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Simplified Sample Processing Combined with a Sensitive One-Tube Nested PCR Assay for Detection of *Pneumocystis carinii* in Respiratory Specimens

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Early diagnosis of *Pneumocystis carinii* pneumonia, a life-threatening complication in immunosuppressed patients, may lower morbidity and mortality. We have developed a one-tube nested PCR assay for the detection of *P. carinii* in respiratory specimens. Four primers were selected from the sequence of the small-subunit rRNA gene of *P. carinii* to amplify a 265-bp fragment, and their specificities for *P. carinii* were confirmed by both theoretical evaluations (by computer-assisted comparison with the sequences in GenBank) and empirical evaluations (with DNA from medically important fungi and diagnostic samples). The assay was optimized for routine diagnostic use. Processing of the clinical samples is rapid and simple (digestion with proteinase K directly in PCR buffer at room temperature in the presence of 10% Chelex 100 and no further purification steps). Bovine serum albumin (1 mg/ml) and glycerol (10%) in the amplification buffer reduced the number of samples inhibitory to the PCR, as assessed by control reactions containing a size-modified target. A total of 749 clinical specimens (312 bronchoalveolar lavage, 403 sputum or induced sputum, and 34 other specimens) from 507 patients (295 human immunodeficiency virus [HIV]-infected and 164 non-HIV-infected patients and 48 patients whose HIV status was unknown) were tested by PCR, and the results were compared with those of an indirect immunofluorescence assay (IFA). Concordant results were obtained for 732 samples (646 negative and 86 positive). There were 17 discrepant results: 12 were PCR positive and IFA negative, and 5 were PCR negative and IFA positive. After resolution of the discrepant results by review of the patients' clinical data, the sensitivity and specificity were 94.8 and 99.1%, respectively, for PCR and 93.8 and 100%, respectively, for IFA. In conclusion, the short procedure time and the technical ease of this PCR assay render it suitable for implementation in routine diagnostic laboratories.

The prognosis for human immunodeficiency virus (HIV)-infected patients is significantly influenced by pulmonary infectious complications, especially *Pneumocystis carinii* pneumonia (PCP) (15, 18). Even though the incidence of PCP has substantially decreased due to chemoprophylaxis, it remains the most frequent AIDS-defining opportunistic infection in Western industrialized countries (9, 12). Early diagnosis of PCP reduces mortality from this complication considerably (19). Routine diagnosis is achieved by demonstrating the parasite microscopically in stained smears, but the sensitivity is heavily dependent on the experience of the center and especially on the staining technique that is applied. The immunofluorescence assay (IFA) has a very good sensitivity and specificity, particularly with bronchoalveolar lavage (BAL) samples and when using a monoclonal antibody that detects both the cyst and the trophozoite stages (3, 21, 22). Performance of staining techniques is less sensitive with sputa and induced sputa, with the IFA reaching a relative sensitivity of detection of *P. carinii* in sputum samples compared to that in BAL samples of 70% (3). The intrinsic sensitivity of DNA amplification by PCR has led several groups to develop protocols for its use to detect *P. carinii*, with good results (see reference 8 for a comparison of six PCR methods). In particular, nested PCR with a second round of amplification in a second tube was shown to be highly sensitive (4, 8). Routine performance of PCR, however, is still hampered by the fact that many protocols are very laborious, particularly when using sputa, they do not include controls in each sample to monitor the occurrence of amplification inhibition, or they need additional hybridization steps to increase the sensitivity and to confirm the specificity. Hence, improvements of the tests are necessary before they can be implemented in routine diagnostic laboratories.

