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ORIGINAL RESEARCH

Development of a Real-time PCR assay for *Pneumocystis jirovecii* on the Luminex ARIES® Platform

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Abstract

Pneumocystis pneumonia (PCP) is an opportunistic infection caused by the fungus *Pneumocystis jirovecii*. Infection with *P. jirovecii* can result in serious illness in patients with a weakened immune system, and can lead to death if it is not properly diagnosed and treated. Direct detection of *P. jirovecii* in lower respiratory tract specimens such as bronchoalveolar lavage (BAL) is preferred for rapid diagnosis, a laboratory service currently not available locally. We report here the development of a diagnostic real-time Polymerase Chain Reaction (PCR) assay using BAL specimens to detect *P. jirovecii*. By targeting the multi-copy mitochondrial large subunit ribosomal RNA gene (mtLSU rRNA) of *P. jirovecii*, assay sensitivity is increased. Primer pairs were designed to include a fluorescent reporter dye-labeled primer with a unique MultiCode® base pair isoC on the 5'end and one unlabeled primer. The performance characteristics were determined on the Luminex ARIES® instrument, combining DNA extraction, amplification and detection into a one-step process. The cassette contains the reagents needed to perform all of the steps including extraction, purification, amplification, and detection, plus a sample processing control. Accuracy, precision, sensitivity, specificity and stability studies were conducted to validate the assay to meet CLIA requirements. The analytical sensitivity was 89.1%, and the analytical specificity was 100%. The assay could reliably detect 200 organisms/mL, crossing thresholds (Ct) and melt temperatures (Tm) were consistent, and no cross-reactivity was observed with other pathogens known to cause respiratory infections. The results demonstrated that these primers are specific to *Pneumocystis jirovecii*. The real-time PCR method using the ARIES® system allowed for rapid and sensitive detection of Pneumocystis pneumonia infections with *P. jirovecii* using clinical respiratory specimens.

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Introduction

Pneumocystis pneumonia (PCP) is a major health concern for immunocompromised individuals, whether due to HIV infection, chemotherapy or solid organ transplantation [1]. PCP is caused by the opportunistic fungal pathogen *Pneumocystis jirovecii*, a pathogen specific to humans, having no other reservoir [2]. Individuals suffering from PCP usually present with non-specific symptoms such as fever, cough, shortness of breath, and fatigue. Sulfa drugs are the most common treatment for infection [3].

A rapid and accurate laboratory test is important for early diagnosis and treatment since any delay in treatment can significantly increase the risk of mortality. This organism has not been successfully cultured to date, so diagnosis depends on other laboratory methods. Traditionally, PCP has been diagnosed with the help of numerous staining methods or fluorescent antibody assays. Detection of nucleic acids from *P. jirovecii* in clinical samples has been evaluated numerous times, with certain authors making the case that molecular detection, such as quantitative PCR, should be the test of choice for PCP [4, 5]. While concerns about colonized versus infected patients have been raised, combining quantitative molecular detection with clinical judgment should reduce false positive results.

We developed and evaluated a real-time PCR assay to detect *P. jirovecii* targeting mitochondrial large subunit ribosomal RNA gene (*mtLSU* rRNA) in lower respiratory tract specimens using the Luminex ARIES® instrument. The objective was to provide a rapid and sensitive test to health care practitioners serving immunocompromised patients in our area, since sending these specimens to an out of state reference laboratory is expensive and takes at least one week for results. The *mtLSU* rRNA target was chosen because of the copy number and previous comparative studies [6]. The Luminex ARIES® is an open-platform instrument which uses MultiCode-RTx® base pairing (isoC:isoG) technology. This instrument was selected because it simplifies the extraction, amplification and detection process without affecting the robustness of diagnostic testing. Selective primers, labeled with fluorescent dyes, facilitate real time detection of both the *mtLSU* rRNA gene target for PCP and an internal control in a single-step. This assay enables rapid diagnosis with great sensitivity and specificity with little hands-on time required.

