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Comparison of PCR and Standard Cytological Staining for Detection of *Pneumocystis carinii* from Respiratory Specimens from Patients with or at High Risk for Infection by Human Immunodeficiency Virus

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The detection of *Pneumocystis carinii* DNA by PCR was compared with routine cytologic staining techniques (CYT). A total of 284 clinical respiratory specimens, including 137 bronchoalveolar lavage (BAL), 63 bronchoalveolar washing, 63 sputum, and 21 induced sputum samples, obtained from patients with or at high risk for human immunodeficiency virus infection were evaluated. Eighty specimens were positive by PCR, and 69 were positive by CYT. PCR was able to detect *P. carinii* in more bronchoalveolar washing specimens (15 versus 11) and in comparable BAL specimens (53 versus 54) compared with CYT. PCR was particularly more sensitive than CYT in detecting *P. carinii* in expectorated sputum (12 versus 4 samples). Of the 19 patients whose respiratory specimens were positive for *P. carinii* by PCR but negative by CYT, 5 had *P. carinii* pneumonia (PCP) confirmed by subsequent BAL and transbronchial or mediastinal lymph node biopsy and 9 had a clinical course highly suggestive of acute PCP. Eleven (58%) of the 19 patients with discordant PCR and CYT results had received prior anti-PCP prophylaxis. In this clinical setting in particular and in the evaluation of sputum specimens, the ability of PCR to detect a low parasitic load suggests that this technique may become an important additional tool, along with current cytological methods, for the detection of *P. carinii*.

Pneumocystis carinii pneumonia (PCP) is the most common opportunistic infection among human immunodeficiency virus (HIV)-infected individuals. Eighty percent or more of patients with AIDS not receiving anti-PCP prophylaxis will ultimately develop PCP (7). More than 100,000 cases of PCP among AIDS patients were projected by 1991 despite the availability of effective chemoprophylaxis (4, 7). Diagnosis of PCP depends upon the identification of the organism by microscopic examination of sputum, pulmonary secretions, and/or pulmonary tissue obtained by bronchoalveolar lavage (BAL) and transbronchial biopsy (TBB) or lung biopsy. BAL, with or without TBB, is the procedure of choice for the evaluation of pulmonary disease in both AIDS and non-AIDS patients (3). The sensitivity of BAL in the diagnosis of PCP using standard cytological strains (CYT) ranges from 86 to 97% (12). When combined with TBB, the sensitivity of the procedure approaches 100% compared with open lung biopsy (12). However, BAL is an invasive technique sometimes associated with different degrees of morbidity (3, 12). BAL is now being supplanted by the examination of sputum, as a result of improvements in the sensitivity of the technique, for the initial detection of PCP. However, the sensitivity of the CYT techniques used for *P. carinii* detection in induced sputum ranges only between 55 and 78% (17). This may be improved by the use of monoclonal antibodies against surface antigens of *P. carinii* (9, 11). Currently, the most commonly employed staining techniques used for the identification of *P. carinii* are the methenamine silver stain, which stains the cyst wall, and the modified Giemsa stain, which stains the parasite at all stages of its life cycle. However, the sensitivity of these stains in detecting *P. carinii* in BAL and sputum specimens is only 81 to 92% (6). Some authors have suggested a possible increase in the diagnostic yield of BAL by combining the methenamine silver stain with the Gram-Weigert stain (5). The development of PCR for *P. carinii*, which amplifies specific DNA sequences, provides a method that may increase the sensitivity of detection of *P. carinii*, particularly when it is present at very low levels (8, 10, 13–16).

In this study we compared the results of PCR with those of CYT in the detection of *P. carinii* in respiratory specimens obtained from patients with or at high risk for HIV infection undergoing bronchial lavage or sputum examination at New York University Medical Center and Bellevue Hospital Center, New York, N.Y.

MATERIALS AND METHODS

Population and sample collection. Respiratory specimens were obtained from 284 patients (279 adults and 5 children) with or at high risk for HIV infection evaluated for pulmonary disease at New York University Medical Center and Bellevue Hospital Center between 1 February and 1 September 1991. Specimens were processed in the pathology laboratory of Bellevue Hospital for routine cytology. Specimens included 84 sputum samples (63 uninduced [S] and 21 induced [IS] by a 30-min inhalation of 3% saline solution via an ultrasonic nebulizer) and 200 BAL fluid samples (137 samples obtained from deep subsegmental bronchi [BAL] and 63 samples obtained from washings of only second-degree branchings of the main bronchi [BW]). In addition, 70 respiratory specimens from patients not at risk for PCP were evaluated. These included 28 sputum specimens obtained from patients with suspected tuberculosis and 42 specimens (including 22 S, 11 BW, and 9 BAL specimens) collected from patients investigated for pulmonary malignancy.

