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ORIGINAL RESEARCH

Real-Time PCR Detection of *Mycoplasma pneumoniae, Chlamydia pneumoniae* and *Legionella pneumophila* in Respiratory Specimens Using the ARIES® System

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Abstract

Background: *Mycoplasma pneumoniae* (Mpn), *Chlamydia pneumoniae* (Cpn), and *Legionella pneumophila* (Lpn) can cause both epidemic and endemic occurrences of acute respiratory disease and are responsible for up to 22% of cases of community acquired pneumonia. Due to the limited availability of FDA-approved molecular diagnostic assays, we developed and evaluated a multiplexed Real-time PCR assay for the detection of these agents in two respiratory specimen types on the Luminex ARIES® instrument. The instrument provides for nucleic acid extraction plus PCR amplification and target detection in the same cassette. The ARIES® instrument generates a cycle threshold value and a confirmatory melt curve value for each reaction, including results for an internal sample processing control. The limit of detection for Mpn, Cpn and Lpn, was 100 CFU/mL, 1000 CFU/mL and 100 CFU/mL, respectively. In addition, accuracy, precision, specificity and stability studies were conducted to validate the assay for diagnostic use. Between November 2016 and June 2017, a total of 836 patient specimens were processed in our reference laboratory, with six positive Mpn and two positive Lpn. No specimens were positive for Cpn during this time period. The availability of a robust multiplex PCR assay greatly enhances the ability to rapidly diagnose infections caused by these three agents causing atypical pneumonia.

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Introduction

Respiratory infections caused by *Legionella pneumophila* (Lpn), *Mycoplasma pneumoniae* (Mpn) and *Chlamydia pneumoniae* (Cpn) may account for up to 22% of all cases of community-acquired pneumonia (CAP) worldwide [1]. These infections may occur in outbreak situations or as sporadic cases. Often referred to as etiologic agents of "atypical pneumonia", Lpn, Mpn and Cpn are not detected during routine microbiologic work-up, and typically require special testing requests. Respiratory fluoroquinolones, or macrolide plus beta lactam class antibiotics, are used to treat patients presenting with community acquired pneumonia initially; thus, a rapid and accurate diagnosis allows for targeted antimicrobial therapy [2].

Diagnosis of these agents has undergone an evolution from detection by isolation in culture [3-5], serologic methods [6], detection of urinary antigen for Lpn [7] and eventually, molecular detection in respiratory specimens [8]. Molecular-based assays were initially reported for Lpn by Edelstein *et. al.*, [9] in 1986, for Mpn by Bernet *et. al.*, in 1989 [10] and for Cpn by Holland et. al. in 1990 [11]. Since that time, additional assays have been described and studies have concluded that due to the lower level of sensitivity of non-molecular detection of these agents, molecular detection in respiratory specimens is the preferred test for rapid diagnosis [12]. This will most often lead to the initiation of appropriate antimicrobial therapy [1]. While macrolides remain the treatment choice for CAP due to Mpn, it is worth noting that resistance has been found in the USA. In a study published in 2015, 13.2% of 91 positive patient specimens demonstrated resistance to macrolides such as erythromycin [13]. Emerging resistance with Mpn reinforces the need for rapid and accurate diagnostics performed directly from respiratory specimens. In this way, treatment failures can be effectively managed based on accurate and specific identification of one or more pathogens.

Subsequently, these three assays have been described in multiplexed format by several investigators [14, 15] and two of these targets, Mpn and Cpn, are currently included in two FDA-cleared assays, the FilmArray Respiratory Panel (Biofire Diagnostics, Salt Lake City, UT) and the NxTAG Respiratory Pathogen Panel (Luminex, Inc., Austin, TX). Recently, CMS has published draft statements about reducing reimbursements for highly multiplexed panels such as these [16], making smaller, targeted panels more appealing to laboratories and health care providers.