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Materials and Methods

Specimens

Archived, de-identified PCP positive and negative clinical specimens were provided by Dr. Scott McClellan, University of Michigan, in order to set assay parameters. These specimens were deemed exempt by the University of Michigan Institutional Review Board (S. McClellan, personal communication). Residual BAL specimens from consenting patients were used for analytical sensitivity, accuracy, precision, stability and specificity studies. Residual BAL specimens were determined to be negative for *P. jirovecii* using this PCR method prior to spiking with known concentrations of *P. jirovecii* DNA. Briefly, specimen processing was done in Bertin tubes (Precellys Lysis Kit, Reference number KT03961-1-006.2, Bertin Corp, Rockville, MD). First, 400 µl of BAL sample were added to the Bertin tube with 400 µL of AL buffer (QIAGEN Catalog number: 19075, Germantown, MS). The Bertin tube was placed on a vortex adaptor and shaken at 10,000 rpm for 5 minutes to disrupt cells. After incubating at room temperature for 10 minutes, the tubes were centrifuged for 2 minutes at 14,000 rpm. Finally, 400 µL of supernatant was added to tubes containing 5 µL of carrier RNA, vortexed, then 405 µL of sample with carrier RNA was used for the assay.

Organisms

Fungal isolates were provided by Dr. Alan Junkins, Norton Healthcare, Louisville, KY. Eleven different fungi were spiked into negative BAL specimens and tested to assess cross-reactivity as follows: *Candida dubliniensis, Candida parapsilosis, Candida glabrata, Candida krusei, Candida tropicalis, Cryptococcus neoformans, Saccharomyces cerevisiae, Histoplasma capsulatum* (yeast form), *Aspergillus fumigatus, Aspergillus versicolor and Aspergillus niger*. *Streptococcus pneumoniae* isolates from the University of Louisville Infectious Diseases Laboratory were tested in the same way to assess cross-reactivity. A single colony was selected from freshly sub-cultured plates using a sterile calibrated loop; the loop was used to inoculate BAL from patient specimens previously determined to be negative for PCP processed as described in the specimen section. College of American Pathologists (CAP) proficiency testing samples (archived IDR-A and IDR-B from 2018 events) containing numerous respiratory viruses and bacteria were used to assess cross-reactivity. For that purpose, 200 µL of proficiency test samples known to contain the following were spiked into negative BAL specimens: Coxsackie virus A9, *Chlamydophila pneumoniae* strain CWL 029, Influenza A California 07/2009 (H1N1), *Bordetella pertussis* A639, Influenza B Massachusetts 2/2012, Coronavirus NL63, Parainfluenza Type 2, RSV Type B CH93(18)-18, *Bordetella parapertussis* A747, Parainfluenza virus Type 4, Echovirus 30 (E-30), Influenza B Strain Brisbane 60/2008, Parainfluenza Type 3, Adenovirus Type 21, *Mycoplasma pneumoniae* M129, Influenza A strain New York 18/2009 (H1N1), and Human metapneumovirus B2. Also, previously characterized bacterial stocks from the University of Louisville Infectious Diseases Laboratory were tested to assess cross-reactivity [7]. *Chlamydia pneumoniae, Legionella pneumophila*, and *Mycoplasma pneumoniae* from each bacterial stock were spiked into BAL, using 160 µL of $1x10^5$ CFU/mL stock as input.

Table 1 Primers used for Luminex ARIES® *P. jirovecii* PCR assay.

Organism	Forward Primer	Reverse Primer	Gene Target	Amplicon Length (bp)
Pneumocystis jirovecii	5'-CAG ACT ATG TGC GAT AAG GTA GAT AGT CG-3'	5'-/56-FAM/iMe-isodC/GGA GCT TTA ATT ACT GTT CTG GGC-3'	Mitochondrial large subunit ribosomal RNA gene	64

Table 2 PCR Thermal profile used for Luminex ARIES® *P. jirovecii* PCR assay.

PCR	
Pre-heat Temperature	95°C
Pre-heat hold time	0 sec
Activation Temperature	95°C
Activation Time	120 sec
Denature Temperature	95°C
Denature Time	5 sec
Anneal Temperature	62°C
Anneal hold Time	7 sec
Extension Temperature	72°C
Extension hold Time	14 sec
Number of PCR steps repeat	3 (from Denature to Extend)
Optical Read Location	Extension
Number of PCR cycles	45
Melt	
Melt Temperature Step	0.5°C
Melt Start Temperature	60°C
Melt final Temperature	95°C
Melt Hold Time	2 sec
Optical read location	Melt hold time

Control Materials

Due to the limited number of clinical samples available for testing, recombinant *P. jirovecii* positive controls were purchased from Zeptometrix (NATtrol *P. jirovecii* Recombinant External Run Control, Catalog number 320679, Buffalo, NY) and EXACT Diagnostics (custom *Pneumocystis jirovecii* run control, Reference number PCPRC, Fort Worth, TX).

