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Detection of *Pneumocystis carinii* DNA in Sputum and Bronchoalveolar Lavage Samples by Polymerase Chain Reaction

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A polymerase chain reaction (PCR)-based assay was developed for the detection of *Pneumocystis carinii* DNA in induced sputum and bronchoscopic alveolar lavage samples. The primer pair was selected from the published sequence of the thymidylate synthase gene of *P. carinii* derived from infected rats. The amplified DNA fragment of 403 bp was detected by agarose gel electrophoresis and by Southern and slot blot hybridization. No positive reaction was seen with DNA from different microorganisms typically found in the respiratory tract. *P. carinii* DNA was demonstrated in 30 of 42 sputum samples from immunosuppressed patients, whereas 21 of 42 sputum samples were positive by indirect immunofluorescence (IFL). Among the 42 patients, 14 were receiving prophylactic chemotherapy. In that group, PCR detected *P. carinii* in nine sputum samples, whereas IFL detected *P. carinii* in only four sputum samples. A positive PCR result was also seen in 5 of 43 IFL-negative bronchoscopic alveolar lavage samples from patients with respiratory symptoms. The PCR assay detected 10 copies of the target DNA, which corresponds to 10^{-18} g of the specific *P. carinii* sequence. The results indicate that PCR amplification in combination with DNA hybridization is specific and is a more sensitive diagnostic method than IFL for the detection of *P. carinii*.

Pneumocystis carinii is a eucaryotic opportunistic pathogen which causes serious pneumonia (*P. carinii* pneumonia [PCP]) in immunosuppressed patients. PCP is the initial disease manifestation in 60% of patients with AIDS and is estimated to ultimately occur in up to 80% of patients with AIDS (1). The infection also occurs in patients receiving immunosuppressive therapy.

Laboratory diagnosis of PCP is dependent on microscopic demonstration of the organism by using conventional cytochemical (i.e., toluidine blue O, Grocott's methenamine silver, or Giemsa) staining. Increased specificity and sensitivity are achieved by immunocytochemical staining (immunofluorescence [IFL]) with monoclonal antibodies (3, 11). Improvement in sensitivity cannot be obtained by in vitro propagation (1). However, the development of the polymerase chain reaction (PCR) (12), a gene amplification procedure by which any specific DNA fragment can be exponentially accumulated in vitro, has made it possible to detect very small numbers of pathogens in clinical specimens. Wakefield et al. (16) have presented clinical applications of a PCR assay based on sequences coding for mitochondrial rRNA of *P. carinii* derived from infected rats. In both sputum (14) and bronchoalveolar lavage (BAL) samples (15), PCR offered a more sensitive technique than cytochemical staining (Grocott's methamine silver staining) for the diagnosis of PCP.

In the present study we established a diagnostic PCR assay based on the amplification of the thymidylate synthase (TS) gene of *P. carinii* derived from infected rats. The amplified sequence of 403 bp was identical to the sequence of the region previously cloned and sequenced by Edman et al. (2), who used degenerated oligonucleotide primers based on conserved regions of different TS amino acid sequences in order to isolate this region of the *P. carinii* genome.

In the present study, the PCR assay was tested on 42 sputum samples as well as 43 BAL samples, all of which were previously tested by IFL. The detection of *P. carinii* DNA in respiratory samples by PCR was compared with the results of detection by IFL and the clinical symptoms of the patients. The influence of chemoprophylaxis against *P. carinii* on the ability to detect the organism was also determined by comparing the results of IFL and PCR.

MATERIALS AND METHODS

Control organisms. *P. carinii* isolated from infected human lung tissue (autopsy material) was used for optimizing the PCR protocol and as an amplification control in all experiments. The organisms were prepared by an enzyme digestion method described previously (5) but that was modified by omitting incubation with DNase.

To determine the specificity of the assay, fungal and bacterial species (*Candida tropicalis*, *Candida glabrata*, *Candida albicans* serotype A, *C. albicans* serotype B, *Candida parapsilosis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Mycobacterium tuberculosis*) colonizing different parts of the human respiratory tract were used. The fungal species and *M. tuberculosis* were obtained as in vitro strains, whereas the human bacterial flora was collected from the upper respiratory tracts of 20 healthy subjects. The bacteria were isolated on blood agar, and the total DNA was extracted as a pool (see below).

