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Performance of a PCR Assay for Detection of *Pneumocystis carinii* from Respiratory Specimens

ANGELA M. CALIENDO,[1,2]* PETER L. HEWITT,[3] JESSICA M. ALLEGA,[1,2] ANNE KEEN,[3] KATHRYN L. RUOFF,[1,2] AND MARY JANE FERRARO[1,2]

Clinical Microbiology Laboratory, Massachusetts General Hospital, Boston, Massachusetts 02114[1]; Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115[2]; and Roche Molecular Systems, Inc., Branchburg, New Jersey 08876[3]

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This study evaluates the performance of a PCR assay for the detection of *Pneumocystis carinii* from respiratory specimens that has been designed for use in the clinical microbiology laboratory. The test includes a simple method for nucleic acid extraction and amplification, a colorimetric probe hybridization technique for detection of amplicons, and an internal control to evaluate for the presence of inhibitors of amplification. Two hundred thirty-two clinical specimens (120 induced-sputum [IS] and 112 bronchoalveolar lavage [BAL] specimens) from 168 patients were tested by both immunofluorescent (direct fluorescent-antibody [DFA]) staining and PCR. Of the 112 BAL specimens, 17 were positive for *P. carinii* by DFA staining and PCR. An additional two specimens were DFA negative and PCR positive. For BAL specimens, the sensitivity and specificity of PCR compared to DFA were 100 and 98%, respectively. Eighteen IS specimens were positive for *P. carinii* by DFA, and 27 were positive by PCR. One of the 18 DFA-positive IS specimens was negative by PCR; this patient had just completed therapy for *P. carinii* pneumonia. Of the 10 specimens that were PCR positive and DFA negative, 4 were from patients who had a subsequent BAL specimen that was positive by DFA and PCR. For IS specimens, the sensitivity of DFA and PCR was 82 and 95%, respectively. The specificity of PCR for IS specimens was 94%. Due to the high sensitivity of PCR for the detection of *P. carinii* from IS specimens, a PCR-based diagnostic test may be a useful screening test and may alleviate the need for bronchoscopy in some patients.

Over the past 20 years *Pneumocystis carinii* has gone from being a rather rare pathogen to being one of the most common causes of pneumonia in immunocompromised hosts. *P. carinii* pneumonia (PCP) occurs more commonly in patients infected with human immunodeficiency virus type 1 (HIV-1) than in patients with malignant neoplasms or organ transplants (7). Without specific prophylaxis, 60 to 80% of HIV-infected individuals will develop PCP during the course of their illness (1, 12). Despite the use of prophylaxis for *P. carinii* infection, PCP remains the most common AIDS-defining opportunistic infection in the United States (3). Without more-effective prevention of HIV-1 infection or prophylaxis against *P. carinii* infection, this organism will remain a major pathogen in immunocompromised individuals and, as a result, a diagnostic challenge for the clinical microbiology laboratory.

The diagnosis of PCP is generally established by morphologically demonstrating *Pneumocystis* organisms from respiratory specimens by a variety of methods, including Giemsa or Giemsa-like rapid stains (e.g., Diff-Quik), Gomori methenamine silver stain, toluidine blue O stain, and fluorescein-conjugated monoclonal antibody (direct fluorescent-antibody [DFA] stain). In patients with AIDS, the diagnosis of PCP can usually be made from induced-sputum (IS) specimens (8). In contrast, for immunocompromised patients with conditions other than AIDS, the IS specimen is rarely PCP positive and the diagnosis requires bronchoalveolar lavage fluid (BAL), bronchial washings, or tissue. The improved diagnostic yield from IS for patients with AIDS has been attributed to higher organism burden (6). A more-sensitive diagnostic test may allow the less-invasive IS specimens to be used in patients with a lower organism burden. This concept is supported by studies that have shown PCR-based amplification assays to have an increased sensitivity compared to DFA (2, 11) and standard staining techniques (4, 9) for IS specimens.

In spite of the increased sensitivity of PCR assays for the detection of *P. carinii*, these assays are not in routine use in clinical microbiology laboratories. Lack of a standardized assay and labor-intensive methods for nucleic acid extraction and detection of amplified products contribute to the difficulty in using PCR-based assays for the detection of *P. carinii* in the clinical laboratory. In this study, we compare the performance of a prototype PCR assay (Roche Molecular Systems, Branchburg, N.J.) with an immunofluorescent staining method for the detection of *P. carinii* from IS and BAL specimens submitted to a clinical microbiology laboratory in a tertiary-care hospital with large numbers of AIDS and immunocompromised patients.

