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Bacteriology

Detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* spp. in clinical specimens using a single-tube multiplex real-time PCR assay☆

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Abstract

A multiplex real-time PCR assay for the detection of *Mycoplasma pneumoniae* (MP181), *Chlamydia* (*Chlamydophila*) *pneumoniae* (CP-Arg), *Legionella* spp. (Pan-Leg), and the human RNase P (RNase P) gene was developed for rapid testing of atypical bacterial respiratory pathogens in clinical specimens. This method uses 4 distinct hydrolysis probes to detect 3 leading causes of community-acquired pneumonia. The assay was evaluated for specificity and sensitivity by testing against 35 related organisms, a dilution series of each specific target and 197 clinical specimens. Specificity testing demonstrated no cross-reactivity. A comparison to previously validated singleplex real-time PCR assays for each agent was also performed. The analytical sensitivity for specific pathogen targets in both the singleplex and multiplex was identical (50 fg), while efficiencies ranged from 82% to 97% for the singleplex assays and from 90% to 100% for the multiplex assay. The clinical sensitivity of the multiplex assay was improved for the Pan-Leg and CP-Arg targets when compared to the singleplex. The MP181 assay displayed equivalent performance. This multiplex assay provides an overall improvement in the diagnostic capability for these agents by demonstrating a sensitive, high-throughput and rapid method. This procedure may allow for a practical and efficient means to test respiratory clinical specimens for atypical pneumonia agents in health care settings and facilitate an appropriate public health response to outbreaks.
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Keywords: Real-time PCR; Multiplex real-time PCR; Community-acquired pneumonia

1. Introduction

Respiratory infections caused by the atypical pathogens *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* spp. collectively account for approximately 15% of all community-acquired pneumonia (CAP) cases (Bartlett and Mundy, 1995; Gupta and Sarosi, 2001). Because these infections are difficult to discern from other causes of pneumonia based on examination, symptoms, or chest X-ray findings, laboratory identification of the etiology is critical to establish the correct course of treatment (Cunha, 2006). *M. pneumoniae* accounts for 15–20% of atypical CAPs, and outbreaks have been reported to occur in 3- to 7-year intervals with varying incidence rates (Foy, 1993; Klement et al., 2006; Waites and Talkington, 2004). Although symptoms are usually mild and individuals often do not seek medical treatment, some patients require hospitalization and severe extrapulmonary disease can develop (Koskiniemi, 1993; Mok et al., 1979; Waites and Talkington, 2004). *C. pneumoniae*, an obligate intracellular bacterium, is frequently misdiagnosed or undetected and is estimated to cause 10–15% of atypical CAP infections (Dowell et al., 2001; Marrie et al., 1996; Menendez et al., 1999; Sopena et al., 1999; Welti et al., 2003). Incidence

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rates may be much higher because approximately 50% of adults have antibodies to *C. pneumoniae* (Birkeback et al., 2000; Grayston, 1989; Tuuminen et al., 2000; Wan and Grayston, 1990). Legionellae account for approximately 2–8% of atypical CAPs, with *Legionella pneumophila* serogroup (Sg) 1 causing approximately 70% of cases, followed by Sg 2, 4, and 6 along with *L. bozemanii*, *L. longbeachae*, and *L. micdadei* (Bartlett and Mundy, 1995; Fields et al., 2002; Gupta and Sarosi, 2001).

Each of these agents are fastidious organisms that require time-consuming procedures, specialized media, and technical expertise to successfully culture (Gupta and Sarosi, 2001). Several commercially available serologic kits exist for detecting infection with *M. pneumoniae* and *C. pneumoniae*; however, none display a high degree of sensitivity or specificity. Moreover, the need for both acute and convalescent patient serum makes this a retrospective test and not ideal for a rapid diagnosis. Urinary antigen tests are routinely used for diagnosis of Legionnaire's disease but only detect *L. pneumophila* Sg1, thereby allowing for approximately 30% of other *Legionella* infections to go undetected (Cunha, 2006; Fields et al., 2002).