Clinical samples. Sputum and BAL samples selected for analysis by PCR were obtained from 1987 to 1991 (Table 1) and were tested for the presence of *P. carinii* by IFL (see below). Most of the sputum samples were obtained from patients with respiratory symptoms, but six of the sputum samples were from asymptomatic human immunodeficiency

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TABLE 1. Clinical data for the material analyzed by PCR

Sample no.[a]	Underlying disorder[b]	Presence of respiratory symptoms
A1–A16	HIV	+
A17	HIV	−
A18	Polymyositis	+
A19	HGG	+
A20	CLL	+
A21	CLL	+
B1–B12	HIV	+
B13–B17	HIV	−
B18	AML	+
B19	CLL	+
B20	—	+
B21	Pancreas cancer	+
C1–C12	Renal-tx	+
C13–C18	Bone marrow-tx	+
C19	Liver-tx	+
C20	Pancreas-tx	+
C21	Lymphoma	+
C22–C23	Lung infiltrates	+
C24	RA	+
C25	Mb. Bechterew	+
C26–C43	—	+

[a] A, sputum samples positive for *P. carinii* by the IFL method; B, sputum samples negative for *P. carinii* by the IFL method; C, BAL samples negative for *P. carinii* by the IFL method.

[b] HGG, hypogammaglobulinaemia; CLL, chronic lymphatic leukemia; AML, acute myeloic leukaemia; —, samples from patients without known immunosuppression; tx, transplantation; RA, rheumatoid arthritis, Mb. Bechterew, morbus Bechterew.

virus (HIV)-infected patients (participating in a study on pentamidine prophylaxis against *P. carinii*). Thirty-four sputum samples were acquired from patients with HIV infection (samples A1 to A17 and B1 to B17 in Table 1) and eight were from non-HIV-infected patients (samples A18 to A21 and B18 to B21 in Table 1).

Five patients had received treatment for PCP between 2 and 14 days before the sampling day (see Table 4). Four of the patients were infected with HIV, and the fifth patient suffered from chronic lymphatic leukemia. Six HIV-infected patients received primary prophylactic nebulized pentamidine against *P. carinii* (no previous episode of PCP), and eight patients received the same drug as secondary prophylaxis (previous episode of PCP).

The BAL samples were from non-HIV-infected patients with or without immunosuppression (samples C1 to C43 in Table 1). The PCR products of the clinical samples were analyzed by agarose gel electrophoresis (AGE) and then by slot blot hybridization (see below). Some PCR products from clinical samples were also analyzed by Southern blotting.

Sample preparation. All sputum and BAL samples containing mucous material were liquefied with 6.3 mM dithiothreitol for at least 5 min at room temperature. Samples were then treated with a 25% final concentration of ethanol for inactivation of HIV, pelleted by centrifugation at 2,000 × *g* for 5 min, and stored at 4°C. Prior to analysis by PCR, the samples were washed once with 0.14 M phosphate-buffered saline (pH 7.4), centrifuged at 2,000 × *g* for 5 min, and resuspended in the same buffer. A volume of 100 μl was used for DNA isolation. In order to break the cyst wall of the parasite, the samples were treated with the following protocol, which was based on one described by Holm et al. (6), with some modifications: 0.25 mg of lyticase (Sigma, St. Louis, Mo.) per ml for 60 min at 37°C in the presence of 1 M sucrose, 0.1 M sodium citrate, and 1 mM Na_2EDTA (pH 8). After centrifugation at 3,000 × *g* for 5 min, the cell pellets were resuspended in a lysis buffer containing 100 mM Tris-HCl (pH 8), 75 mM NaCl, and 25 mM EDTA (pH 8), freeze-thawed once, and digested with 200 μg of fresh proteinase K per ml in 0.5% sodium dodecyl sulfate (SDS) for 60 min at 55°C. The samples were then extracted with saturated phenol by the following triple procedure: extraction with an equal volume of phenol, then with a 25:24:1 mixture of phenol-chloroform-isoamyl alcohol, and finally with a 24:1 mixture of chloroform-isoamyl alcohol. The DNA in the final water phase was precipitated with 2 volumes of ethanol and was resuspended in TE buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA). Five micrograms of tRNA per ml was added as a carrier in the extraction and precipitation procedure. DNAs from the *P. carinii*-infected human lung tissue and the fungal cultures were also isolated by this protocol. The DNA extraction from human pooled bacteria on blood agar and *M. tuberculosis* strain culture was preceded with lysozyme and pronase digestion as described previously (4).