Standard staining procedures for detection of *P. carinii* (Giemsa and modified methenamine silver stains) and PCR were performed on each specimen. PCR was performed without knowledge of the clinical status of the patients or staining results. Clinical data were obtained by retrospective medical chart review.

Sample processing. Respiratory specimens were collected in sterile containers and diluted with 70% ethanol. In addition, sputum specimens were treated with

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TABLE 1. Results of PCR and CYT in detecting *P. carinii* in 284 respiratory specimens obtained from patients with or at high risk for HIV infection

Sample origin	No. of samples[a]						
	Total	CYT+	PCR+	CYT+ or PCR+	CYT+, PCR+	CYT+, PCR−	CYT−, PCR+
BAL	137	54	53	62	45	9	8
BW	63	11	15	17	9	2	6
S	63	2	8	9	1	1	7
IS	21	2	4	5	1	1	3
Total	284	69	80	93	56	13	24

[a] +, positive result; −, negative result.

dithiothreitol (Sputolysin; Behring Diagnostics, La Jolla, Calif.). Specimens were centrifuged and washed in Hanks balanced salt solution (Whittaker Bioproducts, Walkersville, Md.). The remaining part of the pellet was processed for DNA amplification.

Cytology. Slides for CYT were prepared by Cytospin (Shannon) on polylysine-coated slides. Giemsa and modified methenamine silver staining was performed using standard methodology (1). Slides were reviewed by a qualified pathologist.

PCR. The pellets were digested with proteinase K (5 mg/ml; International Technologies, Inc., New Haven, Conn.). Ten microliters of DNA solution was used for each PCR. Remaining DNA was stored at −20°C. The PCR was performed as per the Perkin-Elmer Cetus protocol with a final Mg^{2+} concentration of 2.5 mM. The oligonucleotide primers used were pAZ102-E (5′-GAT GGCTGTTTCCAAGCCCA-3′) and pAZ102-H (5′-GTGTACGTTGCAAAG TACTC-3′), which amplify a 346-bp segment that is specific for a region of the gene coding for the large subunit of mitochondrial rRNA of human *P. carinii* (16). The amplification protocol included one 2-min cycle of denaturation at 94°C followed by incubation at 80°C for 8 min. The amplification was done for 35 cycles consisting each of 2 min of denaturation at 94°C, followed by annealing at 55°C for 2 min and extension at 72°C for another 2 min. Positive (from BAL and lung biopsy specimens obtained from patients with PCP) and negative (all the reaction mixture components minus the DNA template) controls were included in each series of tests.

Amplified products were detected by autoradiography using liquid hybridization with the ^{32}P-end-labelled internal primer pAZ102-L2 (5′-ATAAGGTA GATAGTCGAAAG-3′) after electrophoresis in 12% polyacrylamide gel. A sample was considered positive when DNA with the molecular size expected for the amplified product (346 bp) was visualized on the autoradiographs. Each experiment was performed at least twice.

RESULTS

The results are summarized in Table 1. Of the total 284 specimens tested, 80 (28%) were positive by PCR, compared to 69 (24%) positive by CYT. A total of 93 (33%) samples were positive by PCR or CYT. Of the 69 (81%) samples positive by CYT, 56 were positive by PCR. None of the control samples obtained from patients investigated for suspicion of tuberculosis or malignancy tested positive for *P. carinii* by PCR.

Of the 200 BAL or BW specimens, 65 (33%) were positive by CYT while 68 (34%) were positive by PCR. PCR was positive for 53 BAL specimens, while CYT was positive for 54; 45 of 54 (83%) BAL specimens positive by CYT were also positive by PCR. PCR was positive for more BW specimens than was CYT (15 versus 11); 9 of 11 (82%) BW specimens positive by CYT were also positive by PCR.

Of the 84 sputum specimens (S and IS) tested, 4 (5%) were positive for *P. carinii* by CYT while 12 (14%) were positive by PCR. PCR was positive for more S specimens—8 of 63 (13%)—than was CYT—2 of 63 (3%). One of the two S specimens positive by CYT was positive by PCR also. PCR was positive for more IS specimens—4 of 21 (19%)—than was CYT—2 of 21 (10%). One of two IS specimens positive by CYT was positive by PCR.

