem 2006;52:494–503. [PubMed: 16423901]

Herwaldt LA, Gorman GW, McGrath T, Toma S, Brake B, Hightower AW, et al. A new *Legionella* species, *Legionella feeleii* species nova, causes Pontiac fever in an automobile plant. Ann Intern Med 1984;100:333–8. [PubMed: 6696354]

Joly JR, Dery P, Gauvreau L, Cote L, Trepanier C. Legionnaires' disease caused by *Legionella dumoffii* in distilled water. CMAJ 1986;135:1274–7. [PubMed: 3536052]

Jones TF, Benson RF, Brown EW, Rowland JR, Crosier SC, Schaffner W. Epidemiologic investigation of a restaurant-associated outbreak of Pontiac fever. Clin Infect Dis 2003;37:1292–7. [PubMed: 14583861]

Katz SM. Legionellosis. Boca Raton, Fla: CRC Press; 1985.

Kozak NA, Lucas CE, Winchell JM. Identification of *Legionella* in the environment. Methods Mol Biol 2013;954:3–25. [PubMed: 23150387]

Lee J, Caplivski D, Wu M, Huprikar S. Pneumonia due to *Legionella feeleii*: case report and review of the literature. Transpl Infect Dis 2009;11:337–40. [PubMed: 19392730]

Lo Presti F, Riffard S, Vandenesch F, Reyrolle M, Ronco E, Ichai P, et al. The first clinical isolate of *Legionella parisiensis*, from a liver transplant patient with pneumonia. J Clin Microbiol 1997;35:1706–9. [PubMed: 9196178]

Lowry PW, Blankenship RJ, Gridley W, Troup NJ, Tompkins LS. A cluster of *Legionella* sternal-wound infections due to postoperative topical exposure to contaminated tap water. N Engl J Med 1991;324:109–13. [PubMed: 1984176]

Mercante JW, Winchell JM. Current and emerging *Legionella* diagnostics for laboratory and outbreak investigations. Clin Microbiol Rev 2015;28:95–133. [PubMed: 25567224]

Mitchell SL, Wolff BJ, Thacker WL, Ciembor PG, Gregory CR, Everett KD, et al. Genotyping of *Chlamydophila psittaci* by real-time PCR and high-resolution melt analysis. J Clin Microbiol 2009;47:175–81. [PubMed: 19005152]

Murdoch DR. Diagnosis of *Legionella* infection. Clin Infect Dis 2003;36:64–9. [PubMed: 12491204]

Newton HJ, Ang DK, van Driel IR, Hartland EL. Molecular pathogenesis of infections caused by *Legionella pneumophila*. Clin Microbiol Rev 2010;23:274–98. [PubMed: 20375353]

Palutke WA, Crane LR, Wentworth BB, Geiger JG, Cardozo L, Singhakowinta A, et al. *Legionella feeleii*-associated pneumonia in humans. Am J Clin Pathol 1986;86:348–51. [PubMed: 3751997]

Parry MF, Stampleman L, Hutchinson JH, Folta D, Steinberg MG, Krasnogor LJ. Waterborne *Legionella bozemanii* and nosocomial pneumonia in immunosuppressed patients. Ann Intern Med 1985;103:205–10. [PubMed: 4014902]

Ratcliff RM, Lanser JA, Manning PA, Heuzenroeder MW. Sequence-based classification scheme for the genus *Legionella* targeting the *mip* gene. J Clin Microbiol 1998;36:1560–7. [PubMed: 9620377]

Reingold AL, Thomason BM, Brake BJ, Thacker L, Wilkinson HW, Kuritsky JN. *Legionella pneumonia* in the United States: the distribution of serogroups and species causing human illness. J Infect Dis 1984:149:819. [PubMed: 6726009]

Steinert M, Hentschel U, Hacker J. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol Rev 2002;26:149–62. [PubMed: 12069880]

Svarrer CW, Uldum SA. The occurrence of *Legionella* species other than *Legionella pneumophila* in clinical and environmental samples in Denmark identified by *mip* gene sequencing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect 2012;18:1004–9. [PubMed: 22070605]

Thacker WL, Plikaytis BB, Wilkinson HW. Identification of 22 *Legionella* species and 33 serogroups with the slide agglutination test. J Clin Microbiol 1985;21:779–82. [PubMed: 3998110]