In this study, special emphasis was given to the development of a robust, fast, and simple method of extracting DNA from respiratory materials as well as the optimization of the DNA amplification reaction in order to minimize the effects of inhibitory substances that might be encountered in clinical samples. A new set of PCR primers was used for improved one-tube nested reactions, obviating the need for a contamination-prone step of transferring aliquots of a first round of amplification into other tubes. The results of PCR and IFA with clinical samples were compared in a blinded prospective epidemiological study. Furthermore, because the genetic diversity of *P. carinii* isolates infecting humans has so far been shown to occur at two loci (7, 23), single-stranded conformational polymorphism (SSCP) analysis of the diagnostic PCR amplicon was used to assess whether this fragment is useful as an additional genetic marker for tracking *P. carinii* strains.

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MATERIALS AND METHODS

Specimens. Respiratory specimens were obtained from 507 patients who were evaluated for clinically suspect pulmonary infections admitted to the University Hospital of Zurich between June 1994 and February 1996. A total of 295 of the patients had HIV infections, 43 had undergone organ transplantation, 30 pa-

tients had hematologic malignancies, and 14 patients had received chemotherapy or corticosteroids for other reasons. BAL was performed by fiberoptic bronchoscopy in a standard fashion. Sputum was collected either by spontaneous expectoration or after induction by inhalation of hypertonic saline solution.

Processing of samples for indirect immunofluorescence. Sputum samples (0.5 to 3 ml) were liquefied at 37°C for 5 to 10 min with 7 ml of a solution containing 20 mM dithiothreitol and 20 mM EDTA and centrifuged (2,000 × *g*, 5 min). The sediment was resuspended in 30 μl of 0.25% trypsin in phosphate-buffered saline (PBS), and after 5 min at room temperature, the suspension was diluted with 12 ml of PBS. After centrifugation (2,000 × *g*, 5 min) smears were prepared from the sediment.

BAL samples (500 μl) were mixed with 50 μl of 0.25% trypsin in PBS and were incubated at room temperature for 5 min. Cytocentrifuge preparations of trypsinized BAL fluids (100 to 500 μl per slide) were made by using a Cytospin 2 cytocentrifuge (Shandon).

Indirect immunofluorescence with acetone-fixed smears was performed by using monoclonal mouse anti-*P. carinii* antibodies (clone 3F6; DAKO AS, Glostrup, Denmark) according to the instructions provided by the manufacturer.

Processing of samples for DNA amplification. Aliquots of liquefied clinical samples (1.5 ml of BAL sample and 50 to 500 μl of other specimens) were centrifuged (1,500 × *g*, 5 min) and washed once with TE buffer (Tris-HCl, 10 mM; EDTA, 1 mM [pH 8.0]), and the pellets were stored at −20°C. The thawed pellets were dissolved in 100 μl of PCR buffer (final concentrations, 50 mM KCl, 20 mM Tris-HCl [pH 8.4], 4 mM $MgCl_2$, 5% Tween 20) and digested with proteinase K (100 μg) at room temperature for 45 min by rotating in the presence of 10% (wt/vol) Chelex (100 to 200 mesh; Bio-Rad). After heat inactivation of the proteinase K (94°C, 10 min), 10 μl was used for DNA amplification.

Fungal DNA from in vitro-cultured and lyophilized *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, a *Penicillium* sp., a *Rhizopus* sp., *Saccharomyces cerevisiae* was obtained as described above.

Oligonucleotide primers. Four primers were selected from the sequence of the small-subunit rRNA gene of rat *P. carinii* (2) (GenBank accession no. X12708) for a one-tube nested PCR. The outer primers PC1 (5′-CCAGATTAGCTTTT GCTGATCGCGGG-3′, corresponding to positions 1359 to 1384) and PC2 (5′-TTTACTTCCTCTAAATGACCAAATTTGATC-3′; complementary to positions 1739 to 1768) amplify a 409-bp fragment; the inner primers PC5 (5′-CAG AGCCAGCAAGTTCATTTC-3′, corresponding to positions 1485 to 1506, but with only 3 instead of 4 thymidine residues proximal to the 3′ end, according to our own sequencing results) and PC6 (5′-CCAAATTTGATCAACTTTCCAG-3′, complementary to positions 1729 to 1750 and hence overlapping the outer primer PC2 by 12 residues at the 5′ end) give rise to a 265-bp product.