Within the last decade, improvements for the detection of these agents have been made. Specific real-time PCR assays have been developed to provide a more rapid and reliable method for detecting respiratory pathogens in clinical specimens (Apfalter et al., 2003; Hayden et al., 2001; Mitchell et al., 2009; Winchell et al., 2008). More recently, multiplex (conventional and real-time) PCR assays have also been developed (Gullsby et al., 2008; Loens et al., 2008; McDonough et al., 2005; Miyashita et al., 2004; Welti et al., 2003). Miyashita et al. (2004) designed a multiplex conventional PCR test, followed by Micro-Chip Electrophoresis Analysis, that showed an increase in detection rates over traditional methods. A duplex real-time PCR assay for *C. pneumoniae* and *M. pneumoniae* also showed improved detection rates in clinical specimens when compared to conventional PCR (Gullsby et al., 2008). A real-time multiplex nucleic acid sequence-based amplification assay, targeting the 16S rRNA region of these organisms, has also been used for detection (Loens et al., 2008). Although only a limited number of clinical specimens were tested, specific detection was achieved in this study, albeit with lower sensitivity. This assay also requires the use of molecular beacons that may pose design challenges and extensive optimization.

The current study reports the development and evaluation of a multiplex real-time PCR assay for simultaneous detection of 3 atypical bacterial pneumonia-causing organisms in clinical specimens. To our knowledge, this is the first report of a 4-plex, single-tube, real-time TaqMan® (Applied Biosystems, Foster City, CA) PCR assay for the detection of *M. pneumoniae* (MP181), *C. pneumoniae* (CP-Arg), *Legionella* spp. (Pan-Leg), and human DNA (RNase P). This rapid, reliable, and convenient method allows for early identification of these agents to assist in providing a timely and more appropriate public health response.

2. Materials and methods

2.1. Primer and probe design

The primer and probe sequences for all markers are listed in Table 1. The MP181 and RNase P assays have been described previously (Emery et al., 2004; Winchell et al., 2008). *Legionella* spp. primers and probe (Pan-Leg) were designed manually targeting the *ssrA* gene (GenBank accession no. U68079) and tested against a comprehensive list of *Legionella* species and serogroups (Fields et al., 2002). Primer Express 3.0 (Applied Biosystems, Foster City, CA) was used to design primer and probe sequences for the CP-Arg assay targeting the arginine repressor protein gene of *C. pneumoniae* (GenBank accession no. AE009440.1 for TW-183). The CP-Arg assay was tested against all available isolates of *C. pneumoniae*:AR-388, BR-393, W6, IOL-207, FML-7, FML-12, FML-16,

Table 1
Primers and probes for multiplex real-time detection of *M. pneumoniae*, *C. pneumoniae*, *Legionella* spp., and human DNA

Primer/probe	Sequence (5′→3′)	Gene target	Product (bp)	Multiplex primer/probe final concentration
MP181-F	TTTGGTAGCTGGTTACGGGAAT	*M. pneumoniae*	73	250 nmol/L
MP181-R	GGTCGGCACGAATTTCATATAAG	CARDS Tx (Kannan and Baseman, 2006)		250 nmol/L
MP181-P	HEX-TGTACCAGAGCACCCCAGAAGGGCT-BHQ1			100 nmol/L
CP-Arg-F	CGTGGTGCTCGTTATTCTTTACC	*C. pneumoniae*	74	250 nmol/L
CP-Arg-R	TGGCGAATAGAGAGCACCAA	argR		250 nmol/L
CP-Arg-P	Quas670-CTTCAACAGAGAAGACCACGACCCGTCA-BHQ3			50 nmol/L
Pan-Leg-F	GGCGACCTGGCTTC	*Legionella* spp.	230	125 nmol/L
Pan-Leg-R	TATGACCGTTGATTCGATACC	*ssrA*		125 nmol/L
Pan-Leg-P	FAM-A***C***GT***G***GG***T***TGC-BHQ1[a]			25 nmol/L
RNase P-F	AGATTTGGACCTGCGAGCG	Human RNase P (Emery et al., 2004)	62	250 nmol/L
RNase P-R	GAGCGGCTGTCTCCACAAGT			250 nmol/L
RNase P-P	CalRd610-TTCTGACCTGAAGGCTCTGCGCG-BHQ2			50 nmol/L

[a] Locked nucleic acid probe.

FML-19, K66, TW-183, CM1 CWL-029, TW-2043, TW-2023, CWL-011, CWL-050, and BAL-16. These isolates were obtained from various clinical sources, including throat washings, NP/OP swabs, and BALs.

