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Dual detection of *Legionella pneumophila* and *Legionella* species by real-time PCR targeting the 23S-5S rRNA gene spacer region

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Abstract

Although the majority of cases of Legionnaires' disease (LD) are caused by *Legionella pneumophila*, an increasing number of other *Legionella* species have been reported to cause human disease. There are no clinical presentations unique to LD and hence accurate laboratory tests are required for early diagnosis. Therefore, we designed a real-time PCR assay that targets the 23S-5S rRNA intergenic spacer region (23S-5S PCR) and allows for detection of all *Legionella* species and discrimination of *L. pneumophila* from other *Legionella* species. In total, 271 isolates representing 50 *Legionella* species were tested and the assay was validated using 39 culture-positive and 110 culture-negative patient specimens collected between 1989 and 2006. PCR-positive results were obtained with all 39 culture-positive samples (100% sensitivity). Specimens that tested positive according to 23S-5S PCR, but were culture-negative, were further analysed by DNA sequencing of the amplicon or the macrophage infectivity potentiator (*mip*) gene. In addition to *L. pneumophila*, *Legionella longbeachae*, *Legionella cincinnatiensis* and *Legionella micdadei* were identified in the specimens. The assay showed a 7-log dynamic range displaying a sensitivity of 7.5 CFU/mL or three genome equivalents per reaction. Sixty-one specimens containing viruses or bacteria other than *Legionellae* were negative according to 23S-5S PCR, demonstrating its specificity. Use of this assay should contribute to the earlier detection of respiratory disease caused by *Legionella* species, as well as to increased rates of detection.

Keywords: 23S-5S, *Legionella pneumophila*, *Legionella* species, Legionnaires' disease, real-time PCR
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Introduction

Legionellae are ubiquitous in natural and man-made aqueous environments. To date, at least 52 *Legionella* spp. have been identified (http://www.bacterio.cict.fr/l/legionella.html). Twenty-three species have been found to be associated with human diseases [1,2]. Approximately 80–90% of reported cases of Legionnaires' disease (LD) are attributed to *Legionella pneumophila*; however, all species may cause infection, especially in immunocompromised hosts [1,3,4].

Legionnaires' disease has no unique clinical or radiographic features [5,6], which may lead to inappropriate therapy and a poor prognosis. Therefore, a validated and rapid diagnostic assay is of great importance. Current laboratory criteria for ensuring a confirmed diagnosis of LD involve isolating *Legionellae* by culture and detecting *L. pneumophila* serogroup 1 antigen in urine or seroconversion. Although these methodologies have good specificity, they primarily detect *L. pneumophila*. Non-*pneumophila Legionella* spp. may grow on buffered charcoal yeast extract (BCYE) media, but it usually takes approximately 1–2 weeks of incubation time for identification. Some strains, such as *Legionella*-like amoebal pathogens, are very fastidious and require amoebal co-culture [7], which is laborious and impractical for clinical diagnosis. Therefore, infections caused by non-*pneumophila* species may not be diagnosed.

To address these deficiencies, molecular assays that target the nucleic acid of *Legionellae* have been developed, but their applications in clinical diagnosis are still limited. For example, the proportion of cases diagnosed by PCR and other genotypic methods in Europe from 1995 to 2004 accounts for < 2% of cases of LD (n = 27 244) [8]. Reluctance to use PCR assays for diagnosis is partially attributable to: (i) post-amplification procedures that are laborious, time-consuming

and prone to carry-over contamination and false positivity, and (ii) limited assay optimization and validation. Here, we present a real-time PCR assay that allows rapid detection and differentiation of *L. pneumophila* from 50 non-*pneumophila* species without the need for post-PCR manipulation. The assay has been optimized to ensure sensitivity and specificity and validation has been carried out using culture isolates and clinical specimens. This assay should increase the rate of detection of infection caused by non-*pneumophila* strains of *Legionellae* and contribute to early diagnosis of LD.