Reagents

ARIES® MultiCode® DNA ReadyMix® tubes (Part number: 3697, Luminex, Austin, TX) and ARIES® extraction cassettes (Part number: 50-10026, Luminex) were purchased ready to use. Carrier RNA (QIAGEN part number 1017647, Germantown, MD) was prepared as a 1 µg/µL solution in AVE buffer (QIAGEN part number 1026956, Germantown, MD), aliquots were stored at -20°C. Primer pairs, obtained from Integrated DNA Technologies Inc. (IDT, Coralville, IA), were designed to include a FAM reporter-labeled reverse primer with an isodC on the 5'end, and a second unlabeled forward primer for *P. jirovecii*. The primer set was designed to amplify the mitochondria large subunit (*mtLSU*) rRNA from *P. jirovecii* based on a previous study [8]. Primer designs were evaluated by eye, then screened using IDT's Oligo Analyzer software. Primer sequences are shown in **Table 1**. Each primer was used at a final concentration of 200 nM. Mouse Hepatitis Virus 2 control primers (MHV2, proprietary, Luminex Part Number 3803, Austin, TX) were used to amplify the Sample Processing Control (SPC; confirms extraction, amplification and detects inhibition). The SPC calibrator function allowed for calibration of detected melting temperatures (Tm) based on MHV2 amplification.

Primer Pool Preparation

The *mtLSU* rRNA forward and reverse lyophilized primers were dissolved in Tris-EDTA (TE buffer, Fisher Catalog #BP2473-1, Waltham, MA) to make a 100 µM stock solution of each primer. Further dilution of the 100 µM stock was done with molecular grade water to make 5 µM working stock. To prepare the pooled primer mix, 100 µL of each 5 µM stock primer was combined with 100µL of MHV2 primer stock to make 300 µL total volume.

Table 3 *P. jirovecii* assay Amplification and Melt Curve Settings for ARIES®

Setting	Conditions	*P. jirovecii* *mtLSU rRNA* target FAM channel	SPC MHV2 target AP525 channel
1	Amplification curve Cut off (Ct)	1-40	1-45
2	Melt curve (Tm) Cutoff (°C)	77.4-78.9 (78.15±0.75)	74.7-79.7 (77.2±2.5)
3	Melt curve (Tm) Peak Threshold cutoff (ΔRFU)	-700,000	-150,000

SPC= Sample Processing Control; ΔRFU=Change in relative fluorescent units

Table 4 Assay Sensitivity for *P. jirovecii* in BAL Specimens

Concentration (~Number organisms/mL in BAL)	*P. jirovecii mtLSU rRNA* Ct values (SD)	*P. jirovecii mtLSU* rRNA Tm values (SD)	SPC MHV2 Ct values (SD)	SPC MHV2 Tm values(SD)
$2x10^5$	25.97 (0.45)	77.90 (0.00)	34.23 (1.06)	77.40 (0.26)
$2x10^4$	30.50 (0.82)	78.00 (0.00)	34.43 (0.80)	77.37 (0.32)
$2x10^3$	33.87 (0.29)	78.03 (0.06)	33.73 (0.83)	77.40 (0.30)
$2x10^2$	**37.57 (1.04)**	**77.97 (0.15)**	**33.83 (1.12)**	**77.37 (0.32)**
$2x10^1$	ND	ND	34.87 (1.37)	77.53 (0.31)
$2x10^0$	38.10*	78.00*	35.03 (0.97)	77.40 (0.17)

SPC= Sample Processing Control ;ND=Not Detected; SD= Standard Deviation

*Positive result in one out of three runs.

The complete primer pool was then mixed well by vortexing, spun down and stored in 60 µL aliquots at -20°C.

ARIES® Cassette and Instrument Preparation

Six (6) µL of the mtLSU rRNA pooled primer mix were added to each MultiCode® DNA ReadyMix® tube, then attached to the ARIES® cassette. ARIES® Cassettes (Part Number: 50-10026, Luminex, Austin, TX) contained the extraction reagents. Four hundred (400) µL of processed BAL sample were added to a 1.5 mL micro centrifuge tube along with 5 µL of a 1 µg/mL carrier RNA stock. The tube was vortexed for five seconds prior to transferring the entire volume to the cassette and loaded onto the ARIES® instrument. The extraction, PCR reaction and result generation then proceeded automatically (PCR thermal cycle profile in **Table 2**).