2.2. Singleplex real-time PCR assays

For each singleplex assay, specific TaqMan® probes were labeled with 6-carboxyflourescein (FAM) and were examined separately under previously optimized conditions (Winchell et al., 2008). Briefly, the 25-μL reaction volume contained 12.5 μL of Platinum Quantitative PCR Supermix-UNG (Invitrogen, Carlsbad, CA), an additional 3 mmol/L $MgCl_2$, 1 μL of 10 mmol/L nucleotide mix (Promega, Madison, WI), 1 μmol/L of each primer, 200 nmol/L of probe, 1.25 U of Platinum Taq Polymerase (Invitrogen), and 5 μL of template. Each assay was performed on the ABI 7500 Real-Time PCR system using the following thermocycling conditions: 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.3. Multiplex real-time PCR assay

The multiplex assay used the same primer and probe sequences as the singleplex assays, but all oligonucleotides were pooled in a one-tube reaction. A combined positive control (CPC) containing pooled genomic nucleic acid from *M. pneumoniae* M129, *C. pneumoniae* TW-183, *L. pneumophila* Sg 1, and human nucleic acid (Promega; catalog no. G3041) was used to determine which fluorophores were optimal for each assay. This pool was calibrated to provide a consistent range of Ct values for each target. Table 1 shows the final concentrations of each primer and probe set and the distinct dyes used for each specific probe. The multiplex reaction contains 12.5 μL of PerfeCTa™ Multiplex qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD), the appropriate volume of each primer and probe, and 5 μL of template to a final volume of 25 μL. The assay was performed on the ABI 7500 Real-Time PCR system using thermocycling conditions described above, except the initial activation step is extended to 5 min, as recommended by the manufacturer.

2.4. Analytical specificity

Analytical specificity for each assay was verified using the following comprehensive panel of related respiratory organisms, each at a concentration of 3 ng/μL: *M. pirum*, *M. penetrans*, *M. hominis*, *M. fermentans*, *M. buccale*, *M. arginine*, *M. hyorhinis*, *M. amphoriforme*, *M. facium*, *B. pertussis*, *C. albicans*, *C. trachomatis*, *C. psittaci*, *C. diphtheriae*, *E. coli*, *L. planetarium*, *M. catarrhalis*, *N. elongate*, *N. meningitidis*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *S. pyogenes*, *S. salivarius*, *U. parvum*, *U. urealyticum*, human DNA, human coronavirus, human rhinovirus, human parainfluenza virus 2, human parainfluenza virus 3, human adenovirus, influenza virus A, influenza virus B, respiratory syncytial virus A, respiratory syncytial virus B (Winchell et al., 2008). No cross-reactivity or nonspecific amplification was observed for any of the assays tested with these organisms (data not shown).

2.5. Analytical sensitivity

Analytical sensitivity was established by testing a dilution series of nucleic acids for each assay. *M. pneumoniae* M129, *C. pneumoniae* TW-183, and *L. pneumophila* Sg1 were grown on appropriate media or cells. Nucleic acid extractions were performed using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) following manufacturer's instructions. Nucleic acid concentrations were determined using the NanoDrop® ND-1000 V3.5.2 Spectrophotometer (NanoDrop products, Wilmington, DE). *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila* nucleic acids were diluted to 100 pg/μL followed by 10-fold dilutions down to 1 fg/μL. Human DNA (Promega) was diluted to 1 ng/μL followed by 10-fold dilutions down to 1 fg/μL. All nucleic acid extracts were diluted in nuclease-free water and tested in replicates of 10 by 2 different operators (20 replicates total). These tests were performed for both the singleplex and multiplex assays. Limits of detection were established for each assay and defined as the lowest dilution in which >50% of replicates had positive crossing threshold values (Ct). 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% (Qvarnstrom et al., 2005).

2.6. Clinical specimens

Respiratory clinical specimens were obtained from previous respiratory surveillance studies that included both domestic and international sources along with outbreak investigations within the United States. All specimens were collected from persons presenting with respiratory symptoms. Clinical sensitivity was established by testing, in triplicate, 177 nasopharyngeal and/or oropharyngeal swabs by all 4 singleplex assays as well as the multiplex assay. In addition, 20 clinical specimens (lung tissue, bronchial lavage, sputa, and spleen tissue) were tested, in triplicate, with the Pan-Leg singleplex assay as well as the multiplex assay. Specimens were extracted using the MagNA Pure LC 1.0 or the MagNA Pure Compact instruments (Roche Applied Science, Indianapolis, IN) using the Total Nucleic Acid Isolation Kit following manufacturer's instructions. Both instruments were programmed for the Total NA Serum_Plasma_Blood protocol with a 200-μL sample volume and 100-μL elution volume. Evaluation of clinical sensitivity for the multiplex versus singleplex assays was performed by comparing crossing threshold (Ct) values using the Student's t-test for statistical analysis.