Materials and Methods

Bacterial and viral strains

Viruses and bacteria other than *Legionellae* were used to test the specificity of the real-time PCR, including *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella holmesii*, *Bordetella bronchiseptica*, *Corynebacterium diphtheriae*, *Candida albicans*, *Chlamydophila pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Escherichia coli*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*, *Mycoplasma arginini*, *Mycoplasma buccale*, *Mycoplasma faucium*, *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Mycoplasma lipophilum*, *Mycoplasma orale*, *Mycoplasma penetrans*, *Mycoplasma pirum*, *Mycoplasma salivarium*, *Moraxella catarrhalis*, *Mycobacterium tuberculosis*, *Neisseria meningitidis*, *Neisseria elongata*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus salivarius*, *Streptococcus oligofermentans*, *Streptococcus australis*, *Streptococcus vestibularis*, *Streptococcus sinesis*, *Streptococcus gordonii*, *Streptococcus agalactiae*, *Streptococcus peroris*, *Streptococcus sanguinis*, *Streptococcus parasanguinis*, *Streptococcus infantis*, *Streptococcus mitis*, *Streptococcus cristatus*, *Streptococcus oralis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, influenza A (H1, H3, H5), influenza B, severe acute respiratory syndrome (SARS), respiratory syncytial virus (A, B), human parainfluenza viruses (1, 2, 3), human metapneumovirus, and adenovirus. Each was cultured using appropriate agar, cells and medium.

Legionella strains were grown on BCYE agar at 35 °C with 2.5% CO_2 for 48–72 h. The incubation period for the culture of human specimens was 7 days. The bacteria derived from a single colony were harvested in water and subjected to genomic DNA purification.

Primers and probe design, synthesis and optimization

Primers targeting the 23S-5S rRNA intergenetic spacer region conserved for all *Legionella* species were designed. The sequences of forward and reverse primers were 5′-GTA CTA ATT GGC TGA TTG TCT TGA CC-3′ and 5′-CCT GGC GAT GAC CTA CTT TCG-3′, respectively. Two probes were designed within the amplicon region. One is specific for *L. pneumophila* (5′-CalOrg-ATC GTG TAA ACT CTG ACT CTT TAC CAA ACC TGT GG-3′BHQ); the other recognizes all known *Legionella* species (5′-FAM ATC TC"G" AA"C" T"C"A "G"AA "G"T"G" AAA C-3′BHQ) ("" denotes lock nucleic acid) and is referred to as the genus-wide probe or *Legionella* spp. probe.

All PCR reactions were performed in triplicate using the AgPath-ID One-Step RT-PCR kit (AM1005; Applied Biosystems, Inc., Foster City, CA, USA) and the 7900HT real-time PCR system (Applied Biosystems, Inc.) unless otherwise indicated.

Standard curve analysis and sensitivity determination

Genomic DNA was purified from *L. pneumophila* serogroup 1 using the KingFisher ML instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the InviMag kit (B-Bridge International, Inc., Sunnyvale, CA, USA) following the manufacturers' instructions. Genome equivalent (gEq), or single copy of genome, was calculated using a genome size of 3.397×10^6 bp (GenBank accession no. NC_002942). Ten-fold serial dilutions of the genomic DNA spanning seven orders of magnitude were amplified using optimized primers and probes.

To determine the lower limit of detection (LLOD) for live bacteria, ten-fold serial dilutions of *L. pneumophila* serogroup 1 were streaked on BCYE plates. Bacterial colonies were manually counted. Colony counts of > 120 or < 20 were excluded from analysis. The bacteria were suspended in water and diluted to *c.* 100 CFU/mL. Genomic DNA was extracted as above from 1.5 mL of bacterial suspension. Five per cent of the purified DNA samples (n = 30) corresponding to *c.* 7.5 CFU/mL were amplified using the 7900HT real-time PCR system.

