Comparison to Standard Methods

Virus–Infected Patients with Polymerase Chain Reaction: A Blinded Comparison to Standard Methods

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Pneumocystis carinii pneumonia (PCP) is an important cause of morbidity and death among persons with human immunodeficiency virus (HIV) infection. Polymerase chain reaction (PCR) analysis of respiratory specimens has been investigated as a rapid diagnostic method. We have previously reported on the utility of this technique for diagnosing PCP in HIV-infected patients. In this report we evaluate PCR used in a blinded study design to avoid biases inherent to retrospective and nonblinded studies. The diagnosis of PCP was established on the basis of clinical findings and morphological studies of bronchoalveolar lavage (BAL) and/or lung biopsy specimens before PCR testing. PCR was performed without knowledge of the diagnosis. PCR results were graded from “negative” to 3+ on the basis of intensity of the banding pattern. Forty-seven patients were enrolled in the study, including 18 with proven PCP and 29 with other conditions. PCR was positive at grade 1 or higher for all 18 patients with PCP (100% sensitivity), at grade 2 or higher for 13 patients (72.2% sensitivity), and at grade 1 or higher for 4 of the 29 control patients (specificity of 86.2%). If a grade 2 or higher was required for diagnosis, the specificity improved to 100%. Results were reproducible with testing of a second aliquot for 46 of 47 patients (97.8%). Our findings confirm that PCR is a sensitive and reproducible test for detection of P. carinii in BAL specimens. Problems with false-positive results for control patients, however, limit the applicability of this method.

Despite wide increases in the use of prophylactic measures and decreasing incidence, Pneumocystis carinii remains a major cause of morbidity and death among HIV-infected individuals [1, 2]. Recent autopsy studies rank P. carinii pneumonia (PCP) as 1 of the 2 most frequent causes of death for patients with AIDS [3, 4]. The infection is also an important and sometimes fatal condition in patients with other immunosuppressive disorders. Currently, the diagnosis of PCP relies on identification of the organism in respiratory secretions and often requires invasive procedures.

Although detection of P. carinii by DNA amplification with use of PCR has recently been described, it is not a widely available method and has not been studied in a blinded fashion. PCR appears to have better sensitivity and specificity than standard diagnostic methods, but the lack of well-designed studies and commercially available assays has precluded wide use of this test in clinical practice. In 1 of the first nonblinded studies, Wakefield et al. [5] noted 16 immunosuppressed patients with PCP identified by conventional bronchoalveolar lavage (BAL) staining. All were positive for amplified P. carinii DNA. Liebovitz et al. showed that PCR and staining techniques were associated with similar rates of detection of P. carinii in BAL samples, but PCR was superior with use of bronchial washing samples [6].

In a retrospective study at our institution, Lu et al. compared 6 different PCR methods, using BAL specimens from 30 patients with confirmed PCP and from 20 patients with other diagnoses [7, 8]. They found that the nested internal transcribed spacers (ITS) PCR method yielded positive results in all PCP case patients and was the most sensitive assay for detection of P. carinii. Recently, Helweg-Larsen et al. showed that a new single-round “touchdown” PCR method had high sensitivity and specificity for diagnosis of PCP in persons with AIDS [9]. These and other published studies [10–12] suggest that DNA amplification may be a promising diagnostic tool for detection of this common infection.

Materials and Methods

Patient selection. During the 12-month period from February 1996 to February 1997, 47 consecutive adult patients with documented HIV infection who underwent BAL evaluation for suspected pneumonia were prospectively studied at the Indiana Uni-
University Hospital and Wishard Memorial Hospital (Indianapolis). Thirteen patients refused to participate in the study or could not be enrolled before the 96-h time limit following bronchoscopy; therefore, these patients were not evaluated.

**Specimen collection.** After informed consent was obtained, a 10- to 50-mL sample of BAL was collected from each patient. All specimens were obtained within 96 h and most within 72 h following bronchoscopy. Each sample was given a numerical code and stored by the investigators at −70°C until batch-testing at the end of the study. Blinded PCR was performed on coded specimens at the Indiana University Molecular Diagnostics Laboratory without knowledge of the patient’s identification, diagnosis, or results of other laboratory tests.

**Reproducibility.** To evaluate reproducibility of the test, each BAL sample was separated into 2 aliquots before DNA amplification testing. A total of 94 aliquots were randomly numbered and were tested in individual assays containing 94 specimens.

