Development and Evaluation of a Rapid and Simple Procedure for Detection of Pneumocystis carinii by PCR

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We report the development of a simplified PCR-based assay for the detection of Pneumocystis carinii DNA in clinical specimens. The adoption of a rapid DNA extraction procedure and the introduction of a type of enzyme-linked immunosorbent assay for PCR product detection enabled this procedure to be carried out in a single working day in a clinical microbiology laboratory. The PCR assay was prospectively compared with an immunofluorescent-antibody (FA) staining method for the detection of P. carinii in induced sputum and bronchoalveolar lavage (BAL) specimens. The results of the study showed that, for induced sputum specimens, FA staining had a sensitivity of 78% (32 of 41 specimens) and a specificity of 100% (166 of 166 specimens); PCR was 100% (41 of 41 specimens) sensitive and 98% (162 of 166 specimens) specific. For BAL specimens, FA staining was 100% sensitive (21 of 21 specimens) and 100% specific (133 of 133 specimens), and PCR had a sensitivity of 100% (21 of 21 specimens) and a specificity of 99% (132 of 133 specimens). These results strongly suggest that use of our PCR-based assay could effect clinically useful improvements in the sensitivity of induced sputum specimens for the detection of P. carinii.

Pneumocystis carinii pneumonia (PCP) is one of the most common and serious infectious manifestations of AIDS and is a significant problem in patients not infected with human immunodeficiency virus (HIV) who are receiving immunosuppressive therapy (9).

The diagnosis of PCP in the laboratory has, until now, been dependent on visualization of P. carinii organisms in stained preparations of appropriate respiratory specimens. Conventional cytochemical stains, including Giemsas, methenamine silver (17), and modified toluidine blue O (5); the nonspecific fluorescent stain calcefluor white (1); and specific monoclonal antibody-based fluorescent stains (11) have all been used for this purpose. A number of studies have demonstrated that the sensitivity of immunofluorescent-antibody (FA) staining exceeds those of the other available staining methods (2, 13), and this technique is now well established, at least in the clinical microbiology laboratory, as the “gold standard” for the diagnosis of PCP.

The majority of laboratory diagnoses of PCP are made by using specimens obtained by bronchoalveolar lavage (BAL). Improved techniques for obtaining and processing induced sputa have led some institutions, including ours, to implement this technique as a less invasive alternative to BAL (6). However, even in combination with immunofluorescence staining, the diagnostic sensitivity of this approach is somewhat variable, and some studies have shown that the use of induced sputum specimens is significantly less sensitive than the use of BAL specimens for the detection of P. carinii (4). Primarily for this reason, the use of induced sputum specimens has not yet gained widespread acceptance as a less invasive alternative to the use of BAL specimens for PCR diagnosis.

In the past 2 years, there has been an accumulation of a body of literature describing PCR-mediated amplification of P. carinii genes, including those encoding mitochondrial rRNA (8, 14, 16), chromosomal rRNA (8, 10), and thymidylate synthase (12), as a technique for improving the diagnostic sensitivity of induced sputum. Although those studies reported potentially significant increases in the sensitivity of P. carinii detection by using PCR, the laborious nature of the DNA extraction and PCR product detection methodologies make this approach of questionable utility for clinical laboratories.

The present report described a PCR-based assay for the detection of P. carinii. The assay can be performed in a diagnostic laboratory in a clinically relevant time frame. The report also contains the results of a study prospectively comparing this technique with FA staining for the diagnosis of PCP from both induced sputum and BAL specimens.

MATERIALS AND METHODS

Clinical specimens. A total of 362 specimens (208 induced sputum specimens and 154 BAL specimens), obtained from 205 patients, submitted to our laboratory for FA staining for P. carinii were analyzed by PCR. A total of 112 of these patients were infected with HIV, 73 had malignancies, and the remainder had other diseases. The criteria used for defining patients with PCP were similar to those used by Lipschik et al. (8) and required the detection of organisms by FA staining of sputum, BAL, or transbronchial biopsy specimens.