Clinical specimens

In total, 149 clinical specimens from 67 patients with respiratory disease possibly caused by *Legionella* infection were tested. The specimens were collected over a 17-year period (Table 1). Cultures were performed on BCYE or selective media upon receipt. Retrospective studies were carried out using the remaining specimens, which had been stored at − 80 °C, to validate the 23S-5S PCR assay. Analysis of the *mip* sequence was performed on all culture-negative, but PCR-positive, samples unless otherwise indicated (Table 1) using the 3130XL Sequencer and BigDye X Terminator reagent and purification kit (Applied Biosystems, Inc.). The sequences were aligned with those in the National Center for Biotechnology Information (NCBI) database or the

TABLE 1. Clinical specimens for validating 23S-5S assay

Sample type	Cultures positive (+), *n*	PCR positive (+), *n*	PCR negative (−), *n*	Cultures (−)/PCR (+), *n*	*mip* sequence analysis of PCR+ but culture− samples[a]	Total
Sputum	9	18	40	9	*L. pneumophila* (*n* = 5) *L. longbeachae* (*n* = 2) *L. cincinnatiensis* (*n* = 1) Novel *Legionella* spp. (*n* = 1)	58
Lung tissue	24	31	28	7	*L. pneumophila* (*n* = 6) Undetermined (*n* = 1)[b]	59
Bronchoalveolar lavage	2	3	4	1	*L. micdadei* (*n* = 1)	7
Bronchial swab	2	2	0	0	Not applicable	2
Nasopharyngeal swab	2	2	0	0	Not applicable	2
Blood	0	8	0	8	*L. pneumophila* (*n* = 2) Novel *Legionella* spp.(*n* = 6)	8
Liver	0	0	1	0	Not applicable[c]	1
Trans-tracheal aspirate	0	0	1	0	Not applicable[c]	1
Spleen	0	0	1	0	Not applicable[c]	1
Pleural fluid	0	1	1	1	*L. pneumophila* (*n* = 1)[d]	2
Formalin-fixed and paraffin embedded tissue (FFPE)	0	1	7	1	*L. pneumophila* (*n* = 1)[d]	8
Total number of specimens	39[e]	66	83	27	27	149
Percentage of specimens	26%	44%	56%	18%	18%	100.0%

[a]*mip* sequence analysis for all culture−/PCR+ samples unless otherwise indicated.
[b]No sample left for sequencing.
[c]Not applicable for sequence analysis.
[d]Amplicon sequence analysis only.
[e]Positive for *Legionella pneumophila*.

online *mip*-based *Legionella* identification tool (http://www.hpa-bioinfotools.org.uk/mip_ID.html). Real-time PCR for the human RNase P gene was carried out in parallel to monitor reaction inhibition and DNA integrity.

Results

Detection of *Legionella* spp. in clinical specimens

The 149 patient specimens corresponded to 11 sample types (Table 1). The assay allowed detection of *Legionella* spp. in all 39 culture-positive specimens. Moreover, *Legionellae* were identified in 27 of 110 culture-negative samples. Specimens that were 23S-5S PCR-positive but culture-negative were confirmed by sequencing the amplicon or by amplifying the *mip* gene, which was subsequently sequenced [9]. Among the 27 culture-negative samples, 15 were positive for *L. pneumophila*, two were positive for *Legionella longbeachae*, one was positive for *Legionella cincinnatiensis* and one was positive for *Legionella micdadei*. Amplicon sequence analysis of *mip* or 23S-5S did not reveal significant homology with any known *Legionella* spp. in seven samples, suggesting potentially novel *Legionella* spp. (R. M. Ratcliff, personal communication, 2008). One sample was depleted and no sequence analysis was performed.

Assay design and optimization

One set of primers and two probes were designed within the same region, resulting in a singleplex dual-colour real-time PCR (Fig. 1a). PCR reactions were performed using the primers at working concentrations of 25–200 nM at two-fold intervals. The sensitivity was three orders of magnitude lower if the primer concentration was < 100 nM. The lowest cycle threshold (Ct) occurred with forward/reverse primers at 100 nM and 200 nM, respectively. Increasing the concentration from 200 nM to 800 nM did not improve sensitivity as Ct values remained nearly unchanged ($p < 0.05$). The probes were titrated similarly from 50 nM to 600 nM. Use of the *Legionella* spp. probe at a concentration of 200 nM gave the best performance and increasing the concentration to 400 nM did not reduce the Ct values. The *L. pneumophila*-specific probe performed better at 400 nM. Higher probe concentration resulted in decreased sensitivity (data not shown).