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We report here the development and evaluation of a multiplexed PCR assay for the detection of Mpn, Cpn and Lpn in oropharyngeal swabs (OP) and broncho-alveolar lavage (BAL) fluid specimens on the Luminex ARIES® instrument. The ARIES® is a stand-alone, open-platform instrument in which the assay uses Multicode®-RTx Technology (Luminex, Inc., Austin, TX) with the synthetic base pair isoC:isoG in a probe-free PCR assay. Because of the hydrogen bonding pattern between these two synthetic bases, they can only bind to each other, adding specificity to the DNA amplification process. This closed system greatly simplifies the process of extraction, amplification and detection within a single cassette while remaining robust for diagnostic testing purposes.

Methods

Reagents: Proprietary MultiCode-Rtx® primer pairs for Mpn, Lpn and Cpn (Part numbers: 3927, 3926 and 3925) were obtained from Luminex. Mouse Hepatitis Virus Control Primer Set 5 (MHV5, proprietary sequence, Part Number: 3806) was used to amplify the Sample Processing Control (SPC, used to confirm extraction, amplification and to detect PCR inhibition). The melt calibrator function allowed for calibration of detected melting temperatures (Tm) in the cassette based on MHV5 amplification. ReadyMix® tubes (Part Number 3697, Luminex, Austin, TX) contain all reagents needed to run PCR on the Luminex ARIES® instrument. SnotBuster® was purchased from COPAN (Catalog number 097CE.A, Murietta, CA). Universal Transport Media (UTM) and flocked swabs were purchased from Quidel/Diagnostic Hybrids (Part Number 402C, San Diego, CA).

Primer Pool Preparation: The four primer sets (Mpn, Lpn, Cpn and MHV5) were purchased in 100 µL aliquots. Once thawed, primers were given a quick spin and equal volumes of each primer were combined to make the multiplex primer set. Pooled primers were stored at -20°C in aliquots of 80 µL.

Organisms: Mpn (ATTC®, Catalog number: 15531TT), Cpn (titered clinical isolate) and Lpn (titered clinical isolate) were used for sensitivity and precision studies. Mpn was grown as described previously [17]. Cpn was propagated in Hep2C cells, then harvested, purified and titered as described previously [18]. Lpn was grown on Buffered Charcoal Yeast Extract (BCYE) agar plates and colonies were suspended in saline. Suspensions were adjusted to a 0.5 McFarland Standard (approximately $1.5x10^8$ CFU/mL) prior to diluting in UTM for PCR studies.

Specimens: Sensitivity, accuracy, precision, stability and specificity studies were assessed using BAL fluid and OP swabs which were collected from healthy volunteers and patients with pneumonia. These specimens were used as matrices after testing negative for Mpn, Cpn and Lpn by both our laboratory-validated PCR test and by the ARIES® PCR assay. For each PCR assay, 200 µL of OP swab specimens collected in universal transport media were used. BAL fluid specimens were pre-treated with SnotBuster® before loading into cassettes by adding 200 µL of specimen to a 1.5 mL microtube and 200 µL of SnotBuster®, then mixed by vortexing for 10 seconds. After incubating at room temperature for 15 minutes, the SnotBuster® pre-treated BAL fluid specimens were centrifuged using a Thermo-Fisher mini centrifuge (mySPIN 6) at 2000 x *g* for one minute. After centrifugation, 390 µL (to avoid any pelleted material) of the pre-treated BAL supernatant was used for the PCR assay.

Cassette Preparation: Eight (8) µL of multiplex primer mix were added to each ReadyMix® tube, then attached to the ARIES® cassette. ARIES® Cassettes (Part Number: 50-10026, Luminex) hold the extraction reagents and Sample Processing Control (SPC). The samples to be tested were added to the cassette and loaded onto the ARIES, then the extraction, PCR reaction, and result generation proceeded automatically.