CTT GAC TCA ATT GGA TTG ACT AAA CGC CAG 360
GAA GGT GAT CTT GGT CCC ATT TAT GGG TTT 390 PC 1
CAA TGG AGG CAT TTT GGA GCA GAA TAT ATT 420
GAT TGC AAA ACC AAT TAT ATT GGA CAA GGT 450
GTT GAT CAA TTA GCC AAT ATT ATC CAA AAA 480
ATA CGA ACA TCA CCA TAT GAT CGT CGA CTC 510
ATA **CTT TCA GCA TGG AAT CCT GCA G g c a t a** 540 probe
c t c t t t t a t c a a t t c a a t a t c a t t g c t t a t 570
t a c t a t t t a g AT TTA GAA AAA ATG GCA TTA 600
CCT CCG TGC CAT ATG TTT TG **g t a a t a t t c a** 630
t a t t t a a c c t a t a t a t g a a a c c a t a a a g c t 660
t a c a a c c a c a t a t a g T CAG TTT TAT GTT CA 690
T ATA CCA TCG AAT AAC CAC CGA CCT GAA CT 720
C TCA TGT CAA TTA TAC CAA CGT TCT TGT GA 750
C ATG GGG CTA GGT GTT CCC TTT AAT ATT GC 780 PC 2
A TCG TAT GCT CTA CTA ACA TGC ATG ATT GC 810

FIG. 1. A part of the coding sequence of the TS gene of *P. carinii* (1). Intervening sequences are presented as lowercase letters; coding sequences are in capital letters. The locations of the oligonucleotide primer pair PC1 and PC2 are indicated by continuous underlining. The intermediate oligonucleotide (boldface) was internal to the amplified region and served as a confirmatory probe.

Detection of *P. carinii* by IFL. IFL was performed with a mouse monoclonal antibody to *P. carinii* (9) and fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin as described by Elvin et al. (3).

Amplification. The two oligonucleotide primers PC1 and PC2 as well as the internal probe (Fig. 1) were synthesized by using a Gene Assembler (Pharmacia, Uppsala, Sweden) and were purified according to the manufacturer's protocol. Primer PC1 corresponds to nucleotides 379 to 396 and primer PC2 corresponds to nucleotides 763 to 781 in the published sequence of the TS gene of *P. carinii* derived from infected rats (2). The amplification product consisted of a 403-bp DNA fragment, including two intervening sequences of 45 and 55 bp, respectively (Fig. 1).

The amplification reactions were performed as described by Saiki et al. (12). Briefly, 2.5 U of *Taq* polymerase (Cetus Corp., Emeryville, Calif.) was used in a 50-μl reaction volume with 3.0 mM $MgCl_2$; each primer was used at 0.4 μM, and 2 μl of sample DNA was used. The reaction mixtures were amplified for 40 cycles in an automated PCR

thermal cycler (Hybaid Ltd., Middlesex, United Kingdom). All amplification experiments started with a denaturation step of 5 min at 94°C. Each cycle consisted of 30 s of denaturation at 94°C, 25 s at the annealing temperature of 50°C, and 1 min of extension at 70°C. The final extension step continued for an additional 5 min.

All experiments were performed in parallel with *P. carinii* DNA extracted from the human lung homogenate as a positive control and with water as a negative control. To avoid contamination, the DNA preparation of the samples, the addition of PCR reagents, and the detection procedures were performed in physically separated areas with different sets of micropipettes. Ten microliters of the PCR product was analyzed for the presence of the 403-bp PCR product by AGE on 3% Nusieve–1% agarose (FMC Bioproducts, Rockland, Maine).

