

Rapid Detection of *Pneumocystis carinii* in Bronchoalveolar Lavage Specimens from Human Immunodeficiency Virus-Infected Patients: Use of a Simple DNA Extraction Procedure and Nested PCR

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We report on the development of a rapid nested PCR protocol for the detection of *Pneumocystis carinii* DNA in bronchoalveolar lavage (BAL) specimens in which the protocol included the use of a commercially available DNA extraction kit (GeneReleaser). GeneReleaser enabled us to obtain amplification-ready DNA within 20 min without requiring the purification of the DNA. The nested PCR was performed with the primers pAZ102-E, pAZ102-H, and pAZ102-L2 (A. E. Wakefield, F. J. Pixley, S. Banerji, K. Sinclair, R. F. Miller, E. R. Moxon, and J. M. Hopkin, *Lancet* 336:451–453, 1990.). Results were obtained in about 4 h with the adoption of denaturation, annealing, and extension steps shortened to 20 seconds. The sensitivity of the nested PCR was tested with a *P. carinii* cyst suspension and was found to be less than one cyst (one to eight nuclei). The detection limit was the same with the use of GeneReleaser or proteinase K-phenol chloroform for DNA extraction. The nested PCR assay was prospectively compared with staining with Giemsa and methenamine silver stains for the detection of *P. carinii* in 127 BAL samples from 105 human immunodeficiency virus-infected patients investigated for acute respiratory illness. Twenty-five BAL specimens (20%) were positive by staining and the nested PCR and 25 (20%) were negative by staining and positive by the nested PCR. These 25 BAL specimens with conflicting results were obtained from 23 patients, 82% of whom were receiving prophylactic therapy against *P. carinii* pneumonia (PCP). Only two patients were diagnosed with possible PCP. The final diagnosis was not PCP for 20 patients who were considered to be colonized or to have a low level of infection. This colonization is not of clinical importance but is of epidemiological importance. Our rapid, simple, and sensitive amplification protocol may be performed in clinical laboratories for the routine diagnosis of PCP with BAL specimens.

Pneumocystis carinii is the major cause of pneumonia and is one of the most frequent opportunistic pathogens in human immunodeficiency virus (HIV)-infected patients. The diagnosis of *P. carinii* pneumonia (PCP) has been dependent on the microscopic visualization of the organism in respiratory specimens by staining methods or immunofluorescence assays. Recently, PCR has been reported to be a specific diagnostic method for PCP. The results of the various studies indicate that PCR is more sensitive than the conventional methods for the detection of *P. carinii* in human samples (3, 4, 6, 7, 10, 11, 17, 19, 20, 22, 25, 26, 29, 33, 34, 36, 38, 40). At present, a highly sensitive technique is required for the detection of *P. carinii* in patients receiving chemoprophylaxis, because chemoprophylaxis may decrease the yield of microscopic methods (16, 21). Furthermore, PCR is a precious tool for clarifying the natural history of the disease and the epidemiology of *P. carinii*.

PCR nevertheless requires improvements to become a routine diagnostic test for PCP. Rapid and simple detection and confirmation procedures that avoid the requirement for radioactive reagents and time-consuming hybridization are needed

for clinical laboratories. The present report describes the results of a prospective study comparing conventional staining tests with a rapid nested PCR performed after a simple DNA extraction procedure with the commercially available kit GeneReleaser for the diagnosis of PCP. Staining and nested PCR with the three primers described by Wakefield et al. (38, 39) were performed with bronchoalveolar lavage (BAL) specimens from HIV-infected patients with acute respiratory illness. The conflicting results between staining tests and nested PCR are discussed in relation to the clinical symptoms and follow-up of the patients.

MATERIALS AND METHODS

Clinical specimens. One hundred twenty-seven BAL specimens were prospectively obtained from 105 HIV-infected patients (80 men and 25 women) from 1 March 1995 to 31 December 1995. All patients were investigated for pulmonary disease, characterized by dyspnea, cough, fever, and possibly accompanied by abnormal chest signs and/or abnormal chest radiographs. Precise clinical data were obtained by retrospective medical chart review for the 23 patients (19 men and 4 women) whose specimens yielded conflicting results by staining and nested PCR. These clinical data included CD4⁺ lymphocyte count, serum lactate dehydrogenase level, the organisms identified in the BAL specimens, treatment against other infections, anti-PCP prophylaxis, and clinical outcome. The probability that these patients had PCP was discussed by using the criteria proposed by Huang et al. (13), including the data cited above.

