Performance Characterization of a Real Time PCR Assay for *Pneumocystis jirovecii* in Bronchoalveolar Lavage Fluid and Sputum

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Abstract

Background: *Pneumocystis jirovecii* pneumonia (PJP) affects immunocompromised patients and contributes significantly to mortality. Outcomes depend on early treatment, making timely and accurate diagnosis critical. Typically, PJP diagnosis is through identification of trophozoite or cyst forms in bronchoalveolar lavage (BAL) fluid or sputum, a labor-intensive and insensitive process. Options for more accessible and sensitive molecular detection are limited. It is known that patients may be colonized, which can cast doubt on the clinical significance of low levels of DNA amplification in qualitative result reporting. In this study, we describe a real time (rt) PCR assay utilizing analyte specific reagent primers targeting the mtLSU gene of *P. jirovecii* and correlate amplification with morphological PJP identification.

Methods: IUHPL Clinical Microbiology assessed sputum or BAL fluid from 109 patients with clinical concern for PJP microscopically via fungal stains (GMS, calcofluor white). Comparative rtPCR was conducted as follows. First, 2µL of residual specimen or control were mixed with an 8µL combination of rtPCR mastermix, control DNA, and primer pairs (DiaSorin Molecular). No nucleic acid extraction was performed. Real time PCR was executed and analyzed on the LIAISON MDX (DiaSorin Molecular) platform. Qualitative amplification results and cycle threshold (CT) values were correlated with microscopic methods to establish performance. Chart review was performed to assess the clinical impact of this assay.

Results: *P. jirovecii* was microscopically detected in 26.6% (29/109) of samples, while 31.1% (34/109) exhibited amplification by rtPCR (**Table 1, Table 2**). Agreement between the two methods was 95.4%; rtPCR demonstrated 100% sensitivity and 93.8% specificity in comparison.

Conclusions: Our results indicate that this assay has exceptional negative predictive value (100%), and therefore may be valuable as a screening test. Considering this data alone, the positive predictive value is lower (85.3%). Further examination of the data, however, revealed that 80% (4/5) of discrepant results demonstrated CT values of >34, while the highest CT for a microscopically positive sample was 31.2. Further clinical correlation may establish a CT cutoff that will reduce false positive and potentially clinically insignificant cases.

DiaSorin Results	Standard of Care Results	
	Detected	Not Detected
Detected	29	5
Not Detected	0	75

Table 1 - Summary of Comparative Results

Introduction

Pneumocystis jirovecii pneumonia (PJP) is one of the most prevalent opportunistic infections in immunocompromised patients including HIV-infected patients, organ or bone marrow transplant recipients, and immunosuppressant users [1-6]. *P. jirovecii* can lead to nosocomial infections in hospital units if patients are at high risk for infection or colonization [7]. PJP diagnosis currently relies on the demonstration of trophic forms or cysts after microscopic examination of bronchoalveolar lavage fluid (BALF) or sputum [8]. However, microscopic diagnosis is difficult and has low sensitivity, particularly when fungal burdens are low. Studies have shown that real-time (rt) PCR detection of PJP is a good tool to exclude PJP infections since it has nearly 100% negative predictive value (NPV) [9]. While the high sensitivity of real-time PCR allows for diagnosis in patients with low fungal loads, it also leads to the over detection of *P. jirovecii* DNA in colonized patients which can make it difficult to differentiate between colonization and active infection[10].

Colonization among healthy immunocompetent individuals has created an unclear picture of the clinical conditions and diseases for which *P. jirovecii* is responsible, including sudden infant death syndrome, chronic obstructive pulmonary disease, cystic fibrosis, and other pulmonary syndromes [11 -17]. Additionally, *P. jirovecii* co-infection is common, particularly in immuno-compromised patients. The high sensitivity of rtPCR brings a new challenge in diagnosis in order to discriminate between colonization and active PJP and inform therapeutic decisions.

In rtPCR, the cycle threshold (Ct) value can be used to correlate the fungal burden in a clinical sample and provide information about tissue burden or progression of disease[18]. The more elevated the fungal burden is, the higher the probability that the diagnosis is pneumonia [19-20]. Higher fungal burdens also play an important role as a morbidity and mortality cofactors in patients with a severe pulmonary background [10,17, 21-22]. As a result, the use of quantification of fungal load represents a valuable tool, which could greatly increase the specificity to distinguish active PJP from colonization and possibly to predict patient outcome.

The aim of this study is to evaluate the performance of a real-time PCR-based assay for the detection of *P. jirovecii* DNA in bronchoalveolar lavage fluid (BALF) and sputum samples.

Methods

Sputum or BAL fluids were collected from patients with clinical suspicion for PJP and examined according to our standard of care procedures. The specimen was stained (GMS, calcofluor white) and microscopically examined for the trophic and cystic forms of *P. jirovecii*.