Sensitivity

For analytical sensitivity, the LOD was determined for the *mtLSU* rRNA target in BAL specimens that were negative by the Luminex ARIES® PCR assay described here. The Zeptometrix PCP positive control (400 µL, $2x10^5$ organisms per mL) was used as the initial dilution, followed by 6 ten-fold dilutions in the negative BAL specimen, then tested as described above. The LOD assay was conducted three times independently (**Table 4**).

Accuracy

A panel of 24 (18 BAL, five sputum and one induced sputum) previously known positive and ten (eight BAL, one bronchial wash, and one sputum) known negative patient specimens provided by Dr. Scott McClellan (University of Michigan) were tested. Additionally, 17 spiked positive samples [PCP controls at varying dilutions: nine Zeptometrix controls at $2x10^5$, $2x10^4$, $2x10^3$, $2x10^2$ organisms per mL, and eight Exact Diagnostics controls at 125,000 copies/mL, 12,500 copies/mL, 1250 copies/mL and 125 copies/mL in BAL (**Table 5**)] and 14 negative blinded BAL samples were also tested by different technologists.

Precision

Reproducibility studies to determine precision were conducted using the Zeptometrix PCP control diluted 1:100 ($2x10^3$ organisms/mL) in negative BAL samples. To demonstrate intra-assay and inter-assay precision, positive controls were tested for seven days and negative controls were tested for five days in duplicate, then for 10-15 days in singlet using different technologists. Average and standard deviations were calculated using MS Excel (**Table 6, A&B**).

Table 5 Accuracy Results for the *P. jirovecii* PCR Assay

P. jirovecii	Expected Positive	Observed Positive	% Agreement	% Sensitivity (95% Confidence Interval)	% Specificity (95% Confidence Interval)
Positive (clinical and spiked respiratory specimens)	41	36	87.8	89.13 (76.43-96.38)	N/A
PCP Negative (clinical respiratory specimens)	0	0	100	N/A	100 (85.75-100)

N/A=Not applicable

Table 6A Intra-assay Precision Studies for the *P. jirovecii* PCR Assay

Sample Type	Day 1 *mtLSU* rRNA Ct value	Day 2 *mtLSU* rRNA Ct value	Day 3 *mtLSU* rRNA Ct value	Day 4 *mtLSU* rRNA Ct value	Day 5 *mtLSU* rRNA Ct value	Day 6 *mtLSU* rRNA Ct value	Day 7 *mtLSU* rRNA Ct value
Positive Control 1	30.7	31.4	33.8	34.10	32.7	33.6	32.8
Positive Control 2	32.7	31.9	32.1	31.6	32.2	34.5	33.7
Average (SD)	**31.7 (1.41)**	**31.65 (0.35)**	**32.95 (1.2)**	**32.85 (1.77)**	**32.45 (0.35)**	**34.05 (0.64)**	**33.25 (0.64)**
Sample Type	**Day 1 SPC Ct value**	**Day 2 SPC Ct value**	**Day 3 SPC Ct value**	**Day 4 SPC Ct value**	**Day 5 SPC Ct value**	**Day 6 SPC Ct value**	**Day 7 SPC Ct value**
Negative Control 1	**33.6**	**37.2**	**37.8**	**37.0**	**35.3**	**N/A**	**N/A**
Negative Control 2	**33.0**	**38**	**40.1**	**37.4**	**36.3**	**N/A**	**N/A**
Average (SD)	**33.3 (0.42)**	**37.6 (0.56)**	**38.95 (1.62)**	**37.2 (0.28)**	**35.8 (0.70)**	**N/A**	**N/A**

Ct=Cycle threshold; Tm=Melt temperature; ND=Not Detected;
SPC=Sample Processing Control; N/A=Not Applicable

Table 6B Inter-assay Precision Studies for the *P. jirovecii* PCR Assay

Sample Type	*P. jirovecii mtLSU* rRNA Ct value	*P. jirovecii* Tm value	SPC Ct value	SPC Tm value
Positive Control (n=15)	**32.98 (0.91)**	**77.98 (0.12)**	**35.02 (1.75)**	**76.99 (0.32)**
Negative Control (n=10)	**ND**	**ND**	**36.12 (1.72)**	**77.28 (0.36)**