Specimen processing. BAL specimens were centrifuged at 1,500 × g for 5 min. Smears were made from sediments and were stained with Giemsa (one slide) or methenamine silver (two slides) stain. Slides were examined by personnel exper-

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rienced in identifying *P. carinii* organisms. The remaining portions of the sediments were frozen at -20°C and were then used for DNA amplification. Staining results were transmitted to the patients' clinicians during the study.

DNA extraction and nested PCR. The method described by the manufacturer for the extraction of DNA from whole blood with GeneReleaser (BioVentures Inc., Murfreesboro, Tenn.) was used for the BAL specimens. Briefly, 10 μl of the sediment was added to 20 μl of GeneReleaser and was denatured in a thermocycler (MJ Research Inc., Watertown, Md.) PCR reagents were then added to the same tube. DNA amplification was carried out in a reaction mixture (100 μl) containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl_2 , 0.25 mM (each) dATP, dTTP, dCTP, and dGTP, 0.25 μM (each) the oligonucleotide primers, and 2.5 U of *Taq* polymerase (Promega Corp., Madison, Wis.). The primers described by Wakefield et al. (38, 39) for the amplification of a part of the mitochondrial gene encoding for the large subunit of rRNA were used: pAZ102-E (5'-GATGGCTGTTTCCAAGCCCA-3') and pAZ102-H (5'-GTG TACGTTGCCAAAGTACTC-3'). After an initial 5-min denaturation step, DNA was amplified for 40 cycles with a final extension period of 5 min at 72°C . Each cycle consisted of 20 s of denaturation at 94°C , 20 s of annealing at 56°C , and 20 s of extension at 72°C . The second amplification step was performed with 1 μl of the amplified product of the first step by using pAZ102-E as the external primer and pAZ102-L2 (5'-ATAAGGTAGATAGTCGAAAG-3') (38, 39) as the internal primer. The reaction mixture and the amplification protocol were the same as those for the first PCR, except that the primers were used at 0.75 μM and the annealing temperature was 50°C . To avoid contamination, the DNA extraction procedure and the two amplification procedures were performed in separate rooms within the laboratory, and aerosol-barrier pipette tips were used to handle all reagent transfers. Positive specimens (BAL specimens obtained from patients with microscopically proven PCP) and multiple negative control specimens (water) were included in each experimental run. The two amplified products were subjected to electrophoresis in a 1.5% agarose gel and were visualized after ethidium bromide staining. The expected *P. carinii*-specific bands consisted of 346- and 120-bp fragments. Nested PCR was performed without knowledge of the staining results. The amplification results were transmitted to the patients' clinicians at the end of the study.

Sensitivity experiments. A cyst-rich suspension of *P. carinii* isolated from an open lung biopsy specimen from an HIV-infected patient with PCP was used for the sensitivity experiments. Cysts were counted in 5 μl of air-dried smears stained with methenamine silver. Following extraction with GeneReleaser or proteinase K-phenol chloroform, 10-fold dilutions (10^0 to 10^{-12}) of the parasite preparation were tested by nested PCR.

RESULTS

DNA extraction. Amplification-ready DNA from BAL specimens was obtained directly in the amplification tube in 20 min.

Sensitivity of the nested PCR. The cyst-rich suspension was found to contain 1.8×10^4 cysts/ μl on slide preparations stained with methenamine silver. Amplification products were visible after ethidium bromide staining to a dilution of 10^{-5} after the one-step amplification and to a dilution of 10^{-7} after the two-step amplification. The lower detection limit was already less than 1 cyst/ μl after the one-step amplification. The level of sensitivity of the nested PCR was the same after extraction with proteinase K-phenol chloroform and after extraction with GeneReleaser.

Nested PCR with BAL specimens. Nested PCR results were obtained within 4 h (Fig. 1). The results for the 127 BAL specimens tested are given in Table 1. Staining and nested PCR results were concordant for 102 specimens (80%). Twenty-five (20%) BAL samples were negative by staining and positive by nested PCR. These 25 BAL samples were obtained from 23 patients, for whom a retrospective review of their medical charts was performed. They all presented with pulmonary symptoms. Interstitial pneumonitis was observed on chest radiographs for nine patients and lobar infiltrates were observed for seven other patients. Another pathogen was found in BAL specimens obtained from 12 patients, who then received the corresponding curative treatment. Four additional patients were empirically treated with antibacterial agents. Nineteen patients (82%) had received prior anti-PCP prophylaxis, most of them (60%) with trimethoprim-sulfamethoxazole. This chemoprophylaxis was continued during and after the episode of acute respiratory illness. The follow-up period of the study was 1 year. The outcomes are known for 22

