Detection of *Pneumocystis carinii* in Respiratory Specimens by PCR–Solution Hybridization Enzyme-Linked Immunoassay

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By using a recently developed PCR-solution hybridization enzyme-linked assay (PCR-SHELA), we investigated *Pneumocystis carinii* in bronchoalveolar lavage fluid samples and induced sputa of patients with pneumocystosis. In detecting *P. carinii*, PCR-SHELA proved more sensitive than immunofluorescence staining or a single PCR and significantly more diagnostically specific than a nested PCR. Our data suggest that PCR-SHELA could be used to detect *P. carinii* organisms in respiratory samples, particularly in patients with uncertain diagnoses.

PCR plus hybridization, especially nested or heminested PCR, has considerably increased the sensitivity of diagnostic tests for *Pneumocystis carinii* pneumonia (PCP) in respiratory specimens (4, 7, 8, 12). Several investigators have described the use of a PCR-based assay, consisting of detection of PCR products by an enzyme immunoassay (EIA) for the diagnosis of infectious diseases (3, 13). Recently, Cartwright et al. (1) have used a PCR-EIA to detect *P. carinii* in respiratory samples. Continuing our program of research into the molecular diagnosis of PCP, we assessed the ability of a new PCR-solution hybridization enzyme-linked assay (PCR-SHELA) to detect *P. carinii* in bronchoalveolar lavage fluid and induced sputum of human immunodeficiency virus (HIV)-infected patients suspected of having PCP.

Fifty-nine bronchoalveolar lavage and 66 induced sputum samples were obtained from 125 HIV-infected patients (91 men, 34 women; age range, 24 to 61 years) at the Department of Infectious Disease, Catholic University, Rome, Italy. All patients had no previous history of PCP. Patients underwent bronchoalveolar lavage for acute respiratory illness (fever, cough, shortness of breath) with abnormal chest signs and/or arterial hypoxemia and/or chest X-ray abnormalities, for unexplained pyrexia, or for suspected malignancies. Bronchoalveolar lavage was performed before starting high-dose anti-*P. carinii* therapy by two operators using the same multiple-lobe sampling technique. Sputum was induced by trained respiratory technicians using a protocol described by Ng et al. (5). Specimens were immediately processed and examined blindly, some aliquots were cultured in accordance with standard procedures to detect other pathogens.

The presence of *P. carinii* was tested by morphological staining and DNA detection by PCR. Immunofluorescence staining was performed with a commercial kit with monoclonal antibodies specific for cyst wall antigens (Monofluokit *P. carinii*; Diagnostic Pasteur, Paris, France). Immunofluorescence tests were considered positive if five or more cysts were detected on a smear.

Before PCR, bronchoalveolar lavage and induced sputum specimens were digested for 2 h at 55°C with proteinase K (final concentration, 0.5 mg/ml; Sigma). A PCR amplifying a portion of mitochondrial large-subunit rRNA was used with specific primers (11). In brief, pAZ102-E (5^-GATGGCTGT TTCGAXGCCCA-3^) and pAZ102-H (5^-GTGTAACGTTC AACGTACTC-3^) (12) were used as outer primers for the first amplification round (mt-LSU single PCR) in a reaction volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl2, 0.2 mM each deoxynucleoside triphosphate, 1 mM each primer, and 2.5 U of Taq polymerase (Amplitaq; Perkin-Elmer). A hot-start technique was used. One microliter of the first amplification product was reamplified in a second reaction buffer (25 μl) containing the internal primer pair pAZ102-X (5^-GTGAAATACAAATCGGACTAGG-3^) and pAZ102-Y (5^-TCACCTAATATATATGTGGGAC-3^) (mt-LSU nested PCR) (9, 11). The two amplification products (346 and 263 bp) were visualized by ethidium bromide staining after agarose gel electrophoresis.