Ct=Cycle threshold; Tm=Melt temperature; ND=Not Detected;
SPC=Sample Processing Control

Stability

Specimen stability studies were conducted by incubation of negative BAL samples spiked with known PCP Zeptometrix positive control diluted 1:100 ($2x10^3$ organisms/mL) and stored at 4°C, then assayed daily for 8 days **(Table 7A)**. In addition, nine different known *P. jirovecii* positive clinical BAL samples were pooled and stored at 4°C, then assayed daily for 8 days (**Table 7B**).

Specificity

Eleven different fungi, eight different bacteria and 13 different viruses were spiked into negative BAL and tested in to assess cross-reactivity (**Table 8**).

Data Analysis

Microsoft Excel® was used to calculate standard deviations for replicate experiments, while MedCalc Diagnostic Test Evaluation was used to determine analytic sensitivity and specificity [9]. The User Defined Protocol (UDP) module in the SYNCT® software was used to set assay parameters. The SYNCT® software was installed on an external computer for

Table 7A *P. jirovecii* Stability Studies using positive control spiked into negative BAL

Specimen Type	*P.jirovecii mtLSU rRNA* Ct Average (SD)	*P. jirovecii mtLSU rRNA* Tm Average (SD)	SPC Ct Average (SD)	SPC Tm Average (SD)
BAL	33.24 (0.57)	78.04 (0.09)	34.29 (1.34)	76.95 (0.24)

Ct=Cycle threshold; Tm=Melt temperature; ND=Not Detected;
SPC=Sample Processing Control

Table 7B *P. jirovecii* Stability Studies using pooled clinical *P. jirovecii* positive BAL

Specimen Type	*P.jirovecii mtLSU rRNA* Ct Average (SD)	*P. jirovecii mtLSU rRNA* Tm Average (SD)	SPC Ct Average (SD)	SPC Tm Average (SD)
BAL	34.46 (0.50)	77.93 (0.08)	37.86 (2.20)	77.56 (0.13)

Ct=Cycle threshold; Tm=Melt temperature; ND=Not Detected;
SPC=Sample Processing Control

Table 8 Specificity Studies for the *P. jirovecii* PCR assay

Organism	Matrix	SPC Ct Value	SPC Tm Value	PCR Results for *P. jirovecii*
Fungi				
Candida dubliniensis	BAL	32.4	76.6	Negative
Candida parapsilosis	BAL	33.9	76.9	Negative
Candida glabrata	BAL	34.9	76.9	Negative
Candida krusei	BAL	33.9	76.9	Negative
Candida tropicalis	BAL	35.1	76.9	Negative
Cryptococcus neoformans	BAL	33.3	77.1	Negative
Saccharomyces cerevisiae	BAL	32.8	77.7	Negative
Histoplasma capsulatum (yeast form)	BAL	33.9	76.9	Negative
Aspergillus fumigatus	BAL	34.7	76.6	Negative
Aspergillus versicolor	BAL	36.1	76.4	Negative
Aspergillus niger	BAL	32.1	76.9	Negative
Bacteria				
Chlamydophila pneumoniae strain CWL 029	BAL	36.5	77	Negative
Bordetella pertussis A639	BAL	37.5	77.1	Negative
Bordetella parapertussis A747	BAL	37.6	76.8	Negative
Mycoplasma pneumoniae strain M129	BAL	39.4	76.9	Negative
Chlamydia pneumoniae	BAL	39.3	76.9	Negative
Legionella pneumophila	BAL	38.2	76.7	Negative
Mycoplasma pneumoniae	BAL	36.9	76.9	Negative
Streptococcus pneumoniae	BAL	39.1	77.7	Negative
Viruses				
Enterovirus Coxsackie A9	BAL	36.5	77	Negative
Influenza A strain California 07/2009 (H1N1)	BAL	37.5	77.1	Negative
Influenza B Massachusetts 2/2012	BAL	35.9	76.9	Negative
Coronavirus NL63	BAL	35.9	76.9	Negative
Parainfluenza Type 2	BAL	37.6	77	Negative
RSV Type B CH93 (18)	BAL	37.6	77	Negative
Parainfluenza virus Type 4	BAL	37.6	76.8	Negative
Echovirus 30 (E-30)	BAL	36.7	77.1	Negative
Influenza B Strain Brisbane 60/2008	BAL	39.5	76.9	Negative
Parainfluenza Type 3	BAL	39.5	76.9	Negative
Adenovirus strain Type 21	BAL	39.4	76.9	Negative
Influenza A strain New York 18/2009 (H1N1)	BAL	39.7	76.9	Negative
Human metapneumovirus strain B2	BAL	39.7	76.9	Negative

