Short communication

Evaluation of the performance of DiaSorin molecular *Pneumocystis jirovecii*-CMV multiplex real-time PCR assay from bronchoalveolar lavage samples

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A B S T R A C T

The aim of this study was to evaluate the performance of the DiaSorin Molecular PJ-CMV multiplex real-time PCR (PJ-CMV PCR) assay (DiaSorin Molecular LLC, USA) in bronchoalveolar lavage (BAL) samples compared to direct immunofluorescence assay (IFA) for the detection of *Pneumocystis jirovecii* and assess CMV and *P. jirovecii* co-infection rate in immunosuppressed patients with suspected pneumonia. A total of 125 BAL samples from immunosuppressed patients submitted for PJP-IFA were tested. Surplus samples were saved and further tested by using the PJ-CMV PCR assay. Among the 125 samples, *P. jirovecii* was detected in 31.2% (39/125) and in 40% (50/125) of the specimens using IFA and PJ-CMV PCR respectively. Eleven of the PJ-CMV PCR positive samples were negative by direct IFA for *P. jirovecii*. All samples positive by direct IFA were also positive by PJ-CMV PCR. Using the direct IFA as a gold standard, the PJ-CMV PCR sensitivity, specificity, positive predictive value and negative predictive value for detection of *P. jirovecii* were 100%, 87.2%, 78% and 100%, respectively. However, after reviewing the clinical diagnosis, the specificity and PPV increased to 100%. Of the 50 *P. jirovecii* samples positive by PJ-CMV PCR, 18 (36%) were also positive for CMV by the PJ-CMV PCR. The co-infection rate was found to be 37.5% (6/16) and 35.2% (12/34) in HIV infected and non-HIV infected patients. This study indicated that the DiaSorin Molecular PJ-CMV multiplex real-time PCR assay has higher sensitivity than direct IFA for detection of *P. jirovecii* and provides rapid detection of PJ and CMV infection in BAL samples.

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Pneumocystis jirovecii pneumonia (PJP) is one of the most prevalent opportunistic infections in immunocompromised conditions such as human immunodeficiency virus (HIV) infection, stem cell and solid organ transplantation, and malignancy. Because of the high mortality of the disease and potential adverse effects of the treatment regimens, early and accurate diagnosis of PJP is crucial and essential [1].

Since *P. jirovecii* is a non-culturable causative agent, many clinical microbiology laboratories rely on direct microscopic detection of the cysts of *P. jirovecii* from respiratory samples. However, this approach lacks sensitivity and might be particularly challenging in patients receiving anti-retroviral therapy with low organism load [1]. Because of the low sensitivity of microscopy and immunofluorescence (IFA) assay, several PCR technologies with higher sensitivity have been developed for detection of *P. jirovecii* from clinical samples [2–4].

Cytomegalovirus (CMV) is another common and important cause of opportunistic infection in immunocompromised hosts and is associated with high morbidity and mortality [5]. CMV is often detected from respiratory samples with other pathogenic agents, particularly *P. jirovecii,* in immunocompromised patients [6,7]. CMV has immunomodulatory effects and inhibits the immune response generated against *P. jirovecii* and contributes to the delayed clearance of the *P. jirovecii* from patients [7].

The aim of this study was to evaluate the performance of the DiaSorin Molecular PJ-CMV PCR (DiaSorin Molecular LLC, Biotechnology Cypress, CA, USA) compared to direct IFA for *P. jirovecii* and assess co-occurrence of CMV and *P. jirovecii* infection in immunosuppressed patients.

This study was approved by the Institutional Review Board of Wake Forest Baptist Health.

Bronchoalveolar lavage (BAL) specimens from 35 HIV infected and 90 non-HIV infected immunocompromised patients with

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clinical suspicion of PJP were tested for *P. jirovecii* by direct IFA using the Monofluo *P. jirovecii* IFA test kit (MONOFLUO™ Bio-Rad, Laboratories, USA) according to the manufacturer's instructions. According to the clinical data reviewed, all patients evaluated were considered immunosuppressed and BAL samples were submitted for testing because patients met clinical and radiographic criteria for possible PJP and other infections had been ruled out. The non-HIV infected patients included patients with hemato-oncologic malignancies/stem cell or bone marrow transplant (*n* = 38), solid organ transplant (*n* = 17), and other immunosuppressed conditions (*n* = 35). The remaining (surplus) specimen aliquots were stored at −70 °C until tested by PJ-CMV PCR.