Sensitivity experiments: For sensitivity, the Limit of Detection (LOD, or cutoff) was determined for three targets (Mpn, Cpn and Lpn) in both BAL fluid and OP swab specimen types that were negative by both PCR assays. Mpn, Cpn and Lpn were spiked individually into each specimen type at concentrations of 1×10^5 CFU/mL, 1×10^4 CFU/mL, 1×10^3 CFU/mL, 100 CFU/mL, 10 CFU/mL, and 1 CFU/mL then tested as described above. This sensitivity assay was conducted three times independently.

Accuracy experiments: For the accuracy study, archived patient specimens that were tested previously using an in-house validated laboratory-developed test (LDT) PCR assay were tested on the ARIES® instrument. All the positive samples were previously PCR-positive for Mpn, Lpn or Cpn in our laboratory from patients for whom a pneumonia infection was detected using the LDT PCR. Twenty-three (23) Mpn-positive samples (either OP swab or BAL fluid specimens), nineteen (19) Lpn-positive samples (all BAL fluid specimens) and one Cpn-positive patient BAL fluid specimen were tested. In addition, DNA from eleven (11) different Cpn-positive patients was spiked into ReadyMix® tubes for OP swab specimens, and DNA from ten (10) Cpn-positive patients was spiked into ReadyMix® tubes for BAL fluid specimens, then placed in the extraction cassette containing negative specimen and tested. For negative controls, thirteen (13) negative patient BAL fluid specimens and seven (7) negative patient OP swab specimens were tested.

Precision experiments: Reproducibility studies to determine precision were done using both BAL fluid and OP swab specimen types for all three targets spiked with a concentration of 1×10^3 CFU/mL. One positive control containing all three targets (as well as a negative control) were run on different days in order to obtain ten successful runs with different operators for both OP swab and BAL fluid specimen types previously determined to be negative by both PCR assays.

Stability experiments: Specimen stability studies were conducted by storage of spiked BAL fluid and OP swab specimen types (1×10^3 CFU/mL, in duplicate) at 4°C, and these samples were assayed daily for eight days.

Specificity experiments: Twelve bacterial organisms, including *Enterococcus faecalis, Escherichia coli, Staphylococcus aureus, Proteus mirabilis, Aerococcus viridans, Streptococcus agalactiae, Moraxella catarrhalis, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Klebsiella pneumoniae* and *Burkholderia cepacia*, kindly provided by Dr. Alan Junkins, Norton Healthcare, Louisville, KY were used for specificity determination in both OP swab and BAL fluid specimens. *Legionella bozemanii, L. pneumophila* Togus-1, L. *pneumophila* Bloomington-2, and *L. pneumophila* Dallas 1E were provided by the laboratory of Dr. Richard Miller, Environmental Safety Technologies. Louisville, KY *L.*

pneumophila Knoxville (ATCC® 33153, strain type) and *L. pneumophila* Philadelphia (ATCC® 33152, strain type) were obtained from the American Type Tissue Collection (ATCC, Manassas, VA). A single colony was selected from freshly sub-cultured plates using a sterile loop; the loop was used to inoculate two different specimen types determined to be negative for Mpn, Cpn and Lpn (200 µL of OP swab or BAL fluid). In addition, several respiratory viruses were tested in OP and/or BAL matrices. Five clinical samples from patients previously diagnosed with viral infections using the FilmArray® Respiratory Virus Panel (BioFireDX, Salt Lake City, UT) were tested. Titered respiratory virus samples from Zeptometrix (Buffalo, NY) were tested, including Influenza A/H3 (Catalog #0810238CF, Texas/50/12), Human Metapneumovirus (Catalog #0810156CF, hMPV 3/Type B1), Coronavirus 229E (Catalog #0810229CF), Adenovirus B (Catalog #0810062CF, Type 3/Species B), and Influenza A/H1N1 (Catalog #0810244CF, Brisbane/59/07), all at titers >10^5 and assayed for Tissue Culture Infecive Dose ($TCID_{50}$). OP swab and BAL fluid specimens were tested in duplicate to assess specificity.