Specimen processing and DNA extraction. Prior to FA staining, all induced sputa and mucus-containing BAL specimens were liquefied by treatment with sputolysin (6.5 mM dithiothreitol; DTT) for 3 min at 37°C. All specimens were processed in this manner prior to the extraction of DNA for PCR amplification. Following liquefaction (0.5 to 2.0 ml of specimen), cellular material was pelleted by centrifugation (15,000 × g for 5 min), washed twice with 0.15 M phosphate-buffered saline (PBS; pH 7.2), and then resuspended in 150 µl of lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 20 mM DTT, 1 mg of proteinase K ml⁻¹, 250 U of lytase [Sigma] ml⁻¹ [pH 8.0]). After 15 min of incubation at 37°C, an equal volume of sodium dodecyl sulfate (SDS; 2% [wt/vol]) was added, and

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incubation was continued for an additional 10 min at 37°C. The extracted DNA was then purified by adsorption onto a silica matrix (GeneClean; Bio-101, La Jolla, Calif.) and was eluted into 30 μl of TE buffer (10 mM Tris · HCl, 1 mM EDTA [pH 7.4]).

**PCR amplification.** The previously described primers pAZ102-E and pAZ102-H (obtained as a 5'-biotinylated derivative to enable PCR product detection by enzyme-linked immunosorbent assay [ELISA]) were used to amplify a 338-bp fragment of the mitochondrial rRNA gene of *P. carinii* (16). PCR mixtures consisted of buffer (10 mM Tris · HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.001% [wt/vol] gelatin [pH 8.3]), 200 μM (each) deoxynucleoside triphosphate, 7.5 pmol of each primer, 2.5 U of *Taq* polymerase (AmpliTaq; Perkin-Elmer), and 2 μl of specimen extract in a total volume of 20 μl. After denaturing the template at 94°C for 30 s, DNA was amplified for 35 cycles in a Perkin-Elmer 9600 thermal cycler. Cycles consisted of 10 s at 94°C, 10 s at 55°C, and 10 s at 72°C. After cycling, the reaction mixtures were maintained at 72°C for an additional 10 min and were then chilled to 4°C.

**Prevention of contamination.** Standard procedures were used to prevent the contamination of specimens by amplification DNA. Extraction, amplification, and product detection procedures were carried out in separate areas of the laboratory, aerosol-barrier pipette tips were used for all reagent transfers, and negative extraction and amplification controls were run with each experiment.

**Detection of PCR product by ELISA.** The following ELISA-type assay (PCR-enzyme immunoassay [PCR-EIA]) was developed to facilitate the ease of PCR product detection. Upon completion of amplification, aliquots (10 μl) of the PCR mixtures were added to 40 μl of hybridization buffer (0.15 M NaCl, 15 mM sodium citrate, 10 mM Tris · HCl, 1 mM EDTA [pH 7.5]) and were denatured by heating at 94°C for 5 min. A total of 10 ng of a 5'-digoxygenin-labelled probe (pAZ102-L2 [16]) was then added, and the resultant mixture was incubated for 15 min at 50°C. Samples were transferred to streptavidin-coated microtiter wells (Pierce Chemical Co., Rockford, Ill.) containing 100 μl of dilution buffer (PBS, 0.5% [wt/vol] bovine serum albumin, 0.05% [vol/vol] Tween 20), and the plates were incubated for an additional 30 min at room temperature. Wells were rinsed with washing buffer (PBS, 0.05% [vol/vol] Tween 20), and horseradish peroxidase-labelled anti-digoxygenin antibody was added (100 μl; 1:5,000 diluted in dilution buffer; Boehringer Mannheim, Indianapolis, Ind.). After a final 15-min incubation, wells were again washed, and bound peroxidase was detected colorimetrically by the addition of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide and then by 10 min of incubation at room temperature.

**Detection of PCR product by hybridization.** Conventional solid-phase DNA hybridization (Southern blotting) was used to confirm the presence of *P. carinii* DNA in specimens giving a PCR-EIA-positive, FA staining-negative result. Following agarose gel electrophoresis, PCR products were transferred to Hybond N+ membranes and hybridized at 50°C with 3'-fluorescein-labelled pAZ102-L2 probe, and the presence of hybrids was detected nonradiometrically by using the ECL detection system (Amersham Corp., Arlington Heights, Ill.).