Residual specimen or *Pneumocystis* molecular control (2 µL) was used without nucleic acid purification prior to rtPCR. The remaining reaction components totaled 8 µl for a final reaction volume of 10 µl. All reactions were prepared immediately prior to amplification as master mixes containing all reaction components: (a) *Pneumocystis jurovecii* primer pair, (b) TA master mix, (c) Simplexa Extraction and Amplification Control DNA, (d) Simplexa Extraction and Amplification Control primer pair, and (e) BAL/sputum Sample or Control. Direct amplification reactions using DiaSorin Molecular's *P. jirovecii* primer pair were carried out on the 96-well Universal Disc using the LIAISON MDX instrument. Data collection and analysis was performed with LIAISON MDX Studio software. The Ct value for positive samples was defined as the cycle number at which the fluorescence generated within a reaction crossed the fluorescence threshold.

Positive and negative controls were included with each set of specimens, and processed in the same manner as the patient specimens. The positive control contained a cloned fragment of the *P. jirovecii* genome and the negative control consists of a *P. jirovecii* -free dilution matrix. Each sample or control additionally contained an internal control, which was used to determine whether the amplification successfully occured. An additional set of controls were diluted to known concentrations (36000, 17200, 8600, 4300, 2150, and 1075 copies *P. jirovecii* per mL) and assessed five times in order to establish linearity and limit of detection.

Results

Of the 109 samples included in this study, 26.6% (29/109) demonstrated trophic or cystic forms during microscopic examination, and 31.1% (34/109) exhibited rtPCR amplification (**Table 1**). The Ct values and standard of care results for these 34 rtPCR positive samples are depicted in **Table 2.** The five discrepant samples all had relatively high Ct values in relation to the other positive results, with four of the five having a Ct >34. When considering microscopy as a gold standard, this rtPCR method demonstrated 100% sensitivity and 93.8% specificity.

Patient #	Standard of Care	DiaSorin Molecular	Ct Value	Patient #	Standard of Care	DiaSorin Molecular	Ct Value
1	Not Detected	Detected	36.1	18	Detected	Detected	21.6
2	Not Detected	Detected	35.8	19	Detected	Detected	21.5
3	Not Detected	Detected	35	20	Detected	Detected	21.4
4	Not Detected	Detected	34.1	21	Detected	Detected	20.3
5	Detected	Detected	31.2	22	Detected	Detected	20.2
6	Detected	Detected	30.2	23	Detected	Detected	19.9
7	Not Detected	Detected	29.2	24	Detected	Detected	19.4
8	Detected	Detected	28.4	25	Detected	Detected	19.3
9	Detected	Detected	26.1	26	Detected	Detected	19.3
10	Detected	Detected	25.2	27	Detected	Detected	18.8
11	Detected	Detected	25.1	28	Detected	Detected	18.6
12	Detected	Detected	23.3	29	Detected	Detected	17.7
13	Detected	Detected	23.2	30	Detected	Detected	17.7
14	Detected	Detected	23.2	31	Detected	Detected	17.7
15	Detected	Detected	22.3	32	Detected	Detected	17.1
16	Detected	Detected	21.7	33	Detected	Detected	16.8
17	Detected	Detected	21.7	34	Detected	Detected	16.6

Table 2 – All Specimens in which *P. jirovecii* was Detected
Results are plotted for each patient sample and associated with the respective Ct value. Not depicted are the 75 patient samples in which both methods did not detect *P. jirovecii.*

Results, Continued

In an attempt to correlate the Ct values with the burden of *P. jirovecii* in the sample, specimens at known concentrations were assessed five times each (**Figure 1**). In between 8600 and 36000 copies/mL the Ct correlated somewhat linearly with *P. jirovecii* concentration (R^2=.9537). Two of the previously discrepent samples fell within this linear range (indicating that their likely burden was between 8600 and 36000 copies/mL), while three had lower Ct values (indicating their likely burden was greater than 36000 copies/mL). At lower concentrations amplification was less sensitive, with only 4/5 samples detected at 4300 and 2150 copies/mL and 3/5 detected at 1075 copies/mL.

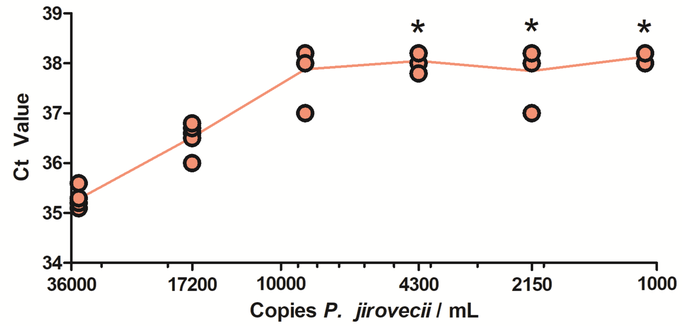

Figure 1 – Correlation of Ct Value and *P. jirovecii* Concentration
Control samples (n = 5) of known concentrations were assessed for *P. jirovecii* amplification at six concentrations (shown on X-axis as $\log_{10}$). The trend line connects mean Ct. An (*) indicates that less than 100% of samples demonstrated amplification.

Conclusions

- Real time PCR demonstrates excellent screening potential to rule out *Pneumocystis jirovecii* pneumonia PJP due to its high NPV (100%).
- Specimens with lower concentrations of *P. jirovecii* may amplify, creating the potential for positive results in colonized or clinically insignificant cases.
- Of our discrepant results, 4/5 (80%) had Ct values of >34. Further study may establish a Ct cutoff in order to improve specificity.

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