Data Analysis: MS Excel was used to calculate standard deviations for replicate experiments, while MEDCALC Diagnostic Test Evaluation was used to determine analytic sensitivity and specificity [19]. The Luminex ARIES® instrument used SYNCT® software and User Defined Protocol (UDP) module to set assay parameters and interpret run results. The SYNCT® software was installed on an external computer for detailed data analysis and editing of Ct and Tm cutoff values. After completing data analysis and PCR assay validation, the cut off values were fixed and locked in the SYNCT® software assay file. Next, the assay file was loaded on the ARIES® instrument for subsequent diagnostic laboratory testing (**Table 1**). In order for a specimen to be called positive for Lpn, Mpn or Cpn, the following conditions had to be met:

1. The SPC for any given specimen must have a valid Ct value and Tm to exclude sample inhibition.
2. The specimen must have Ct and Tm values in the established range for each organism.

Table 1 Amplification and Melt Curve Settings for ARIES®

S.No	Conditions	*Mycoplasma pneumoniae*	*Chlamydia pneumoniae*	*Legionella pneumophila*	SPC
1	Amplification curve Ct Cut off	1-40.0	1-40.0	1-40	1-45
2	Melt curve Tm Cutoff (°C)	77.5±1.0 (76.5 to 78.5)	80.5±1.0 (79.5-81.5)	71.5±1.5 (70.0-73.0)	84.0±1.0 (81.5-86.5)
3	Melt curve Tm Peak Threshold cutoff (ΔRFU)	-250,000	-800,000	-1,300,000	-100,000

ΔRFU=Change in relative fluorescent units

Results

Sensitivity: Sensitivity was expressed as the cutoff for all three targets. All three organisms were serially diluted ten-fold from 1 x 10^5 to 1 x 10^0 CFU/mL for these experiments. Results are represented as mean ± standard deviation, from three replicate, independent experiments. The cutoff results for Mpn, Cpn and Lpn were 100 CFU/mL, 1000 CFU/mL and 100 CFU/mL, respectively (**Table 2**). **Table 2** shows only the cutoff values for BAL fluid specimens, but similar results were observed with OP swab specimens for each organism (data not shown).

Table 2 Assay Sensitivity for Mpn, Cpn and Lpn in BAL Specimens

CONC (CFU/mL) in BAL	Mpn Ct values (SD)	Mpn Tm values (SD)	Cpn Ct values (SD)	Cpn Tm values (SD)	Lpn Ct values (SD)	Lpn Tm values (SD)	SPC Ct values (SD) *MPN run	SPC Tm values (SD) *MPN run
1x10^5	31.5 (0.82)	77.47 (0.06)	31.4 (1.39)	80.7 (0.10)	26.47 (0.84)	71.33 (0.06)	35.67 (2.11)	84.03 (0.23)
1x10^4	35.07 (0.51)	77.53 (0.12)	33.33 (2.63)	80.7 (0.00)	30.50 (0.87)	71.43 (0.21)	35.90 (0.70)	83.90 (0.10)
1x10^3	38.56 (0.15)	77.46 (0.15)	**38.27 (1.02)**	**80.7 (0.10)**	34.27 (1.14)	71.53 (0.06)	35.2 (0.46)	83.9 (0.10)
1x10^2	**40.04 (0.21)**	**77.45 (0.07)**	40.35 (0.07)	ND	**36.83 (2.35)**	**71.50 (0.20)**	35.63 (0.81)	83.9 (0.10)
1x10^1	ND	ND	ND	ND	37.75 (0.49)	71.10 (0.14)	36.6 (1.37)	83.83 (0.10)
1x10^0	ND	ND	ND	ND	ND	ND	35.33 (1.08)	83.93 (0.15)

ND=Not Detected

Accuracy: Twenty-three of 23 previous Mpn-positive samples were likewise positive with the ARIES® assay, giving a 100% sensitivity (**Table 3**). Twenty-one different previously Cpn-positive patient (11 OP swab and 10 BAL fluids) samples were positive using the ARIES® assay, giving a 100% accuracy (**Table 3**). All 19-previous positive Lpn-samples were positive using the ARIES® system as well, giving a 100% sensitivity (**Table 3**). All 19 patient samples negative for both BAL fluid and OP swab specimen types were also negative using the ARIES® system, but the SPC was positive for all samples, indicating there was no inhibition of PCR (data not shown). Sensitivity and specificity are shown in **Table 3**.