DNA amplification. DNA amplifications by PCR were performed with a PTC 200 DNA engine (MJ Research, Watertown, Mass.). Reaction conditions were optimized regarding the concentrations of both primer pairs (from 1 nM to 1 μM) and magnesium (from 1 to 4 mM), the numbers of cycles in both rounds of amplification, and the annealing temperature. Finally, the 100-μl reaction mixtures contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 4 mM $MgCl_2$, 5% Tween 20, 200 μM (each) dATP, dCTP, dGTP, and dUTP, 10% glycerol, 100 μg of bovine serum albumin (BSA; Pharmacia), and 1 U of uracil-*N*-glycosylase (UNG; Life Technologies). Optimal primer concentrations were 2 nM for outer primers PC1 and PC2 and 1 μM for inner primers PC5 and PC6. A total of 2.5 U of *Taq* polymerase (Life Technologies) was added after 10-min incubation steps at 37 and 94°C (to inactivate the UNG). Twenty cycles of 30 s at 94°C and 60 s at 72°C were performed, followed by 30 cycles of 30 s each at 94, 55, and 72°C. The ramp rate from 94°C to the annealing temperature was set to 0.5°C/s.

In order to detect inhibition of the amplification reactions, all specimens were tested in duplicate, with the second reaction being spiked with 10^6 copies of the plasmid bearing the control target. This control target, constructed analogously to the procedure described earlier (10), yields an amplicon of about 450 bp upon amplification with the inner primer pair and is easily discriminated from the *P. carinii*-specific product of 265 bp after gel electrophoresis. A negative control to which no DNA was added was included in all tests.

In addition to our newly developed PCR detection technique, a second *P. carinii*-specific PCR previously described by others was used (24).

Carryover problems of amplicons were prevented not only by use of the dUTP-UNG system but also by using aerosol-guarded pipette tips and by strictly separating the areas of DNA isolation, PCR, and amplicon analysis.

Amplicon production was determined by electrophoresis of 20 μl of the reaction mixtures in ethidium bromide-stained agarose gels (2%).

SSCP analysis. A total of 8 μl of the amplification product was supplemented with 1 μl of NaCl (250 mM), and the mixture was heated to 96°C for 5 min and quenched on ice. A total of 1 μl of loading buffer (30% glycerol in water and 0.25% [each] bromophenol blue and xylene cyanol) was added, and the DNA strands were separated by electrophoresis in 12% polyNat gels (Guest Elchrom Scientific, Cham, Switzerland) at 8 V/cm in the SEA 2000 electrophoresis apparatus (Guest Elchrom Scientific) for 6 h, after which the temperature had risen from 20 to 28°C in the electrophoresis unit, which was cooled with tap water. Single-stranded DNA was visualized by staining with SYBR Green II (FMC, Rockland, Maine) as recommended by the supplier.

Clinical data. PCP was diagnosed if *P. carinii* was detected by IFA staining of sputum or BAL specimens. Patients with IFA-negative sputa and clinically suspected of having PCP underwent bronchoscopical examination if it was clinically indicated and/or were followed at our HIV Clinic or Pulmonary Division. If the only sample available was a sputum sample with a negative IFA result, patients were judged not to have PCP if they did not develop documented PCP or a respiratory illness consistent with PCP. For those patients for whom discrepant results were obtained by IFA and one-tube nested PCR, clinical data were evaluated, including the patients' histories, signs, symptoms, chest X rays, laboratory results, and follow-up observations, as well as the results of studies obtained with additional samples from the patients.