Detection level. Because the DNA preparation from the human *P. carinii*-infected lung homogenate contained both human and *P. carinii* DNAs, we estimated the detection level of the PCR assay by using the 403-bp PCR fragment as a target for reamplification. The DNA concentration was determined by measuring the optical density at 260 nm, and the fragment was diluted 10-fold in 25 μg of human DNA per ml. The human DNA was extracted from clinical sputum samples, which were negative for *P. carinii* by IFL and PCR. This was done in order to mimic the biological conditions in clinical sputum and BAL samples. The reamplified PCR product was then detected by AGE and slot blot hybridization (see above and below).

Analysis of PCR products by hybridization. PCR products (10 μl) were either transferred from agarose gels to nitrocellulose membranes (Schleicher & Schuell, GmbH, Dassel, Germany) by vacuum blotting (Hybaid) or heat denatured and applied to Hybond N membranes (Amersham, Bucks, United Kingdom) with a slot blot apparatus (Millipore, Bedford, Mass.). Both types of membranes were baked for 2 h at 80°C under vacuum and were hybridized to a ^{32}P-labeled probe (Fig. 1, positions 514 to 541) with a specific activity of at least 10^8 cpm/μg of DNA. The hybridization conditions were as follows: 43°C for 2 h in 6× SSC (0.9 M NaCl plus 0.09 M sodium citrate) containing 3× Denhardt solution (0.06% Ficoll 400, 0.06% polyvinylpyrrolidone, 0.06% bovine serum albumin fraction V), 0.5% SDS, 0.1% tetrasodium pyrophosphate decahydrate, and 0.2 mg of sonicated salmon sperm DNA (Sigma) per ml. Filters were washed three times for 5 min each time with 6× SSC–0.1% SDS–0.1% tetrasodium pyrophosphate decahydrate at 20°C and then three times for 5 min each time in the same solution at 43°C; thereafter, the filters were exposed to RPN-6 film (Amersham) at −70°C.

RESULTS

Specificity. We applied the PCR technique by using the primer pair PC1 and PC2 for amplification of DNA from *P. carinii*-infected human lung tissue and clinical respiratory samples. The size of the amplified product as analyzed by AGE (Fig. 2) was 403 bp, as predicted from the TS gene sequence (Fig. 1). When the primer pair was tested for specificity on DNA preparations from 10 different microorganisms, no signs of amplified DNA could be seen in the agarose gels (Fig. 2). Moreover, the *P. carinii* probe (Fig. 1) used to confirm the presence of *P. carinii* was hybridized to the microbial DNA preparations, and no positive reaction could be seen by autoradiography after Southern blot analysis (Fig. 2).