Table 3 Accuracy Results for the Mpn, Cpn and Lpn PCR Assay

Target Organism (Specimen Type)	Expected Positive	Observed Positive	% Agreement	% Sensitivity (95% Confidence Interval)	% Specificity (95% Confidence Interval)
Mpn (10 OP, 1 BAL, and 12 BAL or OP, unspecified)	23	23	100	100 (85.18-100)	100 (83.16-100)
Cpn (11 OP, 10 BAL)	21	21	100	100 (83.89-100)	100 (83.16-100)
Lpn (19 BAL)	19	19	100	100 (82.35-100)	100 (83.16-100)
None (13 BAL, 7 OP)	0	0	100	N/A	N/A

OP=oropharyngeal swab; BAL= brocho-alveolar lavage fluid; N/A=Not applicable

Precision: Mpn, Cpn and Lpn (1 x 10^3 CFU/mL) were spiked into either negative BAL fluid or OP swab samples and used as positive controls by different laboratory technologists on different days. Reproducibility of the resultant Ct and Tm values for four targets (including the MHV5 SPC) did not change over the course of the different testing periods by different operators. The standard deviation (SD) for Mpn, Cpn and Lpn Ct values were less than 2.5, and SD Tm values less than 0.15, on both BAL fluid and OP swab specimens (**Table 4**). Negative controls were negative for all three targets. All internal control results (SPC) indicated no inhibition on either BAL or OP specimen types/matrices (individual run data not shown).

Table 4 Precision Studies for the Mpn, Cpn and Lpn PCR Assay

CONC. (1000 CFU/mL)	Mpn Ct* AVG (SD)	Mpn Tm# AVG (SD)	Cpn Ct AVG (SD)	Cpn Tm AVG (SD)	Lpn Ct AVG (SD)	Lpn Tm AVG (SD)	SPC Ct AVG (SD)	SPC Tm AVG (SD)
BAL	31.47 (1.75)	77.44 (0.10)	33.40 (2.29)	80.67 (0.11)	32.16 (1.29)	71.21 (0.14)	36.33 (1.54)	83.88 (0.14)
OP	31.45 (0.89)	77.47 (0.12)	32.80 (1.60)	80.68 (0.10)	31.63 (1.10)	71.26 (0.11)	35.05 (1.49)	83.88 (0.09)

*Ct=Cycle threshold; #Tm=Melt temperature

Stability: Mpn, Cpn and Lpn (1 x 10^3 CFU/mL of each organism) were spiked into both BAL fluid and OP swab matrices and tested in duplicate for seven days, and in singlet on day eight. After preparing the sample on day one, it was stored in the refrigerator at 4°C (±2°C). The SD for all Ct values over eight days was less than 1.5 for both specimen types. Expected Ct and Tm values for each organism were observed each day of testing, whether they were spiked into BAL fluid or OP swab specimens (data not shown). Thus, specimens demonstrated stability at refrigerated temperatures for up to eight days after collection.

Specificity: Twelve different bacterial organisms and several common respiratory viruses were spiked into BAL fluid and OP

swab matrices and tested Mpn,Cpn and Lpn primers (**Table 5**). In addition, five specimens from patients diagnosed with respiratory virus illnesses were tested using the Mpn, Cpn and Lpn multiplex PCR assay. There was no cross-reactivity observed, and the results demonstrated that these primers were specific to Mpn, Cpn and Lpn. In addition, *Legionella pneumophila* strains of various serotypes and *Legionella bozemanii* were tested to gauge genus, species and serotype detection (**Table 5**). We found that multiple serotypes of Lpn were detected, but not *L. bozemanii*, indicating that *L. pneumophila* serotypes other than 01 could be identified by this assay.