RESULTS

Sample preparation. Using purified *P. carinii* from rats (kind gift of A. Kiderlen, Robert Koch Institut, Berlin, Germany), we evaluated the influence of temperature and duration of incubation on the lysing efficiency of proteinase K in the presence or absence of sodium dodecyl sulfate (SDS). We found that treatment in an SDS-free but Chelex 100-containing buffer for at least 45 min at room temperature was sufficient to obtain positive PCR results. After heat inactivation of the proteinase K, 1/10 (10 μl) of the samples was directly used for PCR as a standard protocol.

DNA amplification. Four primers were selected. The sequences of these primers were deduced from the known sequence of the 18S rRNA gene for use in a one-tube nested PCR. Initially, all primer sequences were compared to GenBank sequences by using FASTA (Wisconsin package) to ensure their uniqueness. Furthermore, the specificities of the primers for *P. carinii* were confirmed by obtaining negative results with DNAs from seven fungal species (*C. albicans*, *A. fumigatus*, *C. neoformans*, *H. capsulatum*, a *Penicillium* sp., a *Rhizopus* sp., and *S. cerevisiae*) from which, in order to prove that amplifiable DNA was indeed extracted, part of the chitin synthase gene was successfully amplified as described previously (11).

The sensitivity of the optimized one-tube nested PCR was tested with serial dilutions of a highly positive BAL specimen and with the cloned control target sequence. Hence, we could detect three cysts (as detected by IFA; trophozoites were not counted) or 100 copies of the control target, respectively. Amplification with only the inner primer pair resulted in a 100-fold reduced sensitivity corresponding to the sensitivity of a single-step PCR with primers derived from the mitochondria RNA gene (24).

Epidemiological evaluation of the PCR detection method. A total of 749 clinical specimens (BAL, sputum, induced sputum, tracheal fluid, bronchial fluid, and pleural effusion specimens) from 507 patients (295 HIV-infected and 164 non-HIV-infected patients and 48 patients whose HIV status was unknown) were tested by PCR and IFA (Table 1). Concordant results were obtained for 732 specimens (646 negative and 86 positive). There were 17 discrepant results: 12 were PCR positive and IFA negative and 5 were PCR negative and IFA positive. Of these five specimens with false-negative PCR results, two specimens had very low pathogen burdens (Table 2). In one BAL specimen and one sputum specimen, a single cyst was detected by IFA. Furthermore, only trophozoites were found in one sample with a false-negative result by PCR. On this basis, the sensitivity of the one-tube nested PCR was 94.5%, its specificity was 98.2%, its positive predictive value was 87.8%, and its negative predictive value was 99.2% (Table 1).

On the basis of clinical data, the discrepant results were interpreted as described below.

(i) IFA negative and PCR positive; probably true positive. Of the 12 IFA-negative, PCR-positive specimens, 6 (from five patients) were considered to be true positive when the clinical data were considered. In two patients, *P. carinii* was detected in

TABLE 1. Detection of *P. carinii* in respiratory specimens from different patient groups by PCR and IFA[a]

Specimen	HIV status of patients	No. of specimens[b]				Sensitivity (%)[c]	Specificity (%)[c]
		PCR^-, IFA^-	PCR^+, IFA^-	PCR^-, IFA^+	PCR^+, IFA^+		
All	All patients	646	12	5	86	94.5	98.2
All	HIV positive	378	9	4	72	94.7	97.7
	HIV negative	220	3	1	14	93.3	98.7
	Unknown	48	0	0	0	NA[d]	100
BAL	HIV positive	72	3	0	26	100	96.0
	HIV negative	169	3	1	13	92.9	98.3
	Unknown	25	0	0	0	NA	100
Sputum	HIV positive	297	6	4	44	91.7	98.0
	HIV negative	35	0	0	0	NA	100
	Unknown	17	0	0	0	NA	100
Other[e]	HIV positive	9	0	0	2	100	100
	HIV negative	16	0	0	1	100	100
	Unknown	6	0	0	0	NA	100