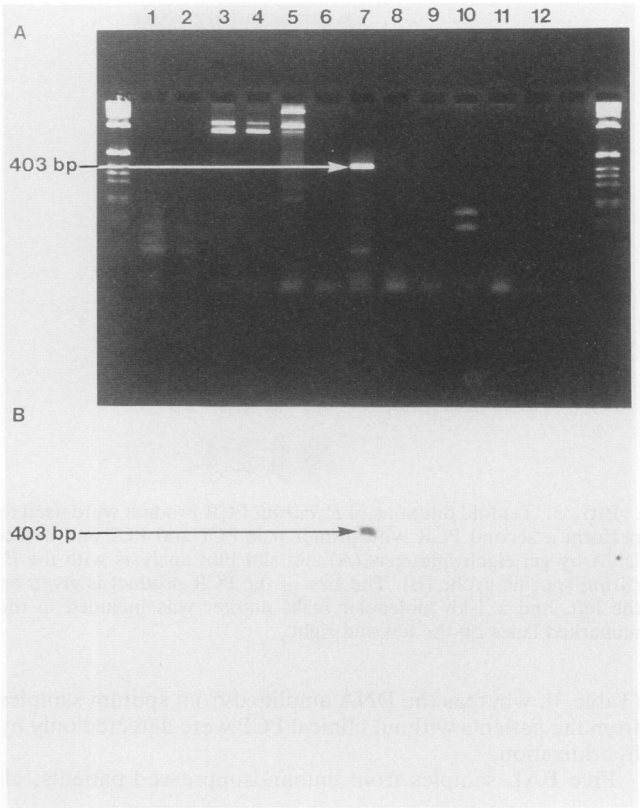

FIG. 2. (A) Gel electrophoresis of amplified negative control DNA by using primer pair PC1 and PC2. The size of the PCR product is shown on the left. Unmarked lanes to the left and right, 1-kb molecular mass markers; lane 1, *C. tropicalis*; lane 2, *C. glabrata*; lane 3, *C. albicans* serotype A; lane 4, *C. albicans* serotype B; lane 5, *C. parapsilosis*; lane 6, *C. neoformans*; lane 7, positive control (*P. carinii* DNA from autopsy material); lane 8, negative control (water); lane 9, *A. fumigatus*; lane 10, human bacterial throat flora; lane 11, *M. tuberculosis*; lane 12, human DNA. (B) Southern blot of the gel in panel A by using the *P. carinii*-specific probe.

Detection level. We were able to detect 10 copies of the target DNA, or 10^{-18} g of *P. carinii*-specific DNA, in a sample volume of 10 μl by slot blot hybridization (Fig. 3). The PCR detection level for AGE was 10 times lower (10^{-17} g).

Detection of the 403-bp sequence in clinical samples. The results of the PCR assay detected by AGE and DNA hybridization (Fig. 4) for all clinical material tested retrospectively in comparison with the results of IFL are given in Table 2. Among the 34 sputum samples from HIV-infected patients, we were able to amplify *P. carinii* DNA from 25 of the samples, of which 19 were detected by AGE. Amplified *P. carinii* DNA was also detected in five of eight sputum samples from non-HIV-infected patients. All 21 IFL-positive sputum samples were positive by PCR: 20 by AGE and 21 by DNA hybridization. Among the 21 IFL-negative sputum samples, PCR detected *P. carinii* DNA in 9 samples: 4 by AGE and 9 by hybridization. The nine sputum samples were from eight HIV-infected patients and one patient with chronic lymphatic leukemia (Table 1). The sputum samples with *P. carinii* DNA detected by AGE were from patients with confirmed PCP (by IFL) or clinically suspected PCP

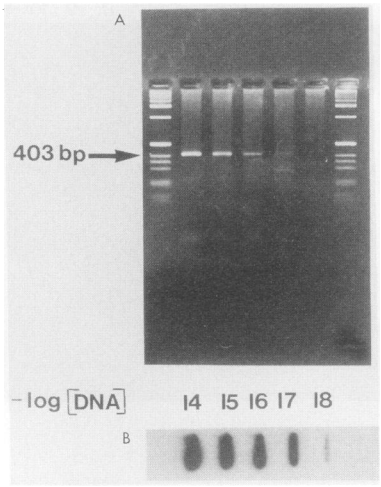

FIG. 3. Tenfold dilutions of *P. carinii* PCR product were used to perform a second PCR with primer pair PC1 and PC2 and detect DNA by gel electrophoresis (A) and slot blot analysis with the *P. carinii*-specific probe (B). The size of the PCR product is given on the left, and a 1-kb molecular mass marker was included in the unmarked lanes on the left and right.

(Table 3), whereas the DNA amplified from sputum samples from the patients without clinical PCP were detected only by hybridization.

Five BAL samples from immunosuppressed patients, all of which were negative by IFL, were positive by PCR; DNA in one of the BAL samples was detected by AGE, and DNA in all five BAL samples was detected by DNA hybridization. These patients were diagnosed with pneumonia caused by *Aspergillus* sp., *Haemophilus influenzae*, *Escherichia coli*, herpes simplex virus type 1, and cytomegalovirus. The amplification results for these patients could not be verified by IFL of previous or additional samples from the same patients. No *P. carinii* DNA was detected in BAL samples from patients without immunosuppression.

When comparing the influence of treatment or anti-*P. carinii* chemoprophylaxis on *P. carinii* detection on the two methods, we found that all sputum samples from patients receiving full treatment were positive by both IFL and PCR (Table 4). PCR was positive by hybridization for 9 of 14 sputum samples from HIV-infected patients receiving prophylaxis, whereas 4 of 14 sputum samples were positive by IFL. In the untreated group, PCR was positive by hybridization for 16 of 23 sputum samples, whereas 12 of 23 sputum samples were positive by IFL.