Table 5 Specificity Studies for the Mpn, Lpn and Cpn PCR assay

Organism	Matrix	SPC Ct Value	SPC Tm Value	Results
E. faecalis	OP	**35.5**	**83.8**	Negative for Mpn, Cpn, Lpn
E. coli	OP	**37.3**	**83.9**	Negative for Mpn, Cpn, Lpn
S. aureus	OP	**36.2**	**83.8**	Negative for Mpn, Cpn, Lpn
P. mirabilis	OP	**35.6**	**83.8**	Negative for Mpn, Cpn, Lpn
A. viridans	OP	**37.3**	**83.8**	Negative for Mpn, Cpn, Lpn
S. agalactiae	OP	**35.0**	**83.8**	Negative for Mpn, Cpn, Lpn
M. catarrhalis	OP	**34.6**	**84.1**	Negative for Mpn, Cpn, Lpn
S. epidermidis	OP	**37.3**	**83.8**	Negative for Mpn, Cpn, Lpn
S. pyogenes	OP	**35.9**	**84.1**	Negative for Mpn, Cpn, Lpn
S. pneumoniae	OP	**35.5**	**84.1**	Negative for Mpn, Cpn, Lpn
K. pneumoniae	OP	**38.1**	**83.9**	Negative for Mpn, Cpn, Lpn
B. cepacia	OP	**37.3**	**83.9**	Negative for Mpn, Cpn, Lpn
Influenza A/H1N1 (Zeptometrix)	OP	**33.5**	**84.2**	Negative for Mpn, Cpn, Lpn
Influenza A/H3 (Zeptometrix)	OP	**38**	**84.3**	Negative for Mpn, Cpn, Lpn
Influenza A/H3 (clinical)	OP	**33.4**	**83.8**	Negative for Mpn, Cpn, Lpn
Rhinovirus/ Enterovirus (clinical)	OP	**35**	**83.5**	Negative for Mpn, Cpn, Lpn
Parainfluenza-4 (clinical)	OP	**33**	**83.6**	Negative for Mpn, Cpn, Lpn
Human Metapneumovirus (clinical)	OP	**37.2**	**83.3**	Negative for Mpn, Cpn, Lpn
Human Metapneumovirus (Zeptometrix)	OP	**34.3**	**84.3**	Negative for Mpn, Cpn, Lpn
Coronavirus OC4 (clinical)	OP	**32.9**	**83.6**	Negative for Mpn, Cpn, Lpn
Coronavirus 229E (Zeptometrix)	OP	**33.5**	**84.2**	Negative for Mpn, Cpn, Lpn
Adenovirus 3/B (Zeptometrix)	OP	**31.7**	**84.3**	Negative for Mpn, Cpn, Lpn
L. bozemanii	OP	**33.6**	**83.8**	**Negative** for Mpn, Cpn, Lpn
L. pneumphila Togus-1; Serogroup 02	OP	**33.2**	**84.4**	Negative for Mpn, Cpn, **Positive for Lpn (Ct 26.9/Tm 72)**
L. pneumophila Bloomington-2; Serogroup 03	OP	**38.3**	**84.4**	Negative for Mpn, Cpn, **Positive for Lpn (Ct 22.4/Tm 71.4)**
L. pneumonphila Dallas 1E; Serogroup 05	OP	**38.5**	**83.4**	Negative for Mpn, Cpn, **Positive for Lpn (Ct 23.7/Tm 71.9)**
L. pneumophila Knoxville; Serogroup 01 (ATCC® 33153)	OP	**38.8**	**84.4**	Negative for Mpn, Cpn, **Positive for Lpn(Ct 19.8/Tm 71.5)**
L. pneumophila Philadelphia; Serogroup 01 (ATCC® 33152)	OP	**35.4**	**84.3**	Negative for Mpn, Cpn, **Positive for Lpn(Ct 20.4/Tm 72)**