Amplification efficiency, reproducibility and sensitivity

Linear regression analysis of the standard curves showed excellent correlation between the Ct value and the copy number of the *Legionella* genome ($R^2 = 0.997$) over seven orders of magnitude. The efficiency of amplification was 100.7% and 102.6% for *L. pneumophila* and the genus-wide assays, respectively (Fig. 1b). The primers target the specific sites, even in the presence of high concentrations of *Legionella* genomic DNA (Fig. 1c).

The analytical sensitivity was assessed by amplifying *L. pneumophila* serogroup 1 DNA at a concentration of 3 gEq per PCR reaction. All samples were positive using the *Legionella* spp. probe ($n = 20$; Ct = 34.59 ± 0.76, coefficient of variation (CV) 2.22%) and 95% of the samples were

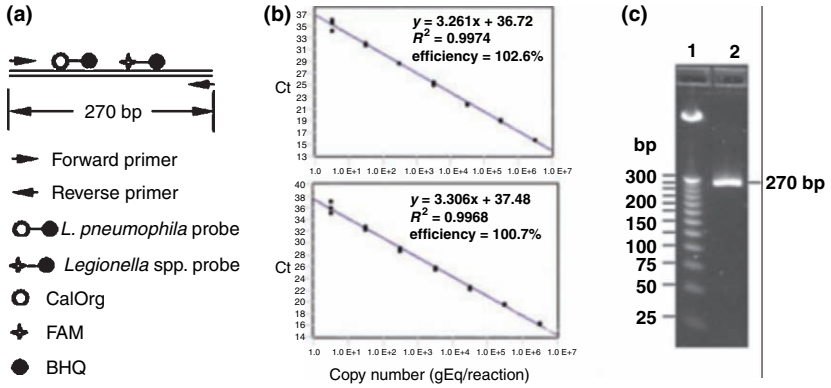

FIG. 1. Assay design and standard curve analysis. (a) Schematic diagram of the design of the primers and probes. The forward/reverse primers were selected in the 23S-5S intergenetic region conserved for all *Legionella* spp. Two TaqMan probes were designed in the antisense strand of the amplicon. One probe recognized all *Legionella* spp. and the other was *Legionella pneumophila*-specific. Both probes are quenched at the 3′-end by black hole quencher (BHQ), but labelled by different fluorescent reporter molecules with distinct emission wavelength. (b) Real-time PCR standard curves generated from *L. pneumophila* probe (upper panel) and *Legionella* spp. probe (lower panel). Seven ten-fold serial dilutions of *Legionella* genomic DNA were prepared for qPCR. The concentration of genomic DNA for each dilution ranged from 3 gEq to 3.0 E + 6 gEq per PCR reaction. The standard curve equation, R^2 value, and amplification efficiency are indicated. (c) Gel analysis of the endpoint PCR product. The amplicon derived from the PCR reaction that has the highest amount of the template (3.0 E + 6 gEq) was analysed on 4% E-gel (Invitrogen Corp., Carlsbad, CA, USA) to assess amplification specificity. Lane 1: 25-bp DNA ladder (Promega Corp., Madison, WI, USA). gEq, genome equivalent; Ct, cycle threshold.

positive using the *L. pneumophila* probe (n = 20; Ct = 35.51 ± 0.96, CV = 2.69%). By contrast, no signals were detected from no-template controls (NTCs) (n > 100) after 40 cycles of amplification. Therefore, the LLOD of the assay was *c.* 3 gEq per PCR reaction. For live bacteria, the assay detected *L. pneumophila* serogroup 1 at 7.5 CFU/mL (n = 30; for the *Legionella* spp. probe: Ct = 33.83 ± 0.52, CV = 1.53%; for the *L. pneumophila* probe: Ct = 37.05 ± 0.92, CV = 2.48%). No amplification was detected in the NTCs (n = 10).