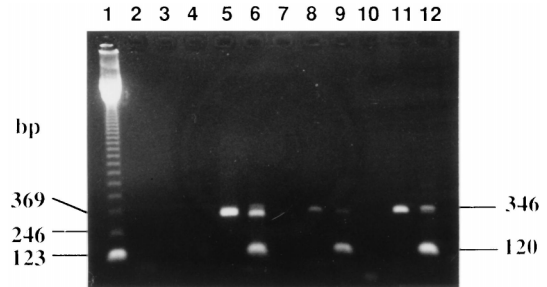


FIG. 1. PCR results after DNA extraction with GeneReleaser. PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Lane 1, molecular mass marker; lanes 2 and 3, negative control (water) for single PCR and nested PCR, respectively; lanes 4 and 5, positive control (*P. carinii* cysts isolated from a lung biopsy specimen) for single PCR and nested PCR, respectively; lanes 6 and 7, BAL specimen negative with stains, single PCR and nested PCR, respectively; lanes 8 and 9, BAL specimen positive with stains, single PCR and nested PCR, respectively; lanes 10, 11, and 12, no template.

patients. No patient developed acute proven PCP, and 16 patients had clinical improvement. Six patients died, one of hemoptysis, one of cytomegalovirus encephalitis, one of disseminated microsporidiosis, and another of hepatic insufficiency. The cause of the death was unknown for the other two patients. Autopsies were not performed. For these 23 patients for whom the results of the staining and nested PCR tests were in conflict, the probability of PCP was discussed by using the criteria proposed by Huang et al. (13). The final diagnosis for 20 patients was not PCP. Eleven patients were diagnosed as having pneumonia caused by other organisms. One of these patients was still receiving atovaquone because he had developed a microscopically proven case of PCP 3 weeks before this second acute respiratory illness. Pulmonary signs disappeared with antibiotic treatment with imipenem and amikacin in combination. For eight patients PCP was excluded on account of one or more of the following: the chest radiograph picture, the presence of purulent sputum, or clinical improvement without specific curative anti-PCP treatment. PCP was a possible diagnosis for only two patients on account of an early death (less than 3 months after the episode of pulmonary illness) without an alternative diagnosis.

The sensitivity, specificity, positive predictive value, and negative predictive value for the nested PCR test were calculated in relation to the staining results and the results of the clinical evaluation. They were 100, 77, 54, and 100%, respectively.

DISCUSSION

In the present study, GeneReleaser was used for the first time to extract DNA from a fungus in human clinical specimens. Our results suggest that GeneReleaser is as effective as proteinase K-phenol chloroform for obtaining amplification-ready DNA from *P. carinii*. Comparable results have recently

TABLE 1. Results of conventional staining tests, simple PCR, and nested PCR for 127 BAL specimens from HIV-infected patients

Stain result	No. (%) of BAL specimens			
	Simple PCR result		Nested PCR result	
	Positive	Negative	Positive	Negative
Positive	24 (19)	1 (0.7)	25 (20)	0 (0)
Negative	18 (14)	84 (66)	25 (20)	77 (60)

been found for *Mycobacterium tuberculosis* (1). GeneReleaser enables specimens to be saved, since 10 μ l of BAL specimen sediment is sufficient. It simplifies and shortens PCR template preparation, avoiding procedures such as digestion with proteinase K, purification with phenol-chloroform, and ethanol precipitation, which may induce contamination and lead to the loss of DNA material. Several investigators did not purify the DNA after proteinase K digestion, but they observed some negative results by PCR with specimens positive by staining (17, 22, 25, 29, 32), probably because endogenous *Taq* polymerase inhibitors were not eliminated. GeneReleaser, an anion-binding resin, prevented such false-negative results in our study by capturing inhibitors. Cartwright et al. (3) developed a rapid DNA extraction procedure using proteinase K, lyticase, and a commercially available purification kit (GeneClean; Bio-101, Inc., La Jolla, Calif.). Their processing time is longer than ours, and many more manipulations are needed. The cost of GeneClean (\$0.36) is higher than the cost of GeneReleaser (\$0.29) for one test. In addition, our procedure requires GeneReleaser as the only reagent. Borensztein et al. (2) developed a filtration technique without DNA extraction, but the processing time is more than 1 h.