Performance with Clinical Specimens: Between November 2016 and June 2017, a total of 836 patient specimens were processed in our reference laboratory, with six positive Mpn and two positive Lpn, giving a 0.72% positivity rate (data not shown). No specimens had detectable Cpn. It was noteworthy that samples rarely had to be retested due to invalid results on the ARIES® instrument. Out of 1064 specimens tested with the multiplex Mpn, Lpn and Cpn PCR assay, only 50 invalid results occurred, giving a 4.7% invalid rate. When respiratory samples did give invalid results, it was typically in BAL fluid specimens with excessive blood or mucus, and diluting 1:2 or 1:4 with saline prior to re-testing gave valid results.

Example readout from ARIES® instrument: The SPC must show the expected amplification and melt curve values (Ct and Tm values) in patient samples that are negative (negative control example, **Figure 1A**). If the patient sample or positive control is positive for any target (Mpn, Cpn or Lpn), the ARIES® result shows the positive sample amplification (Ct value) and melt curve (Tm) data but will not show Ct and Tm values for the SPC (positive control example, **Figure 1B**). This is hardcoded in the software for ARIES® instrument. The SPC amplification and melt curve including Ct and Tm values can be obtained from SYNCT® software, when needed.

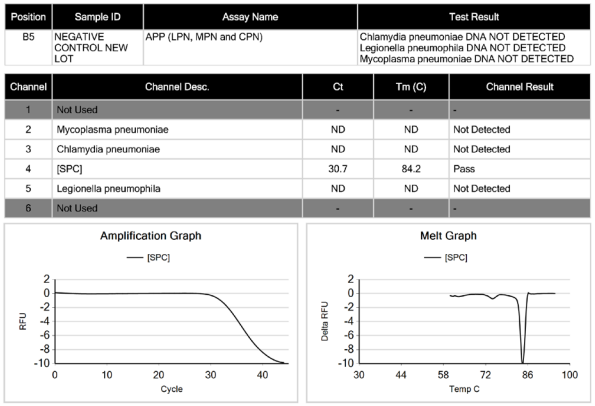

Figure 1A Negative control result. Sample data from ARIES® instrument with a negative control illustrating the amplification curve and melt temperature for the internal control (SPC).

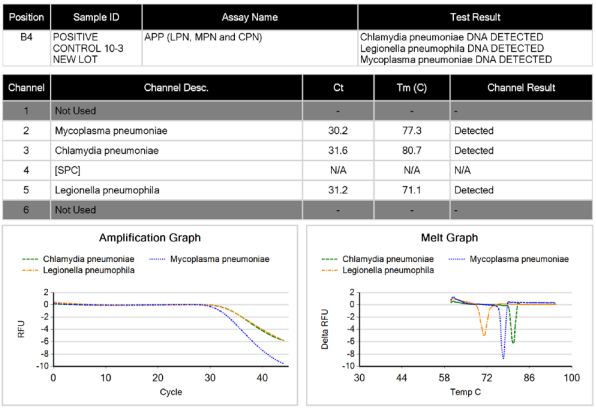

Figure 1B Positive control result. Sample data from ARIES® instrument with a positive control illustrating the amplification curve and melt temperature for Mpn, Cpn, and Lpn (SPC).

Discussion

The Luminex ARIES® instrument performs extraction and generates PCR amplification and melt curves for each target organism, plus the sample processing control, in a single cassette. After addition of primers to ReadyMix® and specimen to the cassette, the PCR amplification is completed in two hours. Due to the limited availability of FDA-approved assays that detect a small panel of atypical pneumonia organisms, we present here the results of our laboratory validation of this multiplex PCR assay that detects Mpn, Cpn and Lpn.