Thacker WL, Benson RF, Schifman RB, Pugh E, Steigerwalt AG, Mayberry WR, et al. *Legionella tucsonensis* sp. nov. isolated from a renal transplant recipient. J Clin Microbiol 1989;27:1831–4. [PubMed: 2671032]

van der Mee-Marquet N, Domelier AS, Arnault L Bloc D, Laudat P, Hartemann P, et al. *Legionella anisa*, a possible indicator of water contamination by *Legionella pneumophila*. J Clin Microbiol 2006;44:56–9. [PubMed: 16390948]

Whiley H, Bentham R. *Legionella longbeachae* and legionellosis. Emerg Infect Dis 2011;17:579–83. [PubMed: 21470444]

Wolff BJ, Thacker WL Schwartz SB, Winchell JM. Detection of macrolide resistance in *Mycoplasma pneumoniae* by real-time PCR and high resolution melt analysis. Antimicrob Agents Chemother 2008;52:3542–9. [PubMed: 18644962]

Yu VL, Plouffe JF, Pastoris MC, Stout JE, Schousboe M, Widmer A, et al. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. J Infect Dis 2002;186:127–8. [PubMed: 12089674]

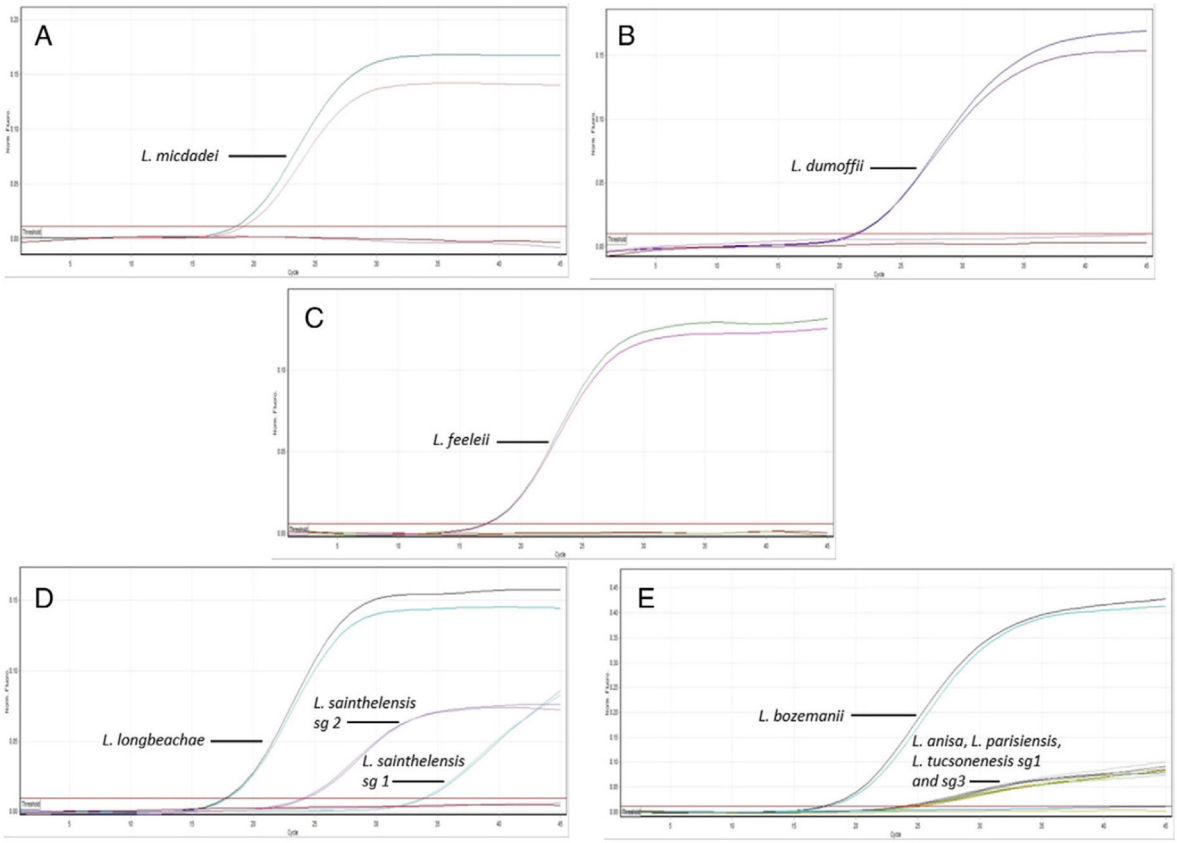

Fig. 1.