3. Results

3.1. Multiplex data analysis

Before performing the multiplex assay, the ABI 7500 instrument is programmed such that each target set was assigned a distinct detector color. Fig. 1A is an example of positive growth curves for all 4 markers using the CPC. The *C. pneumoniae* is displayed in blue, *M. pneumoniae* in brown, Pan-Leg in green, and RNase P in red. Fig. 1B is an example of a no template control reaction showing no amplification in any of the 4 assays. Representative positive clinical specimen results are shown in Fig. 2A–C. A typical negative specimen is shown in Fig. 2D where only RNase P exhibits a positive curve, validating nucleic acid extraction and proper preparation of the master mix.

3.2. Analytical sensitivity and assay efficiency

A comparison of the multiplex versus singleplex assays for all 4 markers including analytical sensitivity, correlation values, and assay efficiencies is shown in Fig. 3. The limits of detection were equivalent for the singleplex and multiplex pathogen-specific assays (50 fg) as well as for the RNase P assay (6 pg). Efficiencies ranged from 82% to 100% depending on the target. The efficiencies for both CP-Arg and Pan-Leg were higher with the multiplex assay versus singleplex at 100% versus 93% and 97% versus 82%, respectively. RNase P efficiencies were identical with each assay (85%), while the MP181 singleplex assay efficiency was higher at 97% versus 90% for the multiplex.

3.3. Primary clinical specimen testing

Table 2 shows the average Ct values for all positive clinical specimens using the multiplex and singleplex assays for all bacterial targets. A total of 197 respiratory specimens were tested using both assays. Of these, 36 specimens tested positive for *C. pneumoniae*, 23 were positive for *M. pneumoniae*, and 17 were positive for *Legionella* spp. The remaining specimens were negative in all 3 replicates for these agents when tested in both assays. All specimens gave amplification curves with the RNase P marker for both the multiplex and singleplex assays with a Ct range of 22.2–32.0 and 23.6–33.0, respectively (data not shown). Statistical