The established limits of detection for Mpn, Cpn and Lpn were 100 CFU/mL, 1000 CFU/mL and 100 CFU/mL, respectively for both BAL and OP specimen types. This is similar sensitivity to previously published studies using NP swabs [20] or NP aspirates [21] in a multiplex PCR assay for Mpn, Cpn and Lpn targets. For example, Miyashita *et al.* [20] found using their multiplex PCR assay with a Micro-Chip Electrophoresis Analysis

System, they could detect 100 copies per mL of Mpn, Cpn and Lpn. When they tested 208 patients with community acquired pneumonia using nasal swabs, the multiplex PCR assay detected 14/15 patients with Cpn, 10/10 with Mpn and 8/8 with Lpn infections [20]. Similarly, Lam et al demonstrated a sensitivity range of 1000 to 10,000 CFU/mL with a multiplex nested PCR assay on the ABI FAST® PCR system [21]. Using 303 clinical specimens (NP aspirates), they found a 48.5% positivity rate for detecting Mpn, Cpn, Lpn or Adenovirus [21]. When previously positive specimens were tested on the ARIES® system with the PCR assay described here, each organism was correctly detected compared to the results from the prior test platform used in our laboratory, giving 100% sensitivity. Precision results were excellent when the same specimens were tested on different days by different testing personnel, as indicated by very small standard deviations.

A panel of twelve different bacterial organisms and several common respiratory viruses were tested using BAL fluid and OP swab matrices. There was no cross-reactivity observed, thereby demonstrating that these primers were specific to Mpn, Cpn and Lpn. Because we tested *L. pneumophila* strains representing serogroups 01, 02, 03, and 05, as well as *L. bozemanii*, we demonstrated primer specificity for *L. pneumophila*; however, we observed broader detection than *L. pneumophila* urine antigen assays that target only serogroup 01. Finally, stability studies indicated that after storage for eight days at 4°C, positive specimens processed by the ARIES® instrument were detected. Future studies could address the stability at -20°C and -80°C for long-term specimen storage.

Taken as a whole, these results indicate that the Luminex ARIES® PCR assay is quite robust. During the validation and subsequent use as a diagnostic PCR assay, a few limitations were noted. For BAL fluid specimens, excess mucus was found to cause invalid results on rare occasions (50/1064, or 4.7%). In these cases, diluting the specimen prior to mixing with SnotBuster® and re-running the diluted specimen eliminated the inhibition observed in the initial specimen. This was not observed with OP swab specimens, which are typically free of excess mucus. The Luminex ARIES® instrument uses SYNCT® software to set parameters and interpret run results. Mastery of this software requires dedicated time and effort; however, technical support, assistance and training is available from the manufacturer. Finally, the ARIES® instrument uses universal cycling conditions to facilitate multiplex PCR assay development. If cycling conditions need to be changed for a particular assay, they can be modified, but then separate assays would need to be performed using different modules on the ARIES® instrument.

The data presented here illustrate the performance characteristics of a multiplex PCR assay for three agents of atypical pneumonia. The MultiCode-Rtx® chemistry enables the detection of almost any nucleic acid target due the highly specific binding of IsoC and IsoG base pairs, without the need for an additional fluorescent-tagged oligo or probe. The format is an improvement over previously published processes due to the elimination of a separate extraction step for clinical specimens. Furthermore, due to the combination of ReadyMix® tubes and cassettes used by the ARIES® instrument, minimal technologist time is required because extraction, amplification and detection occur within the cassette. This format reduces risk of cross-contamination and amplicon contamination significantly and provides both a Ct value and a melt curve value, adding specificity to the assay. The ARIES® system is easy to use with Luminex analyte specific reagents (ASR). Luminex application specialists provide general guidance to aid in new assay development, which is extremely valuable to smaller clinical laboratories with limited resources. As an open PCR platform with the capacity to design primers for multiple targets as well as internal controls, this system offers flexibility for laboratories. This feature is important during a time when reimbursement for highly multiplexed assays is at risk [16], and more targeted panels (like the atypical pneumonia assay described here) are favored currently. In the current climate, it is critical that laboratories calculate cost per test versus reimbursement, either through third party insurance or direct billing for laboratory services, to remain viable. Open platforms, such as the ARIES® system, can help lower direct costs in the face of declining reimbursements.

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