For PCR-SHELA, pAZ102-E and pAZ102-H labeled at the 5' end with digoxigenin were used as primers in the amplification round and pAZ102-L2 (5^-CAATATATCGGACTAGG-3^) (12) labeled at the 5' end with biotin, used as the probe for hybridization, were added at the same time after the beginning of the reaction. Although the 5' biotin-labeled pAZ102-L2 probe was present throughout PCR, it could not take part in the amplification reaction because a 3' tail of dTTP mismatched with the corresponding sequence, thereby preventing oligonucleotide extension. The high annealing temperature also prevented the pAZ102-L2 probe from binding to its target sequence. After the PCR, the amplification products were denatured for 20 min at 99°C and the temperature was then lowered to 48°C to allow pAZ102-L2 to anneal to its complementary product sequence. We used a hot-start technique, and the PCR included 1 cycle of 94°C for 7 min, followed by 30 cycles of 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 1 min; after a denaturation cycle at 99°C for 20 min, the final hybridization was performed at 48°C for 90 min. Ten picomoles of each primer (pAZ102-E and pAZ102-H) was used in a 25-μl reaction volume. Hybridization of the amplification products with various pAZ102-L2 concentrations showed that 0.1 pmol was the optimum concentration for avoidance of background signals. The PCR buffer was the same as that used in the PCR. After amplification and hybridization, the PCR products were diluted by adding 200 μl of phosphate-buffered saline (PBS)—0.05% Tween 20 in ice.
Streptavidin-coated microtiter plates (Boehringer Mannheim, Indianapolis, Ind.) were blocked with sonicated salmon sperm DNA (Sigma Chemical Co., St. Louis, Mo.) for 20 min at room temperature. Wells were washed twice with PBS–0.05% Tween 20, a 100-μl volume of the diluted PCR products was added to each well, and the plate was incubated for 60 min at room temperature. One hundred microliters of peroxidase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) per well diluted 1:500 in PBS–0.05% Tween 20 was added after four washings. After a 60-min incubation at room temperature and four washings, 100 μl of tetramethylbenzidine (Boehringer Mannheim) was added as the substrate; 10 min later, the reaction was stopped by adding 50 μl of 1 M H₂SO₄, and the A₄₅₀ was measured. Each experiment included as positive controls standard end-point dilutions prepared with bronchoalveolar lavage fluid from a PCP patient (4.32 × 10⁶ P. carinii nuclei/ml). PCR-SHELA permitted detection of four P. carinii nuclei/μl with a reproducible absorbance value of >0.490 (cut-off). PCR was diagnosed on the basis of identification of P. carinii organisms by morphological stains, detection of P. carinii DNA by a single PCR in respiratory specimens or lung tissue, and/or clinical findings and therapeutic response. Moreover, if the nested PCR was positive, PCP was diagnosed in patients with clinical suspicion in the absence of an alternative diagnosis, in particular, in those on specific prophylaxis. PCP was excluded when (i) the morphological examination of the bronchoalveolar lavage (not only the induced sputum) sample was negative, (ii) an alternative diagnosis was absent, (iii) resolution of the clinical and radiological picture occurred without anti-P. carinii therapy, and (iv) clinical PCP did not occur in a follow-up for at least 12 months. On the basis of the above criteria, 35 patients were considered to have PCP. In 32 cases, P. carinii organisms were identified in respiratory specimens (17 bronchoalveolar lavage fluid and 15 induced sputum samples). Three patients were classified as having PCP despite negative immunofluorescence staining and single PCR results obtained with their bronchoalveolar lavage fluid. In one of the three patients, who died 2 weeks later, P. carinii was identified in a lung examination. The other two patients were taking primary anti-P. carinii prophylaxis (one dapsone-pyrimethamine and the other aerosolized pentamidine).

Ninety patients were PCP negative but had the following respiratory diagnoses: pneumonia caused by Pseudomonas (16 cases) or Staphylococcus (3 cases) organisms, by Streptococcus pneumoniae (5 cases), by Klebsiella pneumoniae (7 cases), by mycobacteria (24 cases), or by Aspergillus (2 cases) or Cryptococcus (2 cases) organisms and Kaposi’s sarcoma with lung involvement (5 cases). Twenty-six patients had neither opportunistic pathogens nor neoplasia.

Sensitivity, specificity, positive and negative predictive values calculated with a two-by-two table, and 95% confidence intervals were determined. Fisher’s exact test (one sided) was used to determine the significance of differences between the sensitivity and specificity of the four diagnostic techniques. P values of ≤0.05 were considered to indicate statistical significance.