Amplification of un-extracted PJ-CMV BAL samples for use with DiaSorin Molecular reagents was carried out in a total volume of 10 μl of sample without prior centrifugation. The primers allow detection of amplified *P. jirovecii* mtLSU and *CMV UL83* genes in the samples. Reactions contained: 2 μl of BAL samples or external controls, 0.2 μl PJ primer pair (Part number MOL9098), 0.4 μl CMV primer pair (Part number MOL9002), 4 μl TA master mix (Part number MOL9070), 0.2 μl Simplexa Extraction and Amplification Control DNA (Part number MOL9300), 0.2 μl Simplexa Extraction and Amplification Control primer pair (Part number MOL9300), and 1 μl of nuclease free water. Direct amplification reactions using DiaSorin Molecular PJ-CMV primer pairs were carried out on the 96-well Universal Disc using the LIAISON® MDX instrument with dye detection on for CRF610 (PJ), FAM (CMV), and Q670 (Internal control). Data collection and analysis were performed with LIAISON® MDX Studio software. The following cycling conditions were used: 1 cycle at 97°C for 120 s followed by 40 cycles of 97°C for 10 s with a ramp speed of 2°C/s, and 60°C for 30 s with a ramp speed of 2°C/s with capture mode on. The test time of the PJ-CMV PCR assay from sample processing to final result is approximately 60 minutes. Extraction and amplification controls were used to detect PCR failure and/or inhibition. Positive and negative control samples were included in each run. Both CMV and PJ positive molecular controls were purchased from Exact Diagnostics (Exact Diagnostics, Fort Worth, TX). Standard procedures were used to obtain the analytical sensitivity of the test for the detection of *P. jirovecii* and CMV. Briefly, commercially available controls (CMV AD 169-Zeptometrix, Buffalo, NY, and *P. jirovecii* PCPP100-Exact Diagnostics, Fort Worth, TX) were chosen for the limit of detection (LOD) determination. Serial dilutions were prepared in a pooled BAL negative matrix ranging from 5000 copies/mL to 500 copies/mL for CMV and from 2500 copies/mL to 1000 copies/mL for *P. jirovecii*. LOD was defined as the minimum concentration with a detection rate of at least 95% by Probit Analyses. For the analytical specificity assessment, the manufacturer tested a panel of 31 microorganisms (Adenovirus 1, Inactive Influenza A, B, Parainfluenza 1, 2, 3, 4B, Rhinovirus 1A, Respiratory Syncytial Virus A, Metapneumovirus-9, Coronavirus 229E, Enterovirus 71, Herpes simplex virus 1, Cytomegalovirus, *Legionella pneumophila*, *L. longbeachae*, *Bordetella pertussis* ATCC 10380, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Chlamydophila pneumoniae*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Cryptococcus neoformans*, *Mycobacterium tuberculosis*, *M. avium*, *Mycoplasma pneumoniae*, *M. hominis*, *Haemophilus influenzae*, *Candida catenulate*, *C. glabrata*, *C. parapsilosis*,) for *P. jirovecii* and 24 microorganisms (*Anaplasma phagocytophilum*, Adenovirus, Herpes simplex virus 1,2, Human herpes virus 6, 7, 8, BK Virus, Human immunodeficiency virus type 1, 2, Human T-lymphotropic Virus-1, Enterovirus, JC Virus, Epstein-Barr virus, Parvovirus B19, Hepatitis B virus, Hepatitis D virus, Rubella, Varicella zoster virus, *B. henselae*, *B. Quintana*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Toxoplasma gondii*) for CMV known to cause pneumonia to confirm non-reactivity with *P. jirovecii* primers. The organisms were tested at a concentration of 1,000,000 CFU/mL for bacteria and 100,000 TCID50/ml for virus.

LOD was determined to be 2,063 ± 46 copies/mL or 103 copies/Rxn for CMV and 1,826 ± 44 copies/mL or 91 copies/Rxn for *P. jiroveci* in the current study. The assay yielded negative results for the panel of microorganisms other than the target organisms and thus no cross-reactivity was demonstrated. The results for the clinical samples showed that *P. jirovecii* was detected in 31.2% (39/125) and 40% (50/125) of the specimens using IFA and PJ-CMV PCR, respectively. When we compared the results obtained from HIV-infected and non-HIV infected patients, 16 (45.7%) of the 35 HIV infected and 34 (37.7%) of 90 non-HIV infected patients were positive for *P. jirovecii* by PJ-CMV PCR. Eleven of the samples positive by PJ-CMV PCR were negative by IFA. All 39 samples positive by IFA were also positive by PCR. There were no samples that were negative by PJ-CMV PCR and positive by IFA. The Ct for detection of PJ (number of cycles needed for a positive result) obtained with the PJ-CMV PCR was higher than 25 cycles (in 7 samples higher than 30) in the 11 samples negative by IFA suggesting that the PJ organism load was lower in the PJ-CMV PCR positive and IFA negative samples. Using IFA as a gold standard, the PJ-CMV PCR sensitivity, specificity, positive predictive value and negative predictive value for *P. jirovecii* were 100%, 87.2%, 78% and 100%, respectively (Table 1).

Of the 50 *P. jirovecii* positive samples, 18 (36%) were also positive for CMV by the PJ-CMV PCR. The co-infection rate was found to be 37.5% (6/16) and 35.2% (12/34) in HIV infected and non-HIV infected patients with immunosuppressed conditions, respectively.