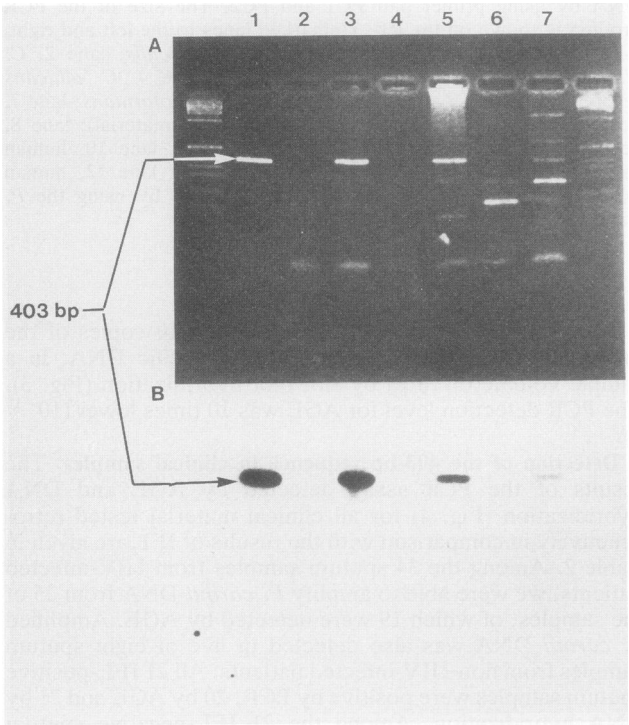

FIG. 4. (A) AGE of clinical sputum samples subjected to PCR. Lane 1, positive control (*P. carinii* DNA from autopsy material); lane 2, negative control (water); lanes 3 and 5, amplified DNA from IFL-positive sputum samples; lanes 4, 6, and 7, amplified DNA from IFL-negative sputum samples; lanes 3, 5, and 7, PCR-positive sputum samples; lanes 4 and 6, PCR-negative sputum samples. (B) Southern blot of the gel in panel A by using the *P. carinii*-specific probe.

TABLE 2. Comparison between PCR (detection by AGE and DNA hybridization) and IFL methods for detection of *P. carinii* in sputum and BAL samples from patients in different categories

Sample	Patient category (no. of patients)	No. (%) positive by the following *P. carinii* detection method:		
		PCR-AGE	PCR-hybridization[a]	IFL
Sputum	HIV (34)	19[b] (56)	25[c] (74)	17 (50)
	Non-HIV (8)	5 (63)	5 (63)	4 (50)
BAL	Immunosuppressed (25)[d]	1 (4)	5 (20)	0
	Immunocompetent (18)[d]	0	0	0

[a] Hybridization by the *P. carinii*-specific probe and detected by autoradiography.
[b] In all, there were 16 IFL-positive and 3 IFL-negative sputum samples.
[c] In all, there were 17 IFL-positive and 8 IFL-negative sputum samples.
[d] Non-HIV infected.

DISCUSSION

The results indicate that PCR for detection of *P. carinii* DNA in clinical respiratory samples is more sensitive than IFL. *P. carinii* DNA was detected in nine sputum and five BAL samples which were *P. carinii* negative by IFL. The increased sensitivity was consistent with the calculated

TABLE 3. Clinical data for patients with PCR-positive and IFL-negative *P. carinii* sputum samples

Patient category (n = 9)	No. of patients	
	AGE	DNA hybridization[a]
Confirmed PCP	2[b]	2[c]
Suspect or possible PCP[d]	2	1
No verified PCP	0	2[e]

[a] Hybridization by the *P. carinii*-specific probe and detected by autoradiography.
[b] Confirmed PCP by IFL after 6 days and 3 months, respectively.
[c] Confirmed PCP by IFL after 5 months and clinical PCP 6 months earlier, respectively.
[d] Three patients with short follow-up times (3, 6, and 7 weeks, respectively) before death. Autopsy was negative or incompletely performed.
[e] Two patients without clinical PCP with 4 and 19 months of follow-up time, respectively.