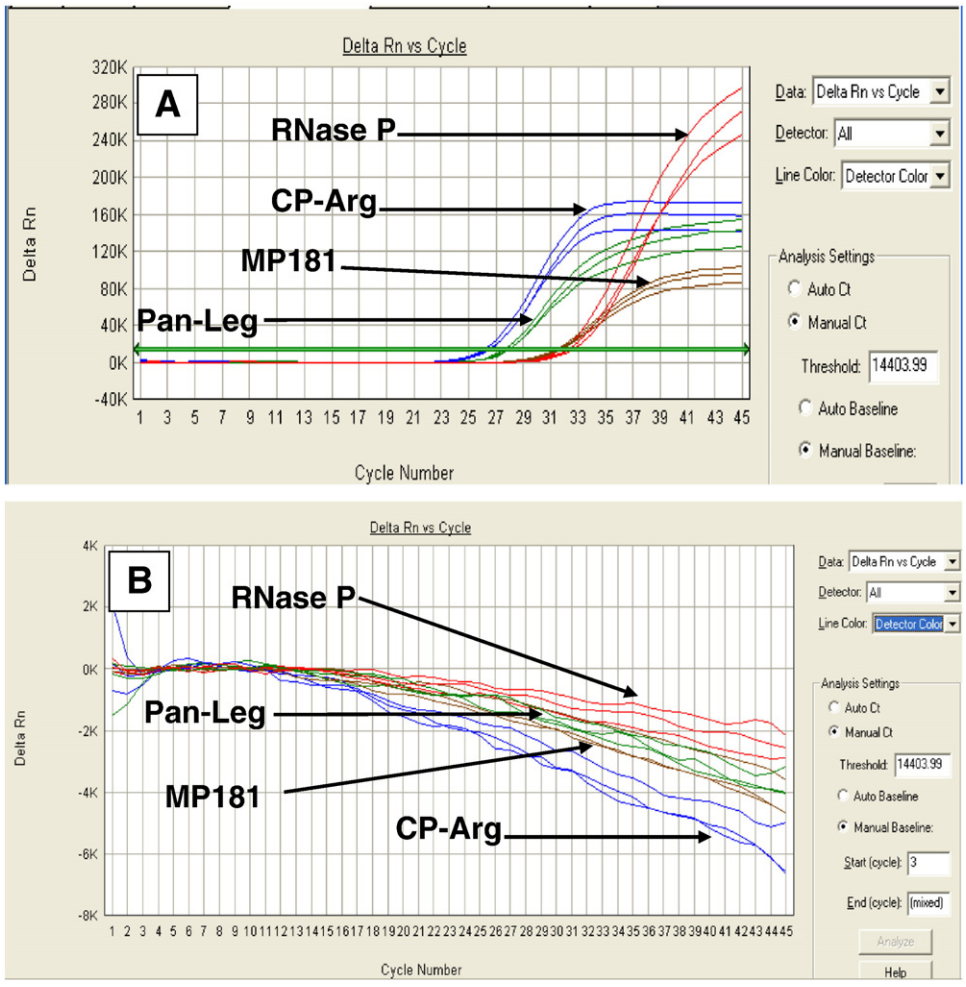

Fig. 1. (A–B) Analysis of CPC and no template control in triplicate using the multiplex assay on the ABI 7500.