MATERIALS AND METHODS

Clinical specimens. A total of 232 (120 IS and 112 BAL) specimens from 168 patients were submitted to the Massachusetts General Hospital Clinical Microbiology Laboratory for detection of *P. carinii* and were tested by both immunofluorescent (DFA) staining and PCR. Specimens were used in the study only if there was adequate volume for PCR testing after the completion of DFA testing. Multiple specimens were tested for 40 patients. Ten of these patients had multiple IS specimens and no BAL specimens, 8 patients had multiple BAL specimens and no IS specimens, and 22 patients had both IS and BAL specimens submitted for testing. The patient population included individuals infected with HIV-1, transplant recipients, and patients immunocompromised due to other illnesses. The diagnosis of PCP required the detection of organisms by DFA staining of IS or BAL specimens. This study was approved by the Massachusetts General Hospital institutional review board.

Specimen processing. IS and BAL specimens were processed by standard methods in our laboratory. Briefly, IS specimens were mixed (1:3) with sputolysin (0.65 mM dithiothreitol [DTT]; Behring Diagnostics Inc.). The mixture was incubated for 10 min at room temperature, mixed, and centrifuged at 1,875 × *g*

* Corresponding author. Mailing address: Clinical Microbiology Laboratory, Gray B526, Massachusetts General Hospital, Boston, MA 02114. Phone: (617) 726-3830. Fax: (617) 726-5957. E-mail: caliendoa@A1.mgh.harvard.edu.

TABLE 1. Comparison of DFA and PCR for the detection of *P. carinii* in respiratory specimens[a]

PCR result	DFA stain result with specimen from:			
	BAL[b]		IS[c]	
	+	−	+	−
+	17	2	17	10
−	0	93	1[d]	92

[a] +, positive; −, negative.
[b] For BAL specimens, the sensitivity of PCR was 100% (17 of 17 specimens detected); the specificity of PCR was 98% (93 of 95 specimens detected).
[c] For IS specimens, the sensitivity of PCR was 94% (17 of 18 specimens detected); the specificity of PCR was 90% (92 of 102 specimens detected).
[d] Patient was without evidence of clinical PCP (see text for details).

for 10 min. After removal of the supernatant, an aliquot of the sediment was used for DFA staining. The quality of the IS specimens was not evaluated. BAL specimens were centrifuged at 1,875 × *g* for 10 min, and a portion of the sediment was used for DFA staining. The remaining IS and BAL sediments were frozen at −10 to −20°C until processed for PCR. BAL and IS specimens were processed for PCR in the following manner. Briefly, 100 μl of IS or BAL sediments was mixed with 500 μl of a respiratory specimen wash solution containing Tris-HCl (pH 8.0), EDTA, and Triton X-100. Following centrifugation at 12,500 × *g* for 10 min, the supernatant was decanted and 100 μl of a respiratory specimen lysis reagent containing NaOH, Triton X-100, EDTA, and sodium azide was added to each cell pellet. The mixture was incubated at 60°C in a dry heat block for 45 min, after which 100 μl of a neutralization reagent containing Tris-HCl (pH 7.5), $MgCl_2$, and sodium azide was added to neutralize the NaOH.

DFA staining. DFA staining for the detection of *P. carinii* was performed with a commercially available murine monoclonal antibody labeled with fluorescein isothiocyanate that reacts with all *P. carinii* forms (Genetics Systems, Inc., Redmond, Wash.). The test was performed according to the manufacturer's recommendation.