**Data collection.** The data were gathered prospectively by review of the medical and laboratory records of the enrolled patients. The following information was collected: symptoms at presentation; history of PCP; chest radiographic abnormalities; current or last known CD4 cell count; current or recent prophylaxis; results of BAL examination; results of transbronchial biopsies; type and duration of specific and/or empirical treatment; and response to medical treatment. In addition, all patients for whom the diagnosis was a condition other than PCP were followed up by review of clinical and laboratory records for a period of 3 months.

**PCR amplification testing.** Nested *P. carinii* ITS PCR was performed as described by Lu et al. [7, 8]. The first PCR was performed with primers 1724F2 (5'-AGTTGATCAAATTTGGTCATTTA-3') and ITS2R (5'-CTCGGACGAGGATCCTCGCC-3'). PCR cycles were as follows: 94°C for 3 minutes; 5 cycles of 94°C for 1.5 minutes, 62°C for 1.5 minutes, and 72°C for 1.5 minutes; and 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. The 40-μL PCR mixture contained 50 ng of template DNA, PCR buffer, 2.5 units of Taq polymerase, 3-mM MgCl2, 0.2-mM dNTP, and 20 pmol of each primer.

The second PCR was performed with primers FX (5'-TTCCGATAGTGAACCTGG-3') and RT2 (5'-CTGAATGGA-GATTTAATTCTTG-3') as follows: 94°C for 3 minutes; 5 cycles of 94°C for 1.5 minutes, 58°C for 1.5 minutes, and 72°C for 1.5 minutes; and 30 cycles of 94°C for 1 minute, 66°C for 1 minute, and 72°C for 1 minute. The reaction mix contained 2 μL of the first PCR product and the same components as those of the first PCR, except that 1.5-mM MgCl2 was used. PCR products were subjected to electrophoresis on a 10% polyacrylamide gel. The gel was then stained with ethidium bromide and observed by ultraviolet illumination. The sample was considered PCR-positive if a DNA fragment of approximately 530 bp was observed.

**Determination of diagnosis.** Diagnoses were established with use of criteria that were agreed upon before the study. All PCR case patients were classified as proven or probable diagnoses. Patients were considered to have proven PCP if they had a compatible clinical syndrome and histologic or cytologic evidence of PCP in a BAL specimen or lung biopsy specimen prepared with Giemsa or Gomori’s methenamine silver (GMS) stain. Patients were considered to have probable PCP if they met either of the following criteria: (1) compatible illness and treatment for PCP for at least 2 weeks, with resolution of the symptoms and receipt of <72 h of treatment for another pulmonary infection, or (2) a compatible illness and treatment for PCP for <1 week, with subsequent PCP occurring within 3 months and receipt of <72 h of treatment for another pulmonary infection.

Patients were classified as having proven community-acquired bacterial pneumonia if they had a new chest radiographic infiltrate and at least 1 of the following: (1) isolation of a bacterial pathogen from blood; (2) isolation of a bacterial pathogen from BAL fluid, bronchial washings, or a biopsy specimen; (3) histopathologic evidence of pneumonia, with demonstration of bacteria by gram stain of the lavage fluid specimen; or (4) direct fluorescent antibody staining or antigen detection positive for *Legionella, Chlamydia*, or *Mycoplasma* species, with no other pathogen identified. Probable community-acquired bacterial pneumonia was defined by a new chest radiographic infiltrate and at least 1 of the following: (1) fever, cough, or pleuritic chest pain; or (2) new onset of purulence of sputum.

**Isolation of Histoplasma capsulatum, Cryptococcus neoformans, or Mycobacterium tuberculosis** was considered diagnostic of pulmonary infection caused by these organisms. Kaposi’s sarcoma was diagnosed by characteristic bronchoscopic or pathological findings. Pathogens that may be more colonizers, such as cytomegalovirus, *Mycobacterium avium*, and *Cryptosporidium*, were considered to be significant only if there were no other pathogens identified and appropriate treatment for these pathogens was initiated. Illnesses that did not meet these criteria were classified as being of unknown etiology. Finally, patients without proven PCP were followed up for 3 months, to exclude other causes of their pulmonary infections.