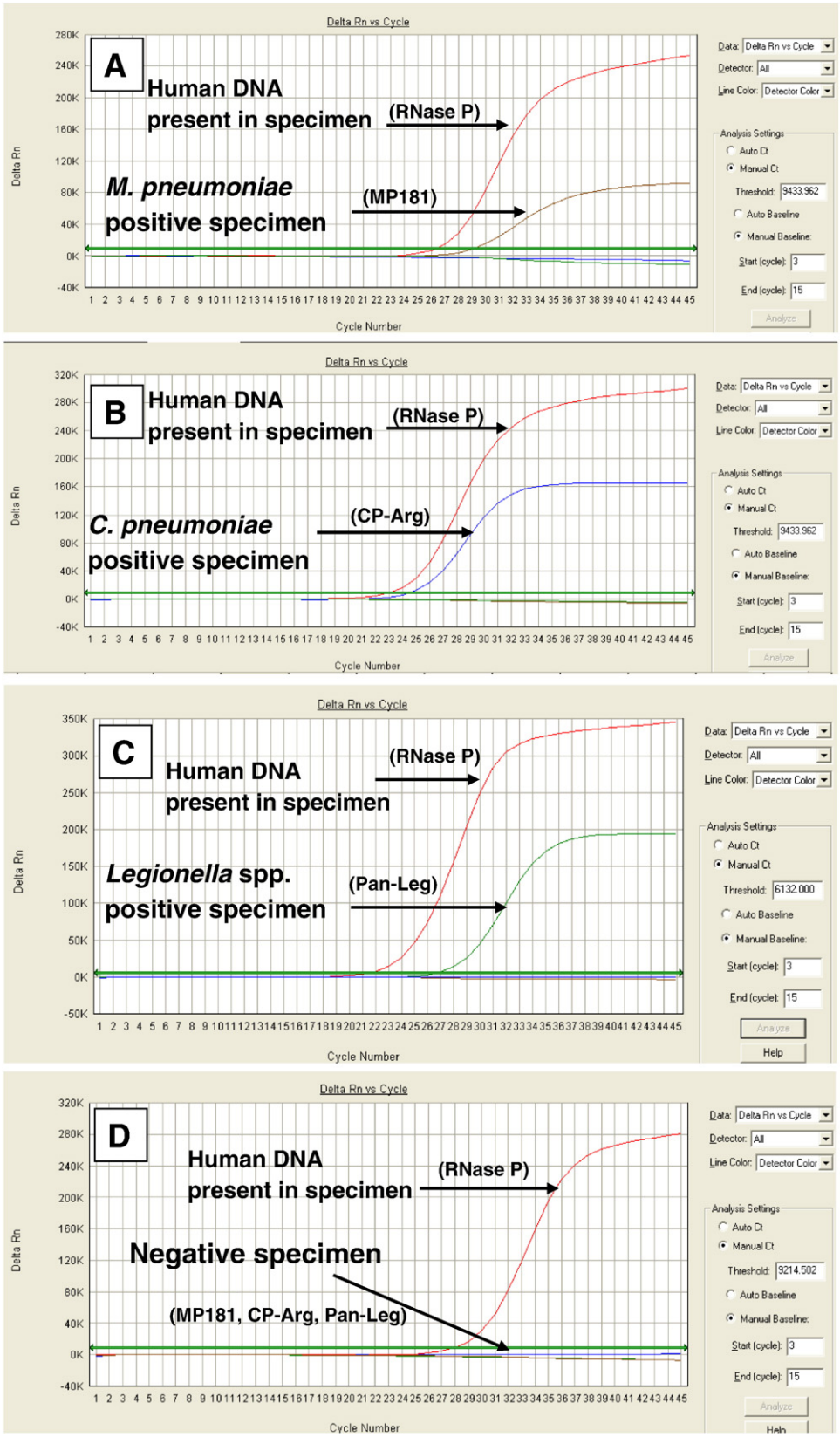

Fig. 2. (A–D) Analysis of representative clinical specimens for MP181 (A), CP-Arg (B), Pan-Leg (C), and a negative specimen (D), using the multiplex assay on the ABI 7500.

Table 2
Comparison of singleplex versus multiplex average real-time PCR Ct values for clinical specimens

Specimen no.	*C. pneumoniae* (CP-Arg)		*M. pneumoniae* (MP181)		*Legionella* ssp. (Pan-Leg)	
	Singleplex	Multiplex	Singleplex	Multiplex	Singleplex	Multiplex
1	37.0	35.0	–	–	–	–
2	28.2	26.4	–	–	–	–
3	32.3	30.2	–	–	–	–
4	38.0	35.9	–	–	–	–
5	35.1	32.8	–	–	–	–
6	33.4	31.2	–	–	–	–
7	35.7	33.1	–	–	–	–
8	35.2	33.0	–	–	–	–
9	35.0	33.1	–	–	–	–
10	35.9	35.4	–	–	–	–
11	25.2	23.8	–	–	–	–
12	33.4	31.4	–	–	–	–
13	37.5	35.5	–	–	–	–
14	30.4	28.2	–	–	–	–
15	31.5	29.4	–	–	–	–
16	24.5	23.3	–	–	–	–
17	32.7	31.7	–	–	–	–
18	36.4	35.3	–	–	–	–
19	39.0	35.2	–	–	–	–
20	26.3	24.7	–	–	–	–
21	30.4	28.9	–	–	–	–
22	23.4	22.4	–	–	–	–
23	39.1	36.0	–	–	–	–
24	33.6	31.3	–	–	–	–
25	37.9	38.1	–	–	–	–
26	37.2	34.9	–	–	–	–
27	36.9	35.8	–	–	–	–
28	44.1[a]	40.4	–	–	–	–
29	39.6[a]	43.7	–	–	–	–
30	38.2[a]	35.6	–	–	–	–
31	38.9[a]	36.0	–	–	–	–
32	38.8[b]	35.9[a]	–	–	–	–
33	38.2[b]	36.7[a]	–	–	–	–
34	38.9[b]	36.7[a]	–	–	–	–
35	39.4[a]	38.0[a]	–	–	–	–
36	–	37.5[b]	–	–	–	–
37	–	–	33.7	34.6	–	–
38	–	–	29.6	29.7	–	–
39	–	–	29.2	29.0	–	–
40	–	–	31.9	31.7	–	–
41	–	–	29.8	29.9	–	–
42	–	–	31.1	36.4	–	–
43	–	–	34.6	34.4	–	–
44	–	–	35.8	36.1	–	–
45	–	–	36.9	36.1	–	–
46	–	–	33.5	33.2	–	–
47	–	–	30.9	31.5	–	–
48	–	–	37.7	38.2	–	–
49	–	–	30.0	28.3	–	–
50	–	–	36.8	36.6	–	–
51	–	–	23.0	22.6	–	–
52	–	–	34.7	35.0	–	–
53	–	–	32.1	32.5	–	–
54	–	–	35.9	35.6	–	–
55	–	–	38.3	40.8	–	–
56	–	–	38.0[b]	40.3[b]	–	–
57	–	–	37.9[b]	39.5[b]	–	–
58	–	–	38.0[a]	38.8[a]	–	–
59	–	–	38.0[a]	37.6[a]	–	–
60	–	–	–	–	31.7	28.6

Table 2 (*continued*)

Specimen no.	*C. pneumoniae* (CP-Arg)		*M. pneumoniae* (MP181)		*Legionella* ssp. (Pan-Leg)	
	Singleplex	Multiplex	Singleplex	Multiplex	Singleplex	Multiplex
61	–	–	–	–	35.6	32.1
62	–	–	–	–	38.4	35.6
63	–	–	–	–	38.2	35.3
64	–	–	–	–	29.9	27.4
65	–	–	–	–	38.4	35.4
66	–	–	–	–	31.4	27.8
67	–	–	–	–	30.5	27.6
68	–	–	–	–	32.5	28.6
69	–	–	–	–	30.0	26.9
70	–	–	–	–	27.6	24.8
71	–	–	–	–	40.3	36.7
72	–	–	–	–	22.5	20.5
73	–	–	–	–	34.9	33.6
74	–	–	–	–	37.3	34.5
75	–	–	–	–	36.4	33.8
76	–	–	–	–	43.5[b]	38.1[b]

– = the specimens were tested for all markers but were negative except as indicated.