detailed data analysis and editing of Ct and Tm cutoff values. After completing data analysis and PCR assay validation, the Ct and Tm cut off values were set and locked in the SYNCT® software assay file. Next, the assay file was loaded on the ARIES® instrument for subsequent diagnostic laboratory testing **(Table 3)**. In order for a specimen to be called positive for *P. jirovecii*, the following conditions had to be met:

1. The SPC for any negative 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 the target organism *P. jirovecii*.

Results

Using a combination of positive and negative patient specimens, and spiked known concentrations of recombinant external positive controls, the performance characteristics of the PCR assay on the ARIES® system were established. The results of this study were highly reproducible, as demonstrated by testing positive and negative controls with different technologists on different days. We obtained consistent results with low standard deviations (**Tables 4, 6A&B and 7A&B**). Analytical sensitivity for the *P. jirovecii* assay was 89.1% (95% CI 76.43-96.38%), while analytical specificity was 100% (95% CI 87.23 -100), (**Table 5**). Stability studies were performed using positive control material spiked into negative BAL and pooled *P. jirovecii* clinical positive BAL samples, stored at 2-8°C for eight days and tested daily for 8 days. Results showed that DNA was detected by the PCR assay under these storage conditions (**Table 7A and B**). Finally, a variety of fungi, bacteria, and viruses that can cause respiratory infections were tested to establish specificity. There was no cross-reactivity observed with the *P. jirovecii mtLSU* rRNA primers using 32 different organisms (**Table 8**). By including the SPC, any inhibition of PCR would have been detected, and none was observed during these studies (**Table 8**).

The assay could reliably detect 3000 copies of *mtLSU* rRNA that equated to 200 organisms/mL of *P. jirovecii* (**Table 4**) in respiratory specimens. It is important to note that *P. jirovecii* is estimated to have 15 copies of the *mtLSU* rRNA gene/organism [10], allowing for more sensitive detection by PCR.

Discussion

Quality laboratory results are dependent on proper specimen collection, transport, storage and processing procedures, as well as adherence to good molecular laboratory techniques to avoid false positive and false negative results. As with any laboratory test, regardless of the method used to diagnose *Pneumocystis* pneumonia, clinical judgment in light of patient presentation and history are the ultimate deciding factors for patient treatment and management [11].

The strengths of this study include the demonstration of the precision, limit of detection using quantitated target DNA and demonstration of specificity. In addition, the ability to adapt the PCR assay to the Luminex ARIES® platform so that extraction, amplification and detection occur in a closed system reduces potential cross-contamination. Some limitations of this study include the small number of clinical samples available for testing and the use of spiked BAL samples to mimic clinical samples. Due to a limited number of available clinical specimens, stability studies were conducted with positive control material in the form of plasmids as well as with pooled positive clinical

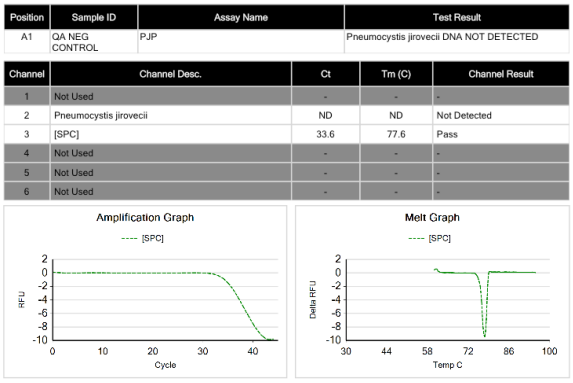

Figure 1A Negative Control Result: Sample data from ARIES® instrument with a negative control illustrating the amplification curve (Ct) and melt temperature (Tm) for the internal control (SPC).