PCR-SHELA detected P. carinii DNA in all 20 bronchoalveolar lavage and 15 induced sputum specimens from patients with PCP at optical densities ranging from >0.55 to 2.7. Bronchoalveolar lavage fluid and sputum samples had similar mean optical densities (Fig. 1). A comparison of the results obtained by immunofluorescence, PCR, and PCR-SHELA with the 35 samples showed that PCR-SHELA and the nested PCR were more sensitive than immunofluorescence staining and the single PCR. In fact, all of the samples were positive by these two techniques, whereas only 32 of the 35 samples were positive by immunofluorescence assay and the single PCR (95% confidence interval, 90.00 to 100.00 versus 76.94 to 98.20). Eight of 90 patients without PCP were positive by the nested PCR; 2 of these were positive by PCR-SHELA also. Therefore, immunofluorescence staining, the single PCR, and PCR-SHELA were significantly more specific in detecting P. carinii than was the nested PCR (100 versus 92%, P = 0.003, 95% confidence interval of 95.98 to 100 versus 83.23 to 96.08; 98 versus 92%, P = 0.05, 95% confidence interval of 92.20 to 99.73 versus 83.23 to 96.08). Immunofluorescence and the single PCR failed to identify P. carinii in three bronchoalveolar lavage specimens from patients with PCP. The nested PCR revealed P. carinii in eight bronchoalveolar lavage fluid samples from patients without PCP (Table 1). The specificity of PCR-SHELA was confirmed by bronchoalveolar lavage specimens from 20 HIV-negative, nonimmunosuppressed patients who underwent bronchoscopy for suspected, but not confirmed, lung neoplasia. These specimens were negative by all tests (data not shown).

To offer immunocompromised patients a better outcome, an immediate need is to improve the sensitivity, specificity, and quickness of diagnostic tests for PCP. The currently used routine immunofluorescence staining test, based on direct morphological identification of the organisms in respiratory specimens, is less sensitive than PCR in recurrent episodes of PCP and in patients who experienced PCP on prophylaxis (10). PCR plus hybridization performed with selected primers and probes (2) appears to be sensitive and specific, yet the use of a
radiolabeled probe limits its application in routine diagnostic protocols. Although a nested PCR overcomes these technical problems, it has another disadvantage. Its high sensitivity complicates clinical interpretation and makes it necessary to exclude other respiratory disorders and laboratory cross-contamination.

In this study designed to find a new means of diagnosing PCP, we used a rapid PCR-SHELA technique previously applied by Qiao et al. to detect the *Leishmania donovani* complex (6). We used the primers targeting the mt-LSU rRNA gene, which have the best diagnostic efficiency in our experience (2), and we included the hybridization step in the amplification program. This technique proved faster and simpler than the PCR-EIA described by Cartwright et al. (1). In accordance with their results, PCR-SHELA yielded a higher sensitivity than immunofluorescence staining and similar specificity with respiratory samples from patients with PCP. Our experiments designed to test the diagnostic efficiency of *P. carinii* detection by PCR-SHELA and single and nested PCRs, all targeting the mt-LSU rRNA gene, showed that PCR-SHELA was more sensitive than the mt-LSU single PCR and more specific than the mt-LSU nested PCR.

The mt-LSU nested PCR revealed *P. carinii* in 8 of the 98 patients without PCP. With bronchoalveolar lavage samples, this positivity could simply depend on “subclinical infection/carryer status,” as has been observed in patients with HIV-related severe immunodeficiency (2, 4, 7). Our finding that two of these nested-PCR-positive samples were also PCR-SHELA positive makes cross-contamination unlikely. A more likely explanation is that the different sensitivities of the two methods (nested PCR, one nucleic per microliter; PCR-SHELA, four nuclei per microliter) led to different results with samples containing few organisms.

In conclusion, our data suggest that PCR-SHELA will be useful for detecting *P. carinii* organisms in respiratory samples. An etiological diagnosis may be crucial in the management of HIV patients with suspicion of PCP in the era of new alternative regimens. Moreover, a molecular technique may be useful for examining noninvasive samples such as induced sputum in outpatient settings and in patients with suspicion of PCP who are receiving specific prophylaxis. The higher specificity of PCR-SHELA than nested PCR allows us to suggest the use of PCR-SHELA for diagnosis of PCP in patients whose diagnoses remain uncertain.

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