[a] Two of three replicates had positive Ct values.

[b] One of three replicates had positive Ct values.

analysis comparing average Ct values was performed to determine if the multiplex assay significantly improved the detection of these agents from clinical specimens. The CP-Arg and Pan-Leg targets showed significant differences between the 2 assays using the Student's t-test with $P < .0002$. The CP-Arg multiplex assay consistently displayed lower Ct values (22.4–38.1) versus the singleplex assay (23.4–39.1). Similarly, the Pan-Leg multiplex assay exhibited lower Ct values, with a range of 20.5–36.7 versus 22.5–40.3 for the singleplex assay. The Student's t-test for the MP181 marker showed no statistical difference between the assays ($P = .030$). Statistical analysis was not performed on specimens that failed to provide positive amplification curves in all 3 replicates with either the singleplex or multiplex assay. Eight specimens tested with the CP-Arg singleplex assay failed to have positive curves in all 3 replicates; however, the multiplex assay was able to detect 4 of these in all 3 replicates. The multiplex assay also detected one additional *C. pneumoniae*-positive specimen (in 1 of 3 replicates) that was negative with the singleplex reaction. The *M. pneumoniae*-specific assay (MP181) showed equivalent sensitivity with both assays detecting less than 3 replicates in the same 4 specimens. Lastly, one Pan-Leg–positive specimen had 1 of 3 replicates positive with both assays, although the Ct value for the multiplex assay (38.1) was significantly earlier than that of the singleplex assay (43.5).

4. Discussion

Although *M. pneumoniae*, *C. pneumoniae*, and *Legionella* spp. have long been established as causative agents for atypical bacterial CAP infections, rapid and reliable diagnosis continues to be a challenge (Niederman et al., 2001; Skerrett, 1999). Our multiplex real-time PCR assay offers a sensitive and specific tool for rapid and simultaneous detection of these agents. This assay provides numerous advantages over existing detection methodologies including singleplex real-time PCR assays for detection of these agents. This multiplex assay increases specimen throughput because all 3 agents, along with a control, can be detected in a single tube. This results in faster turnaround time from specimen receipt to result, a feature that may be especially important during respiratory outbreaks in which the initial etiology is unknown. This assay reduces the potential for error in preparation of both PCR master mix and sample addition. Furthermore, the multiplex assay allows conservation of the specimen extract, leading to greater flexibility and opportunity for follow-up or additional testing. Because less specimen volume is required to perform this assay, the extraction procedure may be modified to decrease elution volume, thereby concentrating the extract to further increase sensitivity.

The sensitivity observed when testing clinical specimens with the multiplex assay was found to be significantly greater in 2 agent-specific assays (CP-Arg and Pan-Leg) and the RNase P. The CP-Arg and Pan-Leg did display superior efficiencies for the multiplex assays compared to the singleplex assays, which may account for the greater sensitivity observed. These data also demonstrate that the intra-assay competition within the multiplex reaction has no negative impact on the detection of these agents. On the contrary, we noticed an overall slight improvement in detection with the multiplex assay. Recent advancements in the formulation of reagents specifically designed for multiplex real-time PCR and improved qPCR instruments have likely added to the success of multiplex assay performance. Collectively, these data support the validity of using the current multiplex assay to test respiratory specimens for these agents.