[a] For test results and diagnostic values of PCR (before resolution of discrepant results), differences were not significant in the McNemar test either for specimen types or for patient groups.
[b] PCR^-, PCR negative; IFA^-, IFA negative; PCR^+, PCR positive; IFA^+, IFA positive
[c] One-tube nested PCR compared with IFA.
[d] NA, not applicable.
[e] Tracheal fluid ($n = 14$), bronchial fluid ($n = 16$), and pleural effusions ($n = 4$).

previous specimens by IFA (PCR was not done because no aliquots were obtained due to very small sample volumes); in one patient a subsequent BAL specimen was positive by IFA and PCR; two samples from one patient collected 2 days apart were positive by PCR only; the fifth patient died 2 months later due to respiratory failure probably due to PCP. Three of these positive results could be confirmed by the second single-step PCR assay.

(ii) IFA negative and PCR positive; probably false positive. Six of the 12 IFA-negative, PCR-positive specimens were considered false positive by the clinicians because the patients improved without anti-*P. carinii* therapy and did not develop PCP during the following 3-month observation period. Interestingly, two of these six false-positive specimens were also positive by the second PCR assay. Thus, either these two specimens were contaminated with *P. carinii* organisms during handling or the patients had subclinical infections.

The amplicons of all 12 specimens with a negative IFA result were indistinguishable in an SSCP analysis with the PCR product from a human clinical sample proven to be infected with *P. carinii* and clearly differed from that from a rat *P. carinii*-infected sample (see Fig. 1 for different patterns of rat and human *P. carinii* isolates).

(iii) IFA positive and PCR negative. All samples false negative by PCR remained negative after additional incubation with proteinase K at 56°C for 2 h (in order to possibly get a more complete lysis of *P. carinii* cells) as well as when tested by the second PCR. Thus, these results probably represent sampling bias of samples with low pathogen numbers.

The performance results for both PCR and IFA are presented in Table 3. The adjusted sensitivity and specificity were 94.8 and 99.1%, respectively, for PCR and 93.8 and 100%, respectively, for IFA. The overall prevalence for *P. carinii* was 15.4%, reaching 21.4% in HIV-positive patients (9.2% in HIV-negative patients).

PCR inhibition. PCR of a first series of 191 samples was performed without BSA and glycerol in the reaction buffer. Strong inhibition of the PCR, as evidenced by the abolished amplification of the control target in the parallel reactions, was observed for 23% of these samples (for 36% of the 102 sputum, 5% of the 83 BAL, and 50% of the 6 other specimens). After adding BSA and glycerol, the corresponding proportions decreased to 8% for all 749 samples tested (13% in sputum, 0% in BAL, and 23% in other specimens). No inhibition was observed after retesting all the inhibitory samples at a 1:20 dilution.

TABLE 2. Quantitation of microscopic findings in BAL and sputum specimens and results of PCR detection

Specimen	No. of microscopically detected *P. carinii* organisms[a]			No. of specimens	No. of PCR-positive specimens
	Clusters[b]	Cysts[c]	Trophozoites[c]		
BAL	0	0	0	266	6
	0	1	0	1	0
	0	2	0	0	0
	0	≥3	0	2	2
	1	NC[d]	NC	7	7
	2	NC	NC	4	4
	≥3	NC	NC	26	26
Sputa	0	0	0	349	6
	NC	1	0	2	1
	NC	2	0	0	0
	NC	≥3	0	13	12
	NC	0	<3	0	0
	NC	0	≥3	2	1
	NC	1	≥3	3	3
	NC	2	≥3	3	3
	NC	≥3	≥3	20	19
	NC	NC	NC	3	3

[a] Indirect immunofluorescence assay was used.
[b] Only clusters in BAL fluid were counted.
[c] Only cysts and trophozoites in BAL fluid were counted when no clusters were detected.
[d] NC, not counted.