Multiplex assay for detection and identification of 9 clinically relevant nonpneumophila *Legionella* isolates. **(A)** *L. micdadei*, **(B)** *L. dumoffii*, **(C)** *L. feeleii*, **(D)** *L. longbeachae, L. sainthelensis* sg 1 and sg 2, and **(E)** *L. bozemanii, L. anisa, L. parisiensis*, and *L. tucsonensis* sg 1 and sg 3. All samples were run in duplicate.

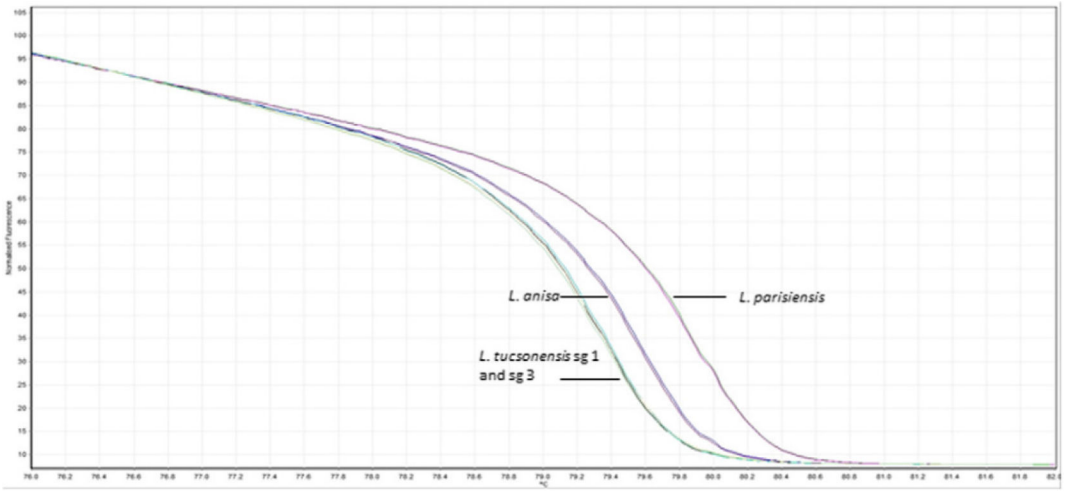

Fig. 2.

Real-time PCR-HRM assay for discrimination of *L. anisa, L. parisiensis*, and *L. tucsonensis* sg 1 and sg 3 isolates targeting the *ssrA* gene.