There are several methods for detection of *P. jirovecii* from respiratory clinical samples including cytologic examination and IFA. Direct IFA test has been widely used and is still in first line for PJP diagnostic in association with pathogen DNA detection. These tests detect both the cystic and trophic forms of *P. jirovecii*. Although the direct IFA tests are more rapid to perform and easier to interpret than cytochemical stains, they might have false-negative results, especially in specimens from non-HIV infected patients since these tend to have fewer organisms present in the specimens [8,9]. These tests also depend upon the skills and experience of the observer in terms of familiarity with the different morphologies of the organisms [10]. An overall 55.5% sensitivity and 98.6% specificity of direct IFA tests has been demonstrated in a meta-analysis article evaluating seven prospective studies [11]. Molecular techniques such as real-time PCR have been used in the diagnostic evaluation of PJP, which have better sensitivity than traditional cytologic stains and IFA [3,4].

In the present study, we evaluated the performance of the PJ-CMV PCR assay on stored BAL samples against IFA for diagnosis of PJP. To our knowledge, this is the first evaluation of the PJ-CMV PCR assay using reagents from DiaSorin Molecular. The PJ-CMV PCR showed a higher sensitivity and specificity at diagnosing PJP in both HIV-infected and non-HIV infected immunocompromised patients compared to the direct IFA test. The higher sensitivity observed in this study is consistent with the results of previous studies on the detection of *P. jirovecii* [2,12,13]. A bivariate meta-analysis study by Lu et al. presented the diagnostic accuracy of *P. jirovecii* PCR techniques, with sensitivity ranging from 96% to 100% and specificity ranging from 87% to 93% [12]. In another review, reported sensitivities ranged from 82% to 100% and specificities ranged between 83% and 100% for PJP diagnosis using PCR assays [13]. A bivariate meta-analysis and systemic review by

Table 1
Comparison of the *P. jirovecii* by direct immunofluorescence (IFA) and the PJ-CMV multiplex real-time PCR assay results (*N* = 125).

		IFA Result		
		Positive	Negative	Total
PJ-CMV multiplex real-time PCR Result	Positive	39	11	50
	Negative	0	75	75
	Total	39	86	125

Fan et al. showed a very high diagnostic accuracy of PCR assays in BAL samples for the diagnosis of PJP with 98.3% sensitivity and 91.0% specificity evaluating 16 published studied [2].

Previous studies that have compared the performance of real-time PCR to IFA have also reported that a small number of samples were positive by real-time PCR but negative by IFA testing due to the higher sensitivity of the PCR [12,14,15]. In this study, eleven samples found positive by PCR were negative by IFA. All 11 of these with potentially false positive results compared to IFA had clinically proven PJP with clinical symptoms. These specimens had an overall higher cycle threshold value compared to samples positive by both methods, which became positive at less than 25 cycles. The specificity increased from 87.2 to 100% and the PPV increased from 78 to 100% once these eleven results were considered true-positive according to patients' clinical diagnostic criteria. One of the advantages of the PJ-CMV PCR assay is that it is designed as a closed system to reduce the potential carry-over contamination from run- to- run with amplicons causing false-positive results. Clinical samples are not open simultaneously during any step in the process. PJ-CMV PCR assay can be also used on other respiratory fluids like sputum or bronchial fluid.

CMV infection has immunomodulatory actions and aggravates the state of immunosuppression. It might lead to higher risk of opportunistic infections with other pathogens such as PJ and significantly influence on the outcome of the PJP infections [16,17]. However, some studies have reported no significant effect of CMV co-infection on the outcome of PJP [5,7,18]. Although the clinical significance of concomitant CMV infection with PJP is poorly understood, some investigators advise that CMV should be monitored closely in PJP infected patients [5]. The PJ-CMV PCR assay detects both of these pathogens in a single sample, eliminating the need for a separate assay for CMV PCR testing in respiratory specimens. Previous studies have reported that 23.4–61.5% of PJP patients also exhibit co-infection with CMV [7,17,19,20]. The percentage of CMV co-infection of this study was found to be 36% of patients, which is consistent with previous studies. There was no significant difference between the HIV infected and non-HIV infected patients in terms of the CMV co-infection.

There are a few limitations to this study. Our initial study question restricted the scope of this study and the data that we had access to. In particular, we compared the PJ-CMV PCR assay against direct IFA, the test routinely used at our laboratory, but not against another molecular test or against a quantitative test that could provide a specific organism load. In addition, although all samples analyzed were from patients meeting clinical criteria of PJP, we do not have further clinical data on the patients.

In summary, our findings demonstrate that the PJ-CMV multiplex real-time PCR assay showed higher sensitivity compared with direct IFA for detection of *P. jirovecii* in BAL samples and might be easily adapted for use in the clinical microbiology laboratories allowing the simultaneous detection of PJ and CMV in a single sample.

Disclosure of interest

Acknowledgments

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