**Results**

**PCP case patients.** Forty-seven HIV-positive patients participated in the study. Eighteen patients had proven PCP; none met the criteria for probable PCP. Two patients received treatment for PCP and other antibiotics simultaneously and did not meet the definition of probable PCP. All proven PCP case patients had radiographic abnormalities, most frequently bilateral infiltration (11 case patients), followed by diffuse infiltration (6 case patients). One patient with proven PCP had a focal unilateral infiltrate. All PCP case patients were diagnosed by GMS or Giemsa stain of the BAL specimen.

Transbronchial biopsy was performed on 12 of these 47 case patients, including 3 PCP case patients, but did not yield a diagnosis of PCP in any case patient who did not have a positive BAL stain. All 3 PCP case patients also were biopsy-positive for *P. carinii*. Of the 18 patients with PCP, 14 (77.7%) were not receiving any prophylactic treatment, and 2 each were treated with dapsone and trimethoprim-sulfamethoxazole, respectively.

PCR testing was blinded, and results were graded from “negative” to +++, according to the intensity of the DNA amplification. Figure 1 illustrates the results of the study, based on the intensity of the DNA amplification. Of the 18 patients with proven PCP, all had positive DNA amplification, graded 1 or
Of the 4 patients with normal radiographs, all had Chlamydia pneumoniae patients. Of the remaining 29 patients, 18 had community-acquired bacterial pneumonia, 3 had fungal pneumonia, 1 had M. avium complex infection, 1 had Kaposi’s sarcoma, 1 had Cryptosporidium species, or Kaposi’s sarcoma (1 each); Unknown, diagnosis not established. Results are classified as negative (−) or positive at grades of 1–3, based on the intensity of amplification.

higher. These results had a sensitivity rate of 100% and a negative predictive value of 100%.

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PCP prophylaxis, and 1 of them had previously had PCP. None of the control patients with positive PCR results for PCP exhibited evidence of PCP during the 3-month follow-up period.

The false-positive rate in the control group was 13.7%, yielding a specificity of 91.4% and positive predictive value of 81.8%. Furthermore, none of the patients with negative PCR results developed PCP within the next 3 months, indicating that none of the negative PCR results were falsely negative.

Reproducibility. Of the 47 enrolled patients, 46 (97.8%) had reproducible positive or negative results, and 39 (82.9%) of the results were reproducible at the same PCR grade. Both assays were positive for all PCP case patients and 3 of the 4 control patients. Results for the PCP case patients were consistently positive, whereas 1 of the 29 control patients was positive initially but negative upon repeat testing.

The other 3 control patients with positive results for the first aliquot also had positive results for the second, and each was of grade 1 on both occasions. The 1 patient with nonreproducible results was a control who had a positive PCR on the first specimen and negative result on the second. The sensitivity and specificity for the second aliquot were 100% and 93.6%, respectively, yielding positive and negative predictive values of 85.7% and 100%.

Discussion

PCP remains a frequent and important opportunistic infection in patients with AIDS. The potential use of PCR in the diagnosis of this infection has been increasingly reported on in the past few years. The data presented here demonstrate both the high sensitivity (100%) and the high reproducibility (97.8%) of this test in the diagnosis of PCP; however, the test was false-positive for 13.7% of control patients. The relevant question is whether the positive results for the 4 control patients represent contamination of the BAL specimen, true PCP case patients with false-negative stains, or P. carinii colonization.

Analysis of 4 control patients with positive results does not support a missed diagnosis of PCP; 1 had a CD4 cell count of 370/µL, another had proven histoplasmosis, and none demonstrated evidence of PCP with follow-up. The positivity of results on 2 occasions (in which separate aliquots of BAL specimens were tested) for 3 of the 4 control patients with positive PCR results makes contamination unlikely. In addition, specimen processing and storage were done at a separate facility from that of the PCR testing, further reducing the chances for contamination.