A total of 24 specimens (9 BAL, 5 BW, 8 S and 2 IS specimens) obtained from 22 patients were positive by PCR and negative by CYT. In order to evaluate whether this discordance reflected reduced detection of *P. carinii* by cytology or possible false-positive PCR, a retrospective review of medical charts was performed. Information was available for 19 of 22 of these patients, accounting for 21 of 24 respiratory specimens. These results are summarized in Table 2.

Eleven of the 19 patients (58%) were receiving anti-PCP prophylaxis (oral trimethoprim-sulfamethoxazole [TMP-SMX] in 7 and aerosolized pentamidine in 4 patients) when their respiratory specimens were collected; 1 of them was receiving secondary PCP prophylaxis with TMP-SMX). Respiratory

TABLE 2. Summary of clinical and laboratory data from 21 respiratory samples obtained from 19 patients who tested positive for PCP by PCR and negative for PCP by routine staining

Collection method	No. of samples							
	Total	From patients with:						
		Prior anti-PCP prophylaxis (no. with TMP-SMX, no. with pentamidine)	Clincal symptoms	Abnormal chest X-ray	Abnormal acetate (>250 U) dehydrogenase	Anti-PCP treatment	Confirmed PCP[a]	Probable PCP[b]
BAL	9	4 (3, 1)	7	9	6[c]	6	3	3
BW	5	2 (1, 1)	4	4	4[c]	3	1	3
S	5	3 (2, 1)	5	2	3	2	1	2
IS	2	2 (1, 1)	2	2	2[c]	1	0	1
Total	21	11 (7, 4)	18	17	15	12	5	9

[a] Three cases were confirmed by positive touch preparations from TBB, one was confirmed by mediastinal lymph node biopsy, and one (S) was confirmed by BAL.

[b] Clinical and roentgenographic findings compatible with PCP with abnormal lactate dehydrogenase values and improvement following anti-PCP treatment (when given).

[c] One patient had abnormal liver function tests.

symptoms (varying from only a cough to a full-blown acute syndrome compatible with PCP) were present in 18 of 19 patients. A roentgenographic picture of interstitial pneumonitis was present in 14 of 19 patients, while 3 additional patients had lobar infiltrates.

Five patients whose samples tested positive by PCR but negative by CYT (one S, one BW, and three BAL samples) had an additional diagnostic procedure performed following the initial negative cytology. Three had TBBs with touch preparations stained by Giemsa and/or silver stain establishing the diagnosis of PCP. A fourth patient had a biopsy of a mediastinal lymph node which confirmed the diagnosis of PCP. The fifth patient had *P. carinii* detected by BAL following an initial negative S examination. Four of them (providing one BW, one S, and two BAL samples) were receiving anti-PCP prophylaxis with TMP-SMX at the time of their hospitalization with suspicion of PCP.

Twelve of the 19 patients (63%) were started on empiric anti-PCP therapy when admitted to the hospital, on the basis of strong clinical and laboratory evidence for *P. carinii* infection. Nine were continued on anti-PCP therapy for 2 to 3 weeks (five after BAL, TBB, or lymph node biopsy confirmed the diagnosis and the other four because of strong clinical suspicion of PCP). Two other patients had received anti-PCP therapy for 7 and 10 days at the time of their deaths due to respiratory failure. The 12th patient was treated for only 5 days with intravenous TMP-SMX with marked improvement in her respiratory and roentgenographic condition. Three of the 12 patients treated for PCP died during the present hospitalization; the autopsy of one of them revealed, as the main cause of death, cytomegalovirus pneumonia. Autopsies were not performed on the other two patients.

Of the seven PCR-positive, CYT-negative patients not treated for PCP, two were outpatients with mild respiratory symptoms. Two patients died during the acute episode. One had evidence of Kaposi's sarcoma on lung biopsy. Autopsies were not performed on either of these two patients. An additional patient (with known cryptococcal disease at the time of bronchoscopy) was lost to further follow-up. Of the other four (providing one BAL and three S samples positive by PCR and negative by CYT), two were diagnosed with cryptococcal pneumonia and *Mycobacterium avium* disease, respectively, prior to being tested for possible PCP. All four patients did not develop acute PCP during a follow-up period of 3 to 7 months. However, three of them continued to receive anti-PCP prophylaxis during the period of the follow-up.

DISCUSSION

We have used a PCR DNA amplification procedure in the diagnosis of *P. carinii* in a large number of respiratory specimens obtained from patients with or at high risk for HIV infection and compared its results with those of CYT used in the diagnosis of the pathogen. These CYT procedures, for practical purposes and despite recognized problems in sensitivity and specificity, as well as marked differences between results at different institutions, are still considered today the "gold standard" in the diagnosis of *P. carinii*. Recent studies, however, suggest that indirect fluorescent-antibody stains are more sensitive than the traditional staining techniques used in the diagnosis of *P. carinii* and may in fact be considered the new gold standards in the detection of this pathogen (9, 11, 13).