PCR amplification. PCR assays were performed by a test developed by Roche Molecular Systems. The primers used were specific for the 18S rRNA gene and amplified a 418-bp sequence of the gene. The sequences of the primers are as follows: upstream primer (PC41), 5′ CGA GAC CTT AAC CTA CTA AAT AGC CAG ATT A 3′; downstream primer (PC22), 5′ AAT GAC CAA ATT TGA TCA ACT TTC CAG CAA 3′. PCR testing on all specimens was performed in duplicate. Fifty microliters of processed specimen was added to 50 μl of master mix containing buffer, AmpliTaq, AmpErase, and an internal control (IC) plasmid DNA. The IC has the same primer binding sites as the *P. carinii* target sequence, and the amplified product is the same size as the *P. carinii*-specific target sequence. The IC DNA is coamplified with *P. carinii* target DNA but does not cross-react with the *P. carinii*-specific detection probe. The IC is detected separately by using a probe specific for the internal control. Cycling parameters were as follows: 50°C for 10 min; 98°C for 20 s, 62°C for 20 s, and 72°C for 45 s for 2 cycles; 94°C for 20 s, 62°C for 20 s, and 72°C for 45 s for 41 cycles; and 72°C for 5 min. Positive and negative controls were included in all runs. Amplicons were detected by probe hybridization in a colorimetric microwell plate detection format. An optical density at 450 nm of <0.35 is considered negative, and a value of ≥ 0.35 is considered positive.

Prevention of contamination. Contamination precautions included use of aerosol barrier pipette tips; use of separate areas of the laboratory for master mix preparation, specimen extraction, and specimen detection; the use of UTP and uracil-*N*-glycosylase in the reaction mixture; and the inclusion of multiple negative controls in each run.

RESULTS

Of the 112 BAL specimens tested, 17 were positive for *P. carinii* by DFA staining and PCR. There were an additional two BAL specimens that were negative by DFA staining and positive by PCR (Table 1). Chart reviews of these two patients at the time of the test result revealed that although both were receiving systemic steroids, neither had clinical evidence of PCP. These two results were therefore scored as false positive by PCR. Compared to DFA, the sensitivity and specificity of the PCR assay for the detection of *P. carinii* from BAL specimens were 100 and 98%, respectively.

Of the 120 IS specimens tested, 18 were positive for *P. carinii* by DFA staining and 27 were positive for *P. carinii* by PCR. Of the 18 DFA-positive specimens, 17 were positive by PCR. The single PCR-negative specimen yielded a positive IC result, ruling out inhibition as the source of the negative result. This specimen was obtained from an HIV-1 positive patient who had just completed a course of therapy for PCP. At the time the specimen was collected, the patient was free of symptoms, therapy for active PCP infection was discontinued, and PCP prophylactic therapy was begun. The patient has been monitored for 3 years on PCP prophylactic therapy and has not had a relapse of PCP.

There were an additional 10 specimens from eight patients that were positive for *P. carinii* DNA by PCR but negative by DFA staining (Tables 1 and 2). Four of these specimens were from three patients who had a subsequent BAL specimen positive for *P. carinii* by both DFA staining and PCR. These were considered true-positive specimens. In these patients, the positive PCR result on the IS specimen would have alleviated the need for bronchoscopy. Of the remaining six specimens that were positive for *P. carinii* by PCR but negative by DFA staining, four were from four patients without clinical evidence of PCP by chart review. Of these four patients, two were HIV-1 seronegative and receiving systemic steroids. One specimen was from an HIV-1 seropositive patient with a CD4 cell count of 12 who presented with a cough and slightly increased lung markings on chest X ray. His symptoms improved with prophylactic doses of trimethoprim-sulfamethoxazole. The final specimen was from a patient with a history of intravenous drug and alcohol abuse and hepatitis C virus infection who presented with profound anemia (hematocrit = 12%). After transfusion for the anemia, the patient developed shortness of breath that responded to diuretic therapy. The patient was HIV-1

TABLE 2. Analysis of specimens with negative DFA and positive PCR results

Patient no. (specimen no.)	Chart review[a]	Disposition
1 (1)	Subsequent BAL specimen positive for *P. carinii* by DFA staining and PCR	True positive
2 (2, 3)	Subsequent BAL specimen positive for *P. carinii* by DFA staining and PCR	True positive
3 (4)	Subsequent BAL specimen positive for *P. carinii* by DFA staining and PCR	True positive
4 (5)	Patient receiving systemic steroids for SLE	False positive
5 (6)	Patient with COPD receiving systemic steroids	False positive
6 (7)	HIV-1 positive patient, CD4 cell count = 12, presented with cough that resolved with prophylactic doses of TMP-SMX	False positive
7 (8)	HIV-1 negative patient with history of IVDU, HCV, with profound anemia, no evidence of PCP	False positive
8 (9, 10)	Patient receiving chemotherapy for NHL, shortness of breath and fevers, no definitive diagnosis made	Indeterminate

[a] Abbreviations: SLE, systemic lupus erythematous; COPD, chronic obstructive pulmonary disease; TMP-SMX, trimethoprim-sulfamethoxazole; IVDU, intravenous drug use; HCV, hepatitis C virus; NHL, non-Hodgkin's lymphoma.

seronegative. The PCR results from these four patients were interpreted as false-positive results.