Accuracy and specificity

Genomic DNA isolated from the 271 strains representing the 50 *Legionella* spp. listed in Table 2 were amplified and detected using the *Legionella* spp. probe and the *L. pneumophila*-specific probe. The *L. pneumophila* probe did not cross-react with any non-*pneumophila* species and accurately detected *L. pneumophila*, despite the presence of high concentrations of genomic DNA.

Total nucleic acid (TNA) was purified from 61 bacterial and viral strains (listed in Materials and methods). The TNA quality was demonstrated by corresponding PCR amplifications. Although the concentration of the templates was relatively high (5 ng, or *c.* 1×10^7 gEq for an organism with a genome size of 3.0×10^6 bp (GenBank accession no. NC_002942), none of the strains reacted with the *Legionella* probes, yielding an analytical specificity of 100%.

Discussion

The urine antigen (UA) test is increasingly used by clinicians in the diagnosis of LD, but it is limited to the detection of *L. pneumophila* serogroup 1 [10]. Extensive use of the UA test has resulted in a decrease in the use of other diagnostic methods, thus reducing the frequency of identification of species other than *L. pneumophila* serogroup 1 [11]. For example, a recent study of population-based surveillance of severe pneumonia in Thailand found no cases of *L. pneumophila* serogroup 1 infection using a UA test, but serological evidence suggested that approximately 5% of cases of pneumonia may be caused by *L. longbeachae* (n = 397) [12]. This underscores the need for diagnostic tests for all *Legionella* spp. In this study, we developed and validated a 23S-5S dual-colour real-time PCR assay. The LLOD of the assay was 3 gEq/reaction for genomic DNA or 7.5 CFU/mL for the live *Legionella* strain. It can distinguish *L. pneumophila* from non-*pneumophila Legionella* spp. and can detect *Legionella* spp. from all culture-positive clinical specimens within 3 h without requiring post-PCR processing steps. We assessed the specificity of the assay by analysing the DNA sequence of the amplicon or the *mip* gene and by assessing the reactivity with the non-*Legionella* bacteria and viruses.

Legionella is not part of the human flora; thus, the presence or absence of *Legionella* DNA in specimens could be

TABLE 2. ***Legionella*** **species used for evaluating the 23S-5S assay**

	Legionella spp.	Strains, *n*
1	*L. pneumophila*	66
2	*L. adelaidensis*	1
3	*L. anisa*	18
4	*L. beliardensis*	1
5	*L. birminghamensis*	5
6	*L. bozemanae*	31
7	*L. brunensis*	1
8	*L. busanensis*	1
9	*L. cherrii*	3
10	*L. cincinnatiensis*	3
11	*L. drozanskii*	2
12	*L. dumoffii*	7
13	*L. erythra*	3
14	*L. fairfieldensis*	1
15	*L. fallonii*	2
16	*L. feeleii*	7
17	*L. geestiana*	1
18	*L. genomo* species	1
19	*L. gormanii*	4
20	*L. gratiana*	1
21	*L. gresilensis*	1
22	*L. hackeliae*	4
23	*L. israelensis*	1
24	*L. jamestowniensis*	2
25	*L. jordanis*	2
26	*L. lansingensis*	2
27	*L. longbeachae*	7
28	*L. londiniensis*	2
29	*L. lytica*	1
30	*L. maceachernii*	1
31	*L. micdadei*	6
32	*L. moravica*	1
33	*L. nagasakii*	2
34	*L. nautarum*	3
35	*L. oakridgensis*	3
36	*L. parisiensis*	2
37	*L. quateirensis*	3
38	*L. quinlavanii*	8
39	*L. rowbothamii*	2
40	*L. rubriluscens*	3
41	*L. sainthelensis*	6
42	*L. santicrucis*	1
43	*L. shakespearei*	3
44	*L. spiritensis*	3
45	*L. steigerwaltii*	1
46	*L. taurinensis*	2
47	*L. tucsonensis*	4
48	*L. wadsworthii*	2
49	*L. waltersii*	1
50	*L. worsleiensis*	2
	Unidentified *Legionella*	31
Total		271