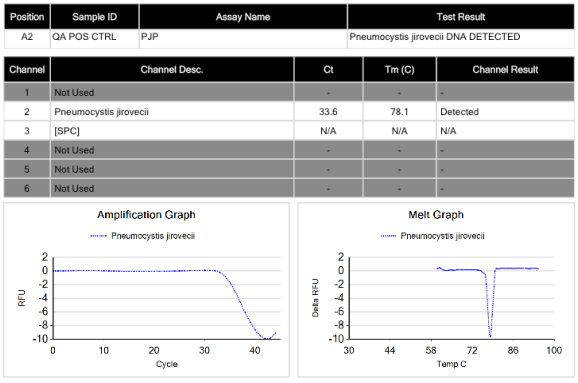

Figure 1B Positive Control Result: Sample data from ARIES® instrument with a positive control illustrating the amplification curve (Ct) and melt temperature (Tm) for *P. jirovecii*.

specimens. Comparison to a "gold standard" assay was also problematic. Numerous publications regarding PCR detection of *P. jirovecii* have used a wide variety of gene targets, methods, instruments, and limits of detection reporting units (i.e. trophic form equivalents [12], plasmid copy/uL [13], DNA copy/uL [11] or cysts/amplification [14], making direct comparisons challenging [15]).

A valid concern with PCP molecular detection assays is that this PCR assay could detect colonized patients as well as patients with infections. While this PCR assay was used in a qualitative manner, the Ct values provided some indication of organism load when *P. jirovecii* was detected [12, 13]. Example instrument results are provided in **Figures 1A** and **1B**. Based on a likely human-to-human transmission route [16-18], detecting colonization may be relevant in certain situations. Setting a cutoff for colonization may not be practical for all situations, however, such as fungal load variability in HIV-infected versus non-infected immunosuppressed patients, or pediatric patients versus adult patients [8, 11, 19]. While microscopic analysis remains a valid laboratory method for determining infection with these organisms, it requires a high level of technical expertise. It also lacks the sensitivity of molecular diagnostics by an estimated factor of 100 [14], thus supporting the need for molecular diagnostic assays for detection of *P. jirovecii*.

Exploration of quantitative PCR assays for *P. jirovecii*, adding other targets (e.g. drug resistance markers or virulence factors), and identifying other high copy number genes such as the mitochondrial smaller subunit (mtSSU) rRNA [10] are future goals to make this assay more sensitive and useful to clinicians. In particular, drug resistance markers are of great interest in an era of antibiotic stewardship initiatives. A study conducted in Chile observed a high prevalence of mutations in the dihydropteroate synthase (DHPS) gene in *P. jirovecii* infected patients, despite a lack of previous exposure to trimethoprim-sulfamethoxazole or dapsone [18]. This study suggested a role for person-to-person transmission. The presence of the DHPS mutation correlated with twice longer duration of mechanical ventilation in this hospitalized group of 56 patients [18], indicating an important area for future study.

The benefits of the ARIES® *P. jirovecii* assay include minimal hands-on time for technologists, one step specimen processing with a robust internal sample processing control, all within a closed system. Results are available within two hours while providing rapid, specific and sensitive results for health care providers. The ability to tailor PCR assays to the local health care community, and to add new targets with guidance from Luminex technical support, adds a high degree of flexibility for future *P. jirovecii* assay improvements. Based on the performance characteristics established by our laboratory, the ARIES® real-time PCR assay meets the CLIA requirements for diagnostic testing using BAL specimens from patients suspected of having PCP. Having the real-time PCR assay available to health care providers managing immunocompromised patients in our community will provide results within 24 hours of receipt, leading to better patient management.