The final two IS specimens that were positive for *P. carinii* by PCR but negative by DFA staining were from a patient receiving chemotherapy for non-Hodgkin's lymphoma. During a 1-month period, the patient was hospitalized twice with shortness of breath and fevers. The patient was initially treated with ciprofloxacin followed by clarithromycin but with minimal improvement of symptoms. The patient was allergic to trimethoprim-sulfamethoxazole, and aerosolized pentamidine therapy for PCP prophylaxis was begun several weeks prior to the first admission. After completion of chemotherapy, the patient continued to receive aerosolized pentamidine and symptoms slowly resolved over several months. Although the physician caring for the patient thought the clinical picture was consistent with PCP, these two specimens were considered false positives in the data analysis since a definitive diagnosis of PCP by DFA staining was not made.

PCP was defined as either an initial or subsequent DFA-positive IS or BAL smear. Of the 10 PCR-positive, DFA-negative IS specimens, 4 were found to be true positives and 6 were found to be false positives. The sensitivity of PCR for the detection of *P. carinii* from an IS specimen after resolution of discrepant results was 95% (21 of 22) and the specificity was 94% (92 of 98). Of the PCR-positive IS specimens, 21 and 6 were positive and negative, respectively, by initial or subsequent DFA staining; of the PCR-negative IS specimens, 1 and 92 were positive and negative, respectively by initial or subsequent DFA staining. In this same group of patients, the sensitivity of the first DFA-stained IS specimen was 82% (18 of 22).

There were five patients diagnosed with PCP from whom follow-up IS specimens were available 2 to 8 weeks after treatment. Four of the patients were diagnosed with PCP based on a positive DFA stain of an IS specimen, and one patient was diagnosed by a positive DFA stain of a BAL specimen. The follow-up IS specimens from all five patients were negative for *P. carinii* by DFA staining. For three of the five patients, the follow-up IS specimen was negative for *P. carinii* DNA by PCR. The follow-up IS specimen from the fourth patient was positive by PCR. However, at the time of the follow-up specimen, the patient presented with cough and shortness of breath and there was a question of recurrent PCP. The final patient had a follow-up IS specimen, collected 2 months after the initial diagnosis of PCP, that was positive by PCR testing. In three of the five patients for whom follow-up IS specimens were available after treatment, the *P. carinii* DNA was cleared from the IS specimen.

We found no evidence of inhibition for the 232 clinical specimens tested by the PCR assay. In addition, there were no discordant PCR results between the duplicate aliquots for the 232 specimens tested.

DISCUSSION

The performance of the assay described here compares favorably with other published studies evaluating in-house PCR assays for the detection of *P. carinii* from respiratory specimens. This study and others (2, 4, 9–11) have shown that PCR testing of respiratory specimens is more sensitive than conventional and DFA staining methods for the detection of *P. carinii*. In this study, the increased sensitivity of PCR was seen for IS specimens only. For BAL specimens, the sensitivity of PCR was similar to that of DFA staining, and the specificity was slightly decreased. There was one IS specimen that was positive for *P. carinii* by DFA staining but negative by PCR. On DFA staining, rare cysts were seen, and it is possible that these were empty cyst walls that did not contain *P. carinii* DNA, as the patient had just finished a course of therapy for PCP, was asymptomatic at the time the specimen was collected, did not receive further treatment for PCP, and did not develop PCP during the subsequent 3 years of follow-up. The improved sensitivity of PCR compared to DFA staining for the detection of *P. carinii* from IS specimens is a key advantage, as it could decrease the need for bronchoscopy. In addition, obtaining an IS specimen is much less costly and puts the patient at lower risk of complications than bronchoscopy. In spite of the improved sensitivity of previously described PCR-based assays for the detection of *P. carinii*, these tests are not used routinely in clinical microbiology laboratories because they are labor-intensive or require radioactive detection methods. In addition, no standardized assay is available. The assay described in this report is easy to perform, requiring 5 to 6 h for completion, with 2 to 3 h of hands-on technical time, depending on the test batch size. The PCR assay has been designed to accommodate both small and large sample batch sizes. Nucleic acid extraction is accomplished with a simple lysis step, and detection of amplified products is done by using a colorimetric microwell plate hybridization format. Due to these performance characteristics, the assay can be incorporated into the daily work flow of a clinical microbiology laboratory without difficulty. In our laboratory, with a batch size of three to six tests per day, the hands-on technical times required to perform the DFA staining and PCR are similar. An advantage of this PCR assay is that it includes an IC, which allows the evaluation of the presence of inhibitors of amplification.