clinically significant. In recent years, molecular methods based on nucleic acid testing (NAT) have been extensively explored for diagnosis of LD. Overall NAT can be assigned to four types of assay: (i) PCR amplification of the whole genome followed by restriction enzymatic digestion and gel analysis [13,14]; (ii) PCR amplification of specific regions of the genome followed by sequencing analysis [15, 16]; (iii) DNA hybridization or fluorescent *in situ* hybridization (FISH) [17–19], and (iv) real-time PCR assays targeting 16S rRNA, the *mip* gene or the 23S-5S intergenetic region [20–23]. A high rate of recombination in the 16S rRNA gene results in multiple heterogeneous copies in the genome, which may lead to erroneous species identification [24,25]. The *Legionella mip* gene is highly variable, which makes designing a consensus probe to recognize all *Legionella* spp. virtually impossible [9]. The relatively conserved 23S-5S region allows the selection of primers and probes to detect *L. pneumophila* and *Legionella* spp. in a singleplex format. Three singleplex real-time PCR assays for identifying and differentiating *L. pneumophila* from other *Legionella* spp. have been reported [20–22]. These tests either require sequencing or melting curve analysis to distinguish *L. pneumophila* from other species, or lack specificity or validation using clinical specimens [20–22].

The availability of suitable patient samples for *Legionella* nucleic acid detection is limited. Oral pharyngeal swabs or nasopharyngeal swabs that are routinely taken from patients with respiratory infections may be less than ideal for *Legionella*-specific PCR testing unless the burden of *Legionellae* is high [26]. Collection of serum or urine samples is simple to perform, but the reported sensitivity varies from 30% to 86% for urine samples [27–29] and is < 60% for serum samples from patients with proven LD [30]. It appears that *Legionella* DNA in serum only peaks in the acute stage of infection [30]. In this study, the sensitivity observed with the archived serum samples was < 30% (data not shown). Bronchoalveolar lavage fluid, bronchial aspirates, lung biopsies, post-mortem tissue specimens and sputum appear to be more suitable for culture and PCR, but most of these specimens are not readily obtainable and are not usually taken for diagnosis [6,31]. Nevertheless, these clinical specimens are valuable for validation of this diagnostic assay. The level of the impurities, such as human genomic DNA, protein and carbohydrates, varies among different types of specimen, and pathogen concentration in a clinical sample is significantly lower than that in laboratory cultures.

Culture provides definitive diagnosis and remains a reference standard for *Legionellae* identification, but its use in the diagnosis of LD continues to decline. The clinical samples used in this study and in others [21–23] were positive only for *L. pneumophila*, suggesting that some non-*pneumophila* species were not detected by culture (*L. cincinnatiensis*, *L. longbeachae* and *L. micdadei*). Serological testing is generally limited to epidemiological studies and is unsuitable for diagnosis of acute LD because seroconversion does not occur until 2 weeks after the onset of illness and may even be absent in some patients [32].

In addition, the serological heterogeneity of *Legionella* spp. and antibody cross-reactions can lead to false-negative or false-positive results [33]. The goal of this study was to develop a molecular assay as a supplemental tool for diagnosis of LD caused by *Legionella* spp.

The absence of unique clinical features or radiographic patterns complicates the diagnosis of LD. Among the 149

lung specimens collected from patients with possible LD, *Legionella* spp. was detected in only 44% of cases. It is possible that the infection in these patients was caused by other pathogens. For example, we detected *Pseudomonas aeruginosa*, *Sphingomonas* spp. and *Eubacterium* spp. in some culture-negative samples using 16S rRNA PCR and sequencing analysis (data not shown).

In conclusion, we have established a sensitive and specific real-time PCR assay that allows the detection of *L. pneumophila* and its differentiation from non-*pneumophila* species. Use of this assay in conjunction with currently recommended diagnostic tests should lead to increased rates of detection of respiratory disease caused by *Legionella* species.

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Transparency Declaration

The authors have no conflicts of interest with commercial entities or any other third parties.

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