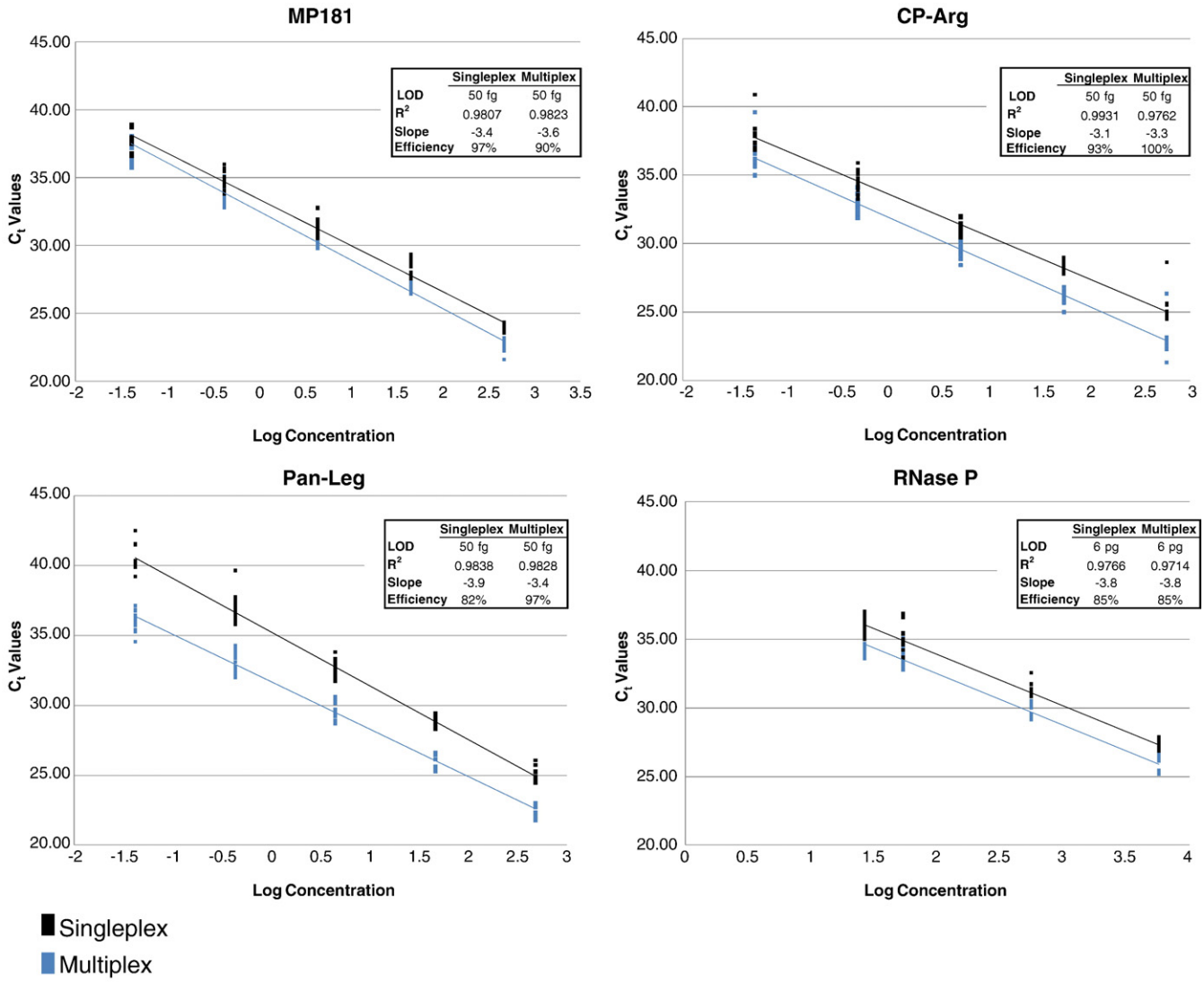

Fig. 3. Comparison of multiplex versus singleplex performance.

These 3 leading atypical bacterial agents of CAP have been targeted previously using various approaches. A multiplex qPCR was performed in 2 separate reactions by Welti et al. (2003) whereby one set contained *M. pneumoniae* and *C. pneumoniae* targets and the other *L. pneumophila* and an internal control. When compared to conventional PCR, these multiplex assays had an overall concordance of >98% when testing 73 respiratory clinical specimens. The use of other chemistries for duplex real-time PCR assays for these agents has also been employed (Gullsby et al., 2008; Loens et al., 2008). To our knowledge, our study is the first report of a single-tube, 4-plex real-time PCR assay that is able to detect these CAP agents along with an internal control (RNase P) in clinical specimens. The use of this assay in surveillance studies may provide greater insight into the etiology and prevalence of atypical bacterial pneumonias among a population while also aiding in the rapid identification of the causative agent for CAP outbreaks. Widespread availability of this and similar assays may lead to a more effective public health response and expedite appropriate treatment of patients.

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