TABLE 4. PCR results for sputum samples in relation to prophylaxis and patient treatment

Patient group (no. of patients)	No. (%) of samples positive by the following *P. carinii* detection method:		
	PCR-AGE	PCR-hybridization[a]	IFL
Untreated (23)	14 (61)	16 (70)	12 (52)
Primary prophylaxis (6)[b]	1 (17)	3 (50)	1 (17)
Secondary prophylaxis (8)[c]	4 (50)	6 (75)	3 (38)
Full treatment (5)[d]	5 (100)	5 (100)	5 (100)

[a] Hybridization by the *P. carinii*-specific probe and detected by autoradiography.
[b] Patients received nebulized pentamidine (300 mg every 2 to 4 weeks), but one exception was a patient who received trimethoprim prophylaxis in 1987.
[c] Patients received nebulized pentamidine (300 mg every 2 to 4 weeks), but one exception was a patient who received pyrimethamine prophylaxis in 1987.
[d] Patients received high doses of trimethoprim-sulfamethoxazole.

detection limit of the PCR assay: 10 trophozoites or approximately 1 to 2 mature cysts of *P. carinii*, assuming that the TS gene is a single-copy gene.

The TS enzyme is present in many different organisms such as animal viruses, bacteria, fungi, and mammals (2). It is therefore likely that the corresponding gene is conserved in various isolates of *P. carinii*, but it may also be similar to sequences in other organisms. The specificity of the intervening sequences of the rat *P. carinii* TS gene (2) made it possible to design a confirmative oligonucleotide probe specific only to the PCR product of *P. carinii*. No false-positive PCR product visible as a band in AGE could be detected after amplification of DNA from fungal and bacterial organisms by using primer pair PC1 and PC2 (Fig. 1). The specificity of the probe is indicated by the positive reaction with amplified *P. carinii* DNA and the negative reactions with DNAs from the control microorganisms when analyzed by the Southern blotting technique (Fig. 2).

Since diagnostic confirmation of *P. carinii* infection cannot be achieved by in vitro propagation, IFL serves as the reference method for detection of the organism. The nine PCR-positive sputum samples which were IFL negative are likely to be true positives because only two samples were from patients without verified PCP (Table 3). Amplified DNA in these latter two sputum samples was detected by weak signals by DNA hybridization, whereas the hybridization signal was weak in only one of the four sputum samples from patients with verified PCP (data not shown).

Although there was a limited amount of sputum samples that could be analyzed and two patients received a prophylactic regimen (trimethoprim and pyrimethamine, respectively, which are no longer considered adequate prophylaxis; see Table 4), the PCR assay seems to be even more advantageous than IFL for detection of *P. carinii* in sputum samples from HIV-infected patients receiving prophylactic treatment against *P. carinii*. Detection rates were similar by the two methods for sputum samples from treated and untreated patients. Our results are consistent with the indication that induced sputum samples examined for *P. carinii* by IFL have a lower diagnostic yield for HIV-infected patients who have received previous aerosolized pentamidine prophylaxis than for patients without anti-*P. carinii* chemoprophylaxis (7, 8).

By the PCR assay described here, we also detected *P. carinii* DNA in 5 (C1 to C2, C13, C21, and C24; Table 1) of 43 BAL samples, all of which were *P. carinii* negative by IFL. The five BAL samples derived from patients with immunosuppressive disorders were also infected with other opportunistic lung pathogens. The clinical diagnosis for the patients with PCR-positive BAL samples suggests that a concomitant *P. carinii* infection is possible but not of apparent clinical importance.

The increased sensitivity of detection of *P. carinii* DNA by PCR based on primer sequences from the TS gene is consistent with the previous DNA amplification results obtained by Wakefield et al. (14, 15). However, they compared the PCR method with silver staining, which is less sensitive than our reference method (IFL) for detection of *P. carinii* (3, 11). We conclude that the application of PCR described here is specific and more sensitive than IFL for detection of *P. carinii*.

In order for *P. carinii* PCR to become a generally used assay in clinical laboratories, the DNA extraction, detection, and confirmation procedures in the current protocol need to be simplified. We are testing new DNA extraction methods, but so far none have shown results as good as those obtained by standard phenol extraction. For confirmation of the specificity of an amplified DNA fragment, restriction analysis is a simple and less costly alternative to time-consuming DNA hybridization. Another approach, which is easy to perform and standardize, is the reversed dot blot technique (13), in which the PCR product is nonisotopically labeled and hybridized to an immobilized probe.

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