TABLE 3. Diagnostic values of PCR and IFA after resolution of discrepant results on the basis of further patient data

Specimen	HIV status of patients[a]	Sensitivity (%)		Specificity (%)		Positive predictive value (%)		Negative predictive value (%)	
		PCR	IFA	PCR	IFA	PCR	IFA	PCR	IFA
All	All patients	94.8	93.8	99.1	100	93.8	100	99.2	99.1
All	Positive	95.1	92.7	99.2	100	96.3	100	99.0	98.4
	Negative	93.3	100	98.7	100	82.4	100	99.5	100
BAL	Positive	100	96.3	97.3	100	93.1	100	100	98.7
	Negative	92.9	100	98.3	100	81.3	100	99.4	100
Sputum	Positive	92.5	90.6	99.7	100	98.0	100	98.7	98.3
	Negative	NA[b]	NA	100	100	NA	NA	100	100
Other[c]	Positive	100	100	100	100	100	100	100	100
	Negative	100	100	100	100	100	100	100	100

[a] Because no *P. carinii*-positive results were obtained for patients with unknown HIV status (see Table 1), the corresponding calculations were not done for this patient group.
[b] NA, not applicable.
[c] Tracheal fluid ($n = 14$), bronchial fluid ($n = 16$), and pleural effusions ($n = 4$).

SSCP analysis. SSCP analysis could discriminate the amplicons of *P. carinii* from humans and rats (Fig. 1). Furthermore, the amplicons obtained with two samples (samples 1866 and 2262) from two patients were distinct from both of these patterns.

DISCUSSION

In attempts to improve the early diagnosis of PCP, several PCR assays have been developed. In their comparison of six existing PCR methods, Lu and colleagues (8) showed that nested PCR assays performed better than nonnested methods with regard to sensitivity (100% with 30 positive BAL specimens) and specificity (as assessed with fungal DNA). In addition, Skot and colleagues (17), when evaluating their two-tube nested PCR for the detection of *P. carinii*, showed that amplicon detection by nested PCR with ethidium bromide-stained gels as the only method was as sensitive as detection after additional Southern hybridization.

All these nested PCR assays, however, involve extended template preparation and purification methodologies, and they need a contamination-prone transfer of aliquots of the first reaction mixture to new tubes which are used for the second reaction. Furthermore, some of these assays were evaluated only with BAL specimens, not with sputum specimens. We have developed a new, complete assay by combining a simple sample processing procedure and the first one-tube nested PCR for the detection of *P. carinii* in clinical samples that includes internal controls to detect false-negative results due to the presence of strong PCR inhibitors. The sensitivity (94.8%) and specificity (99.1%) of this assay are very good, as evaluated with samples from a large population of patients.

The sample preparation procedure is simple, with a low number of manipulations and a short processing time. The initial washing step with TE buffer lyses erythrocytes (which are well-known PCR inhibitors) and also, probably, bacteria. The Chelex 100 that is included in the proteinase K digestion step was shown to be effective in lysing cells (1, 26) and was the most effective means of eliminating the inhibitory effects of DNA polymerase present in sputa (13). Furthermore, our sample preparation method does not include compounds that are known to inhibit *Taq* polymerase (e.g., SDS [16]) and that must be removed or diluted before DNA amplification. This procedure therefore might be of value for the diagnosis of infections due to organisms other than *P. carinii*.

Optimization of reagent concentrations, particularly the relative concentrations of the inner and outer primers, is necessary in a one-tube nested PCR. The concentration of the outer primers was reduced to 2 nM, which is comparable to findings of earlier studies (25), in which a 10 nM outer primer concentration was used in a one-tube nested PCR for the detection of *Mycobacterium tuberculosis*. The BSA (1 mg/ml) and glycerol (10%) in the amplification buffer reduced the number of samples inhibitory to the PCR. Because DNA itself at a high concentration (16) can be inhibitory and such an excess of bacterial or host DNA may be present in, e.g., sputa