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References

1. Roux A, Canet E, Valade S, Gangneux-Robert F, Hamane S, Lafabrie A, et al. *Pneumocystis jirovecii* pneumonia in patients with or without AIDS, France. Emerg Infect Dis. 2014 Sep;20(9):1490–7.
2. Cushion MT, Stringer JR. Has the name really been changed? It has for most researchers. Clin Infect Dis. 2005 Dec;41(12):1756–8.
3. "Pneumocystis pneumonia Treatment," CDC. https://www.cdc.gov/fungal/diseases/pneumocystis-pneumonia/, accessed 10.16.2018.
4. Larsen HH, Masur H, Kovacs JA, Gill VJ, Silcott VA, Kogulan P, et al. Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing *Pneumocystis carinii* pneumonia. J Clin Microbiol. 2002 Feb;40(2):490–4.
5. Doyle L, Vogel S, Procop GW. Pneumocystis PCR: It Is Time to Make PCR the Test of Choice. Open Forum Infect Dis. 2017 Sep;4(4):ofx193.
6. Robberts FJ, Liebowitz LD, Chalkley LJ. Polymerase chain reaction detection of *Pneumocystis jiroveci*: evaluation of 9 assays. Diagn Microbiol Infect Dis. 2007 Aug;58(4):385–92.
7. Marimuthu S, Wolf LA, Summersgill JT. Real-Time PCR Detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* in Respiratory Specimens Using the ARIES® System. The University of Louisville Journal of Respiratory Infections. 2018;2(1):3.
8. Fauchier T, Hasseine L, Gari-Toussaint M, Casanova V, Marty PM, Pomares C. Detection of *Pneumocystis jirovecii* by Quantitative PCR To Differentiate Colonization and Pneumonia in Immunocompromised HIV-Positive and HIV-Negative Patients. J Clin Microbiol. 2016 Jun;54(6):1487–95.
9. MEDCALC Diagnostic Test Evaluation. https://www.medcalc.org/calc/diagnostic_test.php, accessed 9/28/2018.
10. Valero C, Buitrago MJ, Gits-Muselli M, Benazra M, Sturny-Leclère A, Hamane S, Guigue N, Bretagne S, Alanio A. Copy number variation of mitochondrial DNA genes in *Pneumocystis jirovecii* according to the fungal load in BAL specimens. Frontiers in microbiology. 2016 Sep 12;7:1413.
11. Moodley B, Tempia S, Frean JA. Comparison of quantitative real-time PCR and direct immunofluorescence for the detection of *Pneumocystis jirovecii*. PLoS One. 2017 Jul;12(7):e0180589.
12. 12. Alanio A, Desoubeaux G, Sarfati C, Hamane S, Bergeron A, Azoulay E, et al. Real-time PCR assay-based strategy for differentiation between active *Pneumocystis jirovecii* pneumonia and colonization in immunocompromised patients. Clin Microbiol Infect. 2011 Oct;17(10):1531–7.
13. Botterel F, Cabaret O, Foulet F, Cordonnier C, Costa JM, Bretagne S. Clinical significance of quantifying *Pneumocystis jirovecii* DNA by using real-time PCR in bronchoalveolar lavage fluid from immunocompromised

patients. J Clin Microbiol. 2012 Feb;50(2):227–31.
14. Ribes JA, Limper AH, Espy MJ, Smith TF. PCR detection of *Pneumocystis carinii* in bronchoalveolar lavage specimens: analysis of sensitivity and specificity. J Clin Microbiol. 1997 Apr;35(4):830–5.
15. Dalpke AH, Hofko M, Zimmermann S. Development and evaluation of a real-time PCR assay for detection of *Pneumocystis jirovecii* on the fully automated BD MAX platform. J Clin Microbiol. 2013 Jul;51(7):2337–43.
16. Le Gal S, Pougnet L, Damiani C, Fréalle E, Guéguen P, Virmaux M, et al. *Pneumocystis jirovecii* in the air surrounding patients with Pneumocystis pulmonary colonization. Diagn Microbiol Infect Dis. 2015 Jun;82(2):137–42.
17. Nevez G, Chabé M, Rabodonirina M, Virmaux M, Dei-Cas E, Hauser PM, et al. Nosocomial *Pneumocystis jirovecii* infections. Parasite. 2008 Sep;15(3):359–65.
18. Ponce CA, Chabé M, George C, Cárdenas A, Durán L, Guerrero J, et al. High Prevalence of *Pneumocystis jirovecii* Dihydropteroate Synthase Gene Mutations in Patients with a First Episode of Pneumocystis Pneumonia in Santiago, Chile, and Clinical Response to Trimethoprim-Sulfamethoxazole Therapy. Antimicrob Agents Chemother. 2017 Jan;61(2):e01290-16.
19. Takahashi T, Goto M, Endo T, Nakamura T, Yusa N, Sato N, et al. *Pneumocystis carinii* carriage in immunocompromised patients with and without human immunodeficiency virus infection. J Med Microbiol. 2002 Jul;51(7):611–4.