The length of each of the steps of the nested PCR was shortened to 20 s, instead of the 1 or 2 min used in other studies (9–11, 23, 32, 33), without a loss of sensitivity (15). The enzyme-linked immunosorbent assay for PCR product detection developed by Cartwright et al. (3) also seems interesting, provided that it becomes automated.

The primers described by Wakefield et al. (38, 39) were chosen because some investigators have shown that mitochondrial rRNA gene PCR is the most specific and sensitive single-step PCR for the detection of *P. carinii* (5, 23). The increased sensitivity (up to 100 times) that we observed between single-step and two-step PCRs is equivalent to the increased sensitivity produced by hybridization (30, 39) and confirmed the findings of other investigators (9–11). The results of our single-step PCR coupled with agarose gel electrophoresis (less than one cyst/ μ l) are consistent with those of De Luca et al. (5) (five nuclei/ μ l), whereas one cyst contains up to eight nuclei.

P. carinii was detected by nested PCR with 25 specimens which were negative by staining. Such a discrepancy has already been reported by other investigators who used different PCR protocols and different amplification targets. Such results have not been considered false-positive PCR results, and some interpretations have been proposed. The high degree of sensitivity of PCR has permitted the detection of *P. carinii* several weeks before (22, 26, 29, 39) or after (3, 22, 25, 26, 28, 29) an episode of microscopically proven PCP. These findings are consistent with those for experimental pneumocystosis, which have demonstrated that PCR becomes positive before the results of cytologic and histologic studies are positive (30, 31) and remains positive much longer (35). Two of our patients had PCP 3 weeks and 5 months, respectively, before their specimens tested nested PCR positive and staining negative.

Another explanation for the PCR-positive and staining-negative results is that anti-*P. carinii* prophylaxis may reduce the number of organisms, which then escape detection by stains (17, 19, 25, 26, 29, 33). The yield from BAL has been reported to be decreased for patients receiving aerosolized pentamidine (16, 21), and systemic agents like trimethoprim-sulfamethoxazole may further reduce this yield (8). Yet *P. carinii* does not completely disappear (14). The influence of chemoprophylaxis may be reflected by the finding that 82% of our patients whose specimens yielded conflicting results by the two tests had received prior prophylaxis at the time of the bronchoscopy.

Finally, in previous studies conflicting results have been re-

lated to subclinical colonization or to a low level of infection in asymptomatic patients or in patients with a clinical course inconsistent with that of PCP (9–11, 17–19, 22, 24, 25, 26, 29, 33, 34, 39). For 90% of our patients, the final diagnosis was not acute PCP and the outcome was recovery for 74% of the patients without curative anti-PCP treatment. The last fact completely rules out an obvious infection with *P. carinii*. An autopsy was not performed on the six patients who died, but there was no clinical evidence that PCP was the cause of death. It is difficult to distinguish between colonization or low level of infection in our patients because they had pulmonary symptoms that are frequently associated with bacterial, viral, or fungal organisms and that are cured by treatment with antimicrobial agents. On the other hand, 88% of those who recovered continued their anti-*P. carinii* prophylaxis, which might play a role in the recovery. Carriage has especially been studied by investigators who used PCR with induced sputum specimens (3, 4, 6, 7, 17, 20, 22, 26, 36, 40). Leigh et al. (19), in particular, have demonstrated different *P. carinii* carriage rates in HIV-infected patients, and these carriage rates were inversely related to their CD4 counts. All of our 23 patients were severely immunosuppressed, as attested to by their CD4 counts of less than 200×10^6 /liter, and the frequency of *P. carinii* carriage that we found (20%) was the same that Leigh et al. (19) observed in a population with a similar clinical condition. Before the use of PCR, the notion of carriage had already been evoked in lung transplant recipients. Cysts were observed by methenamine silver staining (12) in BAL samples from 88% of the patients, whereas the prevalence of symptomatic pulmonary infection was 35%. *P. carinii* carriage was found by PCR in other organ transplant recipients (20, 24, 25) and in patients with hematological malignancies (24, 40).

In conclusion, we have developed a rapid, simple, and sensitive PCR which may be performed in clinical laboratories for the routine diagnosis of PCP with BAL specimens. Our results confirm that the detection of *P. carinii* by PCR in AIDS patients may not be of apparent clinical importance and may be considered colonization or a low level of infection. The role of pulmonary carriage in the airborne transmission of *P. carinii* infection may be investigated by applying our nested PCR protocol to induced sputum samples, oropharyngeal samples (37), or nasopharyngeal aspirates (27) taken from HIV-infected patients and other immunosuppressed patients.

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