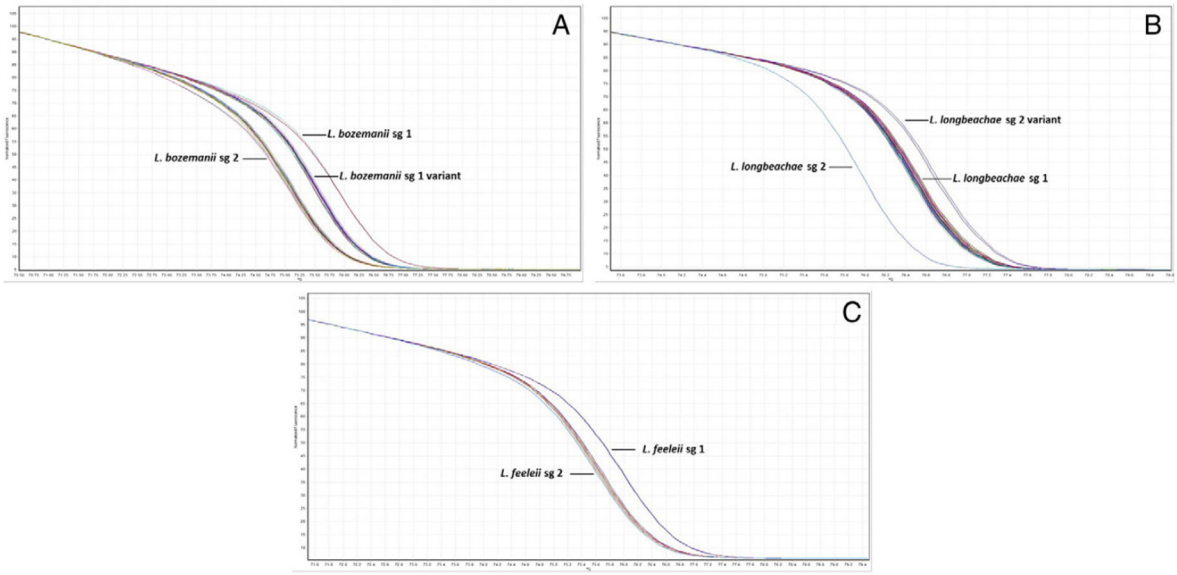

Fig. 3.

HRM profiles for subtyping of *Legionella* spp., targeting nucleotide differences in the *mip* gene. **(A)** Subtyping of 26 *L. bozemanii* isolates, **(B)** subtyping of 77 *L. longbeachae* isolates, and **(C)** subtyping of 11 *L. feeleii* isolates.

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, L. parisiensis, L. tucsonensis* sg 1 and 2	*gyrB*	HQ717438	125 nmol/L
bozemanii-R	CATGCAAACCACCCGATACT				125 nmol/L
bozemanii-P3	Q705-AAATTTACCACCGGCGTGAAGCAC-BHQ3				25 nmol/L
dumoffii-F	CAGGAAAGCGCGACATCTAT	*L. dumoffii*	*legS2*	EU107519	250 nmol/L
dumoffii-R	ATCCAGCTCGTTCGCAATAA				250 nmol/L
dumoffii-P	HEX-TGGAAACCCTCAATGGTCCGTTCT-BHQ1				50 nmol/L
feeleii-F	AACCGGTTTATCGGTCTTT	*L. feeleii*	*figA*	AY753535	125 nmol/L
feeleii-R	ATCAACCAGCTTGTCTCG				125 nmol/L
feeleii-P2	TEX615-AGCTTGAGAATTTGATGGATTATCACTCGC-BHQ2				25 nmol/L
LLB-F1	CTGCAGAAGTTGCTGATTGTG	*L. longbeachae, L. sainthelensis* sg 1 and 2	*ligB*	AY512558	125 nmol/L
LLB-R1	GACGTGGCGAATGACTTATCT				125 nmol/L
LLB-P1	Q670-TGTCGCCAAGAAGTTGTATCTCATGCT-BHQ3				25 nmol/L
micdadei-F	TGACAAGTGAGAGCAAGAGTT	*L. micdadei*	*migB*	AY512559	125 nmol/L
micdadei-R	GTATCTATTCCGACAGCGATAGG				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, L. tucsonensis* sg 1 and 2	*ssrA*	HG525464	250 nmol/L
tmRNA-HRM-R	GGTCATCGTTTGCATTTATATTTA				250 nmol/L

Table 2

Strains used for evaluating specificity and sensitivity of the real-time PCR multiplex assay.

Strains	No. of isolates tested
L. pneumophila sg. 1–17	22
L. adelaidensis	1
L. anisa	43
L. beliardensis	1
L. birmighamensis	2
L. bozemanii	28
L. brunensis	1
L. busanensis	1
L. cherrii	1
L. cinannatiensis	1
L. drozanskii	1
L. dumoffii	21
L. erythra	1
L. fairfieldensis	1
L. fallonii	1
L. feeleii	11
L. geestiana	1
L. genomo species	1
L. gormanii	1
L. gratiana	1
L. gresilensis	1
L. hackleliae	2
L. impletisoli	1
L. israelensis	1
L. jamestowniensis	1
L. jordanis	1
L. lansingensis	1
L. longbeachae	80
L. londiniensis	1
L. lytica	1
L. maceachernii	1
L. micdadei	21
L. moravica	1
L. nagasakiensis	2
L. nautarum	1
L. oakridgensis	2
L. parisiensis	2
L. quateirensis	1
L. quinlavanii	2

Strains	No. of isolates tested
L. rowbowthamii	1
L. rubriluscens	1
L. sainthelensis	20
L. santicrucis	2
L. shakespearei	1
L. spiritensis	2
L. steigerwaltii	1
L. taurinensis	1
L. tucsonensis	3
L. wadsworthii	1
L. waltersii	1
L. worsleiensis	1
L. yabuchiae	1
Total	300