Our results indicate that PCR and CYT have overall comparable rates of detection of *P. carinii* in BAL samples, while PCR is superior to CYT in the detection of *P. carinii* in BW and sputum. *P. carinii* was detected in 24 specimens that were cytology negative. That these were not detected by cytology and not false-positive PCR assays is suggested by the absence of false-positive PCR results in the negative control group, the clinical follow-up of five patients with PCR-positive, CYT-negative results who had further diagnostic tests all confirming the diagnosis of PCP, and possibly by the clinical and laboratory course highly suggestive of acute PCP in nine additional patients. The failure of cytology to detect *P. carinii* in these samples may be due to the lack of specificity of cytochemical stains, uncertainty of the sensitivity of immunochemical stains, and the potential variabilities in the antigenic structure of the *P. carinii* isolates. Of particular significance was the finding that 58% patients with positive PCR and negative CYT had received prior anti-PCP prophylaxis at the time of the testing for PCP. The anti-PCP prophylaxis may contribute to the underdetection of *P. carinii* by CYT by reducing the number of organisms present in the respiratory tract. TMP-SMX is today considered to be superior to pentamidine in the initial anti-PCP prophylaxis in HIV-infected patients, at least in those with fewer than 100 $CD4^+$ lymphocytes per mm^3. From this point of view, our positive PCR and negative CYT detections in patients on TMP-SMX prophylaxis may be viewed as falsely positive while those in patients on pentamidine prophylaxis may be more easily considered true-positive detections. Nevertheless, four of five patients with positive PCR and negative CYT results whose diagnosis was definitively confirmed by an additional diagnostic procedure received TMP-SMX prophylaxis prior to their hospitalization. Therefore, it is extremely difficult to decide, on the basis of our limited data, which prophylaxis regimen provided better protection against PCP.

Why *P. carinii* was not detected in all the cytology-positive specimens (13 missed) is unclear at the present time. This may be a result of dilution (PCRs were performed with at least 1/20 of the cellular material with which cytology was performed) or loss of DNA during processing and storage. We did not use in our study the time-consuming phenol-chloroform DNA extraction as used by all other authors reporting on *P. carinii* detection by PCR; it is possible that by using this method the specificity of our detection could have been improved.

PCR was significantly more sensitive than CYT in detecting the presence of *P. carinii* in sputum (in expectorated and IS samples as well). This suggests that PCR is more sensitive in detecting low levels of *P. carinii* that may be found in sputum. This may be due to differences of proportion between trophozoites and cysts (the presence of DNA-containing forms [trophozoites] less easily detectable by CYT could explain the better results obtained when using PCR) in upper and lower airways.

The detection of *P. carinii* by PCR in a number (5 of 19) of patients with negative cytology and a clinical course inconsistent with acute PCP raises, in addition to the problem of possible false-positive results due to an assay which could be too sensitive, the question of asymptomatic carriage or subclinical infection with *P. carinii*. The asymptomatic carriage of *P. carinii* has been recently described in animal models (8). Most of these five patients, however, were on anti-PCP prophylaxis when *P. carinii* was detected and were maintained on the same regimen following the procedure, suggesting that a low level of infection may persist during prophylaxis. Increased recognition of this changing presentation of the disease, associated with early diagnosis and treatment, may have beneficial consequences upon outcome by reducing the incidence of severe disease or secondary complications of this infection. The frequency of asymptomatic *P. carinii* carriage in patients at risk

for HIV infection, on the other hand, cannot be assessed from our results.

In summary, PCR represents a highly sensitive technique for the detection of *P. carinii* in the respiratory secretions of HIV-infected patients. However, it is possible that sometimes the increased sensitivity of the assay will lead to falsely positive results or divert the clinician's attention to a pathologic process that may be irrelevant to the patient's current problem. Nevertheless, its main advantage in comparison with CYT resides in its superior ability to detect *P. carinii* in sputum and possibly in respiratory specimens obtained from patients on anti-PCP prophylaxis, in which the parasitic load may be small. In this clinical setting, PCR may provide a useful addition to current cytologic methods used for the detection of *P. carinii*. In addition, because of its increased sensitivity in detecting small amounts of parasite, the use of the PCR technique may also clarify the natural history and epidemiology of *P. carinii* infection, including the important question of the asymptomatic carriage of the pathogen in healthy and HIV-infected patients.

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