**Determination of the relative sensitivities of PCR-EIA, agarose gel electrophoresis, and hybridization for detection of PCR product.** PCR-amplified product was purified from agarose gels by electroelution and was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was then precipitated, dried under vacuum, and resuspended in TE buffer. The concentration of DNA was determined by measuring the absorbances of the solutions at 260 nm by using a UV spectrophotometer, and appropriate dilutions were prepared in TE buffer. Aliquots of these dilutions were subjected to agarose gel electrophoresis, PCR-EIA, and hybridization to determine the relative sensitivities of these methods for product detection. A slot blot procedure was used for hybridization; DNA was denatured by heating (95°C, 5 min) and was then applied to Hybond N+ membranes via a slot blot manifold (Life Technologies Inc., Gaithersburg, Md.). Membranes were subsequently probed, and hybrids were detected as described above.

**FA staining.** Detection of *P. carinii* by FA staining was performed by using a commercially available fluorescein isothiocyanate-conjugated murine monoclonal antibody directed against the major glycoprotein component of the cell wall of this organism (Genetic Systems, Redmond, Wash.). Induced sputum and BAL specimens were prepared for staining as described previously (6).

**RESULTS**

**DNA extraction.** The DNA extraction procedure described here enabled us to efficiently extract amplifiable DNA from clinical specimens in approximatley 60 min. The addition of DTT and lyticase, a partially purified enzyme preparation known to have activity against the cell wall of *P. carinii* (7), to liquefied clinical specimens containing *P. carinii* resulted in a rapid loss of cell wall antigen. The reactivities of the organisms with the fluorescent monoclonal antibody were abolished after approximately 5 min of incubation with these agents (data not shown). Neither DTT nor lyticase when used alone completely abolished the immunofluorescence even after prolonged incubation (30 to 60 min). The use of lyticase and DTT in combination with protease K and SDS enabled us to shorten considerably the length of time necessary for *P. carinii* DNA extraction from respiratory specimens. In addition, the use of silica adsorption to purify extracted DNA obviated the need for organic extraction and ethanol precipitation and further simplified and shortened PCR template preparation.

**Sensitivity of product detection by PCR-EIA.** PCR-EIA was capable of detecting 4 ng of PCR product; this amount of amplified DNA reproducibly resulted in an absorbance value of >0.15 (Fig. 1). Controls containing no DNA routinely gave absorbance values of <0.1. Agarose gel electrophoresis was somewhat less sensitive than PCR-EIA, with 20 ng being the minimum amount of DNA necessary to give a clearly visible band. As expected, slot blot hybridization was the most sensitive detection method, and a signal was obtained with only 0.4 ng of product. As a result of these studies, 0.15 was established as the minimum absorbance value required to designate a patient specimen as positive by PCR.

**Detection of *P. carinii* in induced sputum specimens.** The results of our prospective comparison of PCR-EIA with FA staining for the detection of *P. carinii* in induced sputum specimens are given in Table 1. Forty-one induced sputum specimens were obtained from patients with a positive FA staining result for *P. carinii* from either induced sputum or BAL specimens. Of these, 32 (78%) were positive by FA staining and 41 (100%) were positive by PCR-EIA. Of the 167 induced sputums obtained from patients without FA staining-proven PCP, 163 (98%) were negative by PCR-EIA. PCR-EIA of induced sputum specimens had a positive predictive value of 91% (100% for FA staining) and a negative predictive value of 100% (95% for FA staining). Induced sputum specimens from 13 patients had FA staining-negative, PCR-EIA-positive results (assay absorbance values for FA staining-negative specimens varied between 0.18 and 0.90). All 13 of these specimens
were confirmed as true-positive PCR results by Southern blotting. Eleven of the 15 specimens showed a band of the appropriate size on gel electrophoresis, and the remaining 2 specimens required hybridization for detection. For nine of these patients, concomitant BAL specimens were positive for *P. carinii* by FA staining, and thus were regarded as true positives. Of these nine patients, four were HIV-positive patients receiving anti-*P. carinii* prophylaxis, three were HIV-infected patients not receiving anti-*P. carinii* prophylaxis (<5 years of age), and two were patients with malignancies receiving immunosuppressive chemotherapy. Two patients that had PCR-positive, FA staining-negative induced sputum specimens did not undergo bronchoscopy. Both of these patients were infected with HIV and were receiving anti-*P. carinii* prophylaxis, and both subsequently developed FA staining-proven PCP at 4 and 6 weeks, respectively, after the original PCR-EIA-positive specimen was obtained. For the remaining two patients, FA staining-negative BAL specimens were obtained at the same time that the discrepant induced sputum specimens were obtained. One was a non-HIV-infected patient who had been diagnosed with PCP 4 months earlier, and the second was an HIV-positive patient who had been receiving anti-*P. carinii* prophylaxis for 4 years with no prior or subsequent (approximately 2 months) history of PCP.

Detection of *P. carinii* in BAL specimens. Twenty-one FA staining-positive BAL specimens were obtained, and all of these were positive by PCR-EIA (Table 2). Of the 133 negative specimens, 132 were negative by both FA staining and PCR-EIA. Thus, PCR had a sensitivity of 100% and a specificity of 99% for the detection of *P. carinii* in BAL specimens. The single PCR-EIA-positive FA staining-negative BAL specimen (absorbance value, 0.27) was obtained from the non-HIV-infected patient with a prior history of PCP whose induced sputum specimen had also been PCR positive, and was confirmed to be PCR positive by Southern blotting.

**DISCUSSION**

The development of expedient, simple DNA extraction procedures and convenient product detection assays is essential if nucleic acid amplification-mediated detection of *P. carinii* is to become a more widely accessible diagnostic test. Our simple, yet efficient, DNA extraction procedure affords considerable savings in time and labor over previously published protocols for extracting DNA from *P. carinii* (8, 10, 12) since it obviates the need for phenolic extraction and ethanol precipitation. The microtiter plate format used in the PCR-EIA is one familiar to the majority of diagnostic laboratorians and, in addition to convenience and speed, offers sensitivity and specificity comparable to those of conventional methods for detecting products of PCR amplification. DNA extraction, amplification, and product detection can be comfortably completed within a single working day. This would seem to be the maximum allowable time if a PCR assay for the detection of *P. carinii* in specimens obtained by minimally invasive means is to be of significant clinical utility.

The results of our prospective study comparing PCR-EIA with FA staining for the detection of *P. carinii* confirm and extend the findings of previous studies, strongly indicating the potential of PCR for improving the diagnostic value of induced sputum specimens. PCR-EIA of induced sputum specimens detected all documented cases of PCP, whereas FA staining was positive only 78% of the time, with the remaining 22% of

![FIG. 1. Sensitivity of agarose gel electrophoresis (A) slot blot hybridization (B), and PCR-EIA (C) for detection of PCR product. The absorbance values shown for PCR-EIA are the means of three independent determinations. The solid line intersecting the bar chart in panel C represents the minimum absorbance value (0.15) required to designate a specimen as PCR-EIA positive. Lanes 1, negative control; lanes 2, 500 ng; lanes 3, 100 ng; lanes 4, 20 ng; lanes 5, 4 ng; lanes 6, 0.8 ng; lanes 7, 0.4 ng.](image)

**TABLE 1.** Comparison of PCR and FA staining for detection of *P. carinii* in induced sputum specimens

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<thead>
<tr>
<th>PCR result</th>
<th>FA staining result (no. of sputum specimens)</th>
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<tr>
<td></td>
<td>Positive</td>
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<td>Positive</td>
<td>32</td>
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<td>Negative</td>
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<tr>
<td>Total</td>
<td>32</td>
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^b For PCR, the sensitivity was 100% (41 of 41 specimens) and the specificity was 98% (163 of 167 specimens). For FA staining, the sensitivity was 78% (32 of 41 specimens) and the specificity was 100% (167 of 167 specimens).

^b Four patients did not have FA staining-documented PCP when PCR-positive, FA-negative sputums were obtained; thus, these specimens were deemed to have false-positive PCR results.

**TABLE 2.** Comparison of PCR and FA staining for detection of *P. carinii* in BAL specimens

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<tr>
<th>PCR result</th>
<th>FA staining result (no. of BAL specimens)</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
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<tr>
<td>Positive</td>
<td>21</td>
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<td>Negative</td>
<td>0</td>
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<td>Total</td>
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^b For PCR, the sensitivity was 100% (21 of 21 specimens) and the specificity was 99% (132 of 133 specimens). For FA staining, the sensitivity was 100% (21 of 21 specimens) and the specificity was 100% (133 of 133 specimens).

^b This patient did not have FA documented PCP when this specimen was obtained, therefore this was deemed a PCR false positive.
cases of PCP requiring bronchoscopy. Of perhaps greater significance from a diagnostic standpoint is that, even using our stringent criteria for defining PCP, our PCR assay was 98% specific with induced sputum specimens. Of the four patients with false-positive induced sputum specimens, two patients developed PCP shortly after the discrepant specimen was obtained, and one patient had been positive for PCP 4 months earlier, suggesting that these results represent clinical rather than analytical false positives. Not surprisingly, the patient populations in which PCR appeared to be superior to FA staining in making a diagnosis of PCP from induced sputum specimens were primarily those that, a priori, might be expected to have a lower organism burden. Six of the 13 patients in this category were HIV infected and were receiving anti-*P. carinii* prophylaxis and 3 were non-HIV-infected patients on immunsuppressive chemotherapy. The four remaining patients with FA staining-negative, PCR-positive sputum specimens were HIV-infected children less than 5 years old, a population in which the lack of patient compliance during sputum induction might result in a less than optimal specimen being obtained. Given the growing number of patients in each of these categories, it seems reasonable to assume that PCR will become increasingly valuable as a method for detecting *P. carinii* in specimens obtained by noninvasive means.

Previous investigators have differed in their opinions regarding the comparative relevance of PCR-positive specimens in which amplified *P. carinii* DNA is detectable by agarose gel electrophoresis versus those in which hybridization is necessary to visualize a PCR product. Several studies, however, have shown that the high degree of sensitivity achievable with hybridization results in an unacceptable number of clinically insignificant false-positive specimens (8, 12, 14), particularly BAL specimens. Given the potential problems with the clinical specificity of PCR detection of *P. carinii* in BAL specimens and the fact that staining of BAL specimens has been shown to be a highly sensitive and specific method for the diagnosis of PCP (3, 15), it seems unlikely that PCR detection of *P. carinii* in BAL specimens will prove to be of more than marginal diagnostic utility. Consequently, we performed the PCR-EIA on BAL specimens primarily to document further the high predictive value of positive and negative PCR-EIA results rather than to suggest that this technique be applied to the diagnosis of PCP from BAL specimens. The PCR-EIA is intermediate in sensitivity between electrophoresis and conventional hybridization, and our results with BAL and induced sputum specimens appear to support the hypothesis that the use of a less than maximally sensitive detection method is necessary to optimize the clinical utility of a PCR assay for *P. carinii*. We detected all FA staining-positive BAL specimens using PCR-EIA, but only one specimen, from a previously *P. carinii*-positive patient, was positive by PCR but negative by FA staining, giving PCR a specificity of 99%. In an institution that usually continues empiric anti-*P. carinii* therapy after a negative BAL specimen is obtained, 3 of 105 patients with FA staining-negative PCR-negative BAL specimens subsequently developed PCP during a 2- to 9-month follow-up period. This result again demonstrates the value of an FA staining-negative BAL specimen in excluding a diagnosis of PCP and further illustrates the clinical sensitivity and specificity of our PCR assay.

In conclusion, the PCR-EIA offers a comparatively rapid and simple approach for using nucleic acid amplification technology to diagnose PCP. In view of the excellent clinical sensitivity and specificity of this technique on induced sputum specimens we envisage the use of PCR as an adjunct to FA staining for the detection of *P. carinii* in this type of specimen and anticipate that this will result in a significant decrease in the number of patients requiring an invasive procedure for diagnosing respiratory disease caused by this opportunistic pathogen. Although PCR-EIA also had a high degree of sensitivity and specificity for the detection of *P. carinii* in BAL specimens, the lack of evidence for a gain in clinically useful sensitivity when this technique is used in addition to FA staining strongly suggests that only in rare, problematic, cases could PCR have any utility in diagnosing PCP from BAL specimens.

### REFERENCES