There are several important issues regarding the use of a PCR assay as a diagnostic test for PCP. Since *P. carinii* is a ubiquitous organism, there remains a concern that when testing with a method as sensitive as PCR, *P. carinii* DNA may be detected in people without evidence of disease. In addition, in HIV-infected patients who often have repeated episodes of PCP, will a PCR-based assay be able to discriminate between true reinfection and slow clearance of organisms? In this study *P. carinii* was detected in patients without evidence of clinical disease. However, another study of a PCR-based assay for the detection of *P. carinii* from IS specimens examined the significance of a positive PCR in the absence of disease by monitoring patients over time (5). There were eight patients for whom the PCR assay was positive for *P. carinii* DNA but who had no evidence of clinical disease. Six of these patients developed PCP within 164 to 352 days of the positive PCR result. The remaining two patients were begun on PCP prophylaxis therapy, which may have prevented the development of PCP. These results suggest that detection of *P. carinii* by PCR in asymptomatic patients may be an early sign of clinical PCP and may identify a group of patients at high risk of developing PCP in the future. In the study described in this report, specimens obtained from patients with a positive PCR and negative DFA result were not monitored to determine if they subsequently developed PCP.

The apparent decreased specificity of the PCR assay compared with DFA staining for the detection of *P. carinii* in IS specimens needs to be weighed against the increased sensitivity as well as the ability to use an IS specimen rather than a BAL specimen in the PCR assay. It is also possible that the high sensitivity of the PCR assay on IS specimens will allow the use of expectorated sputum specimens for the diagnosis of PCP. This would even further reduce the cost of specimen collection. Studies evaluating the sensitivity of PCR for the detection of *P. carinii* from expectorated sputum specimens are needed.

Though the number of patients with follow-up specimens was small, three of five were able to clear the *P. carinii* DNA in an IS specimen within 8 weeks of initiating treatment. Although further studies are needed, these results may indicate that a PCR assay will be useful for the diagnosis of recurrent disease. PCR also offers the advantage of determining if *P. carinii* DNA is present after treatment for PCP. While current staining methods do not distinguish *P. carinii* cysts from empty cyst walls that lack DNA, PCR could be useful in determining if cysts that persist after treatment contain genetic material.

Due to the high sensitivity of PCR for the detection of *P. carinii* from IS specimens, PCR may be a useful screening test. In this study, there were 25 patients with 26 IS specimens for whom PCP was diagnosed on the basis of a positive DFA stain from either an IS or BAL specimen. IS was positive by PCR with 25 of the 26 specimens. However, depending on the prevalence of PCP, there may also be high numbers of false-positive results with the PCR assay. For example, with a sensitivity of 95%, a specificity of 94%, and a prevalence of infection of 10%, the predictive value of a positive PCR test would be 64%. In this situation, if PCR were to be used as a screening test, a positive result would need to be confirmed by DFA staining, although, based on the study of Elvin et al. described above (5), many patients with a positive PCR and negative DFA stain may go on to develop clinically apparent PCP. Nevertheless, DFA staining will likely remain useful when a diagnostic result for a single patient is needed within a few hours. A negative PCR test on an IS specimen could rule out PCP without the need for bronchoscopy, since the negative predictive value of the test is very high (99%). This is an advantage of PCR testing of IS specimens compared to DFA, especially in a low-prevalence population. Each laboratory will need to calculate its own positive and negative predictive values of the PCR test based on their prevalence rate before the clinical utility of the PCR test can be assessed.

In summary, this study has shown that a prototype PCR assay for the detection of *P. carinii* has a high sensitivity and specificity and can be adapted for use in the clinical microbiology laboratory.

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