If results of 2+ or higher were required to consider a specimen to be positive, the specificity would have improved to 100%, but the sensitivity would have dropped to 72.2%. In this study, the sensitivity of the standard cytological methods was 100%, which prevented us from demonstrating improvement in sensitivity with use of PCR. Although the study was designed to permit identification of case patients with false-negative cy-

![Figure 1. Relation of PCR result (grade) to clinical diagnosis: PCP, Pneumocystis carinii pneumonia; CAP, community-acquired pneumonia; Other, etiology: fungal (3), Mycobacterium avium complex, Cryptosporidium species, or Kaposi’s sarcoma (1 each); Unknown, diagnosis not established. Results are classified as negative (−) or positive at grades of 1–3, based on the intensity of amplification.](https://academic.oup.com/cid/article-abstract/30/1/141/320320)
Some of the earlier studies have shown PCR technique to be more sensitive than the standard diagnostic methods [10–12]. Helweg-Larsen and co-workers recently reported a single-step “touchdown” PCR method that showed high sensitivity and specificity in the testing of BAL and oral wash specimens from patients with AIDS-related PCP, but the study was not blinded and the control patients were not persons with HIV infection or active pulmonary disease [9].

Rabodonirina et al., using a rapid nested PCR method, reported excellent sensitivity (100%) but suboptimal specificity (77%) in testing BAL specimens from HIV-infected subjects [13]. Their study was not blinded, and clinical diagnoses were established retrospectively. Other recent reports have shown PCR to have sensitivities and specificities of 90%–100% in examinations of BAL specimens (mostly from persons with HIV infection), but those studies, for the most part, were neither blinded nor prospective and used predefined criteria for classification of case patients as PCP or control [14–17].

Testing was blinded in 1 study that reported the sensitivity and specificity of PCR to be 100%, but the report did not indicate whether patients were evaluated prospectively or whether diagnoses were made without knowledge of the PCR result [14]. Furthermore, the diagnosis of PCP could have been established on the basis of the PCR result, thus making it difficult to accurately identify false-positive PCR results. Moreover, the control patients were inappropriately selected, since they were patients who underwent bronchoscopy for lung cancer rather than HIV-infected individuals under evaluation for suspected PCP.

Deficiencies in study design limit the applicability of many of these earlier observations. None of these studies prospectively enrolled patients in a trial in which predetermined criteria were used for classification of case patients and results were tested and analyzed in a blinded manner. These deficiencies permit introduction of bias into the analysis and interpretation of results. In addition, none of these studies assessed reproducibility. Therefore, results of prior studies are difficult to interpret, preventing accurate assessment of the diagnostic value of PCR for diagnosis of PCP.

In our study, we carefully avoided such bias. First, we established our diagnostic criteria before the study was begun and strictly adhered to those criteria in the classification of case patients and analysis of results. Case patients were classified before PCR testing, and no case or control patient was reclassified on the basis of PCR results. Second, clinical and laboratory data were not provided to the molecular diagnostics laboratory prior to analysis of the results of the study; thus the blinding was maintained until the results were complete. To the best of our knowledge, this is the first blinded prospective study carefully designed to evaluate the role of PCR in the diagnosis of PCP. Third, 2 aliquots were tested from each patient to allow assessment of reproducibility.

Nevertheless, our study also has its limitations. First, the small size of this study limits our ability to fully assess the accuracy of PCR for diagnosis. Second, failure to perform biopsies in all case patients and to repeat bronchoscopies in those with negative findings prevents definitive exclusion of PCP in case patients with negative cytological results.

A question arises as to the possible failure to diagnose PCP in patients whose PCR results were positive but whose BAL cytological findings were negative. Do such case patients represent false-positive PCR or false-negative cytological results? To address this question in the most rigorous manner, all patients would be required to undergo lung biopsy to exclude false-negative cytologies, as well as follow-up bronchoscopy to identify case patients in which the diagnosis was missed initially. Although this would be optimal, such an approach is not feasible.

We made an assumption that case patients of PCP that were missed at the initial bronchoscopy and not treated appropriately would progress within 3 months, permitting accurate diagnosis. No such case patients were identified in this study. Alternatively, case patients with positive PCR but negative cytological findings could represent airway colonization by P. carinii rather than “false-positive” PCR results. We have no way to exclude such a possibility but question its clinical relevance, since none of these patients developed clinically apparent PCP with follow-up. Again, however, the small size of our study precludes adequate assessment of this possibility.

In summary, our findings confirm that PCR is a sensitive and reproducible test for detection of P. carinii in BAL specimens but suggest that it is less specific than cytology. The false-positive results for control patients limits the applicability of this method of diagnosis. If higher quantitative results are required for diagnosis, the specificity improves at the expense of sensitivity, and the test becomes inferior to the traditional methods for diagnosis that are based on special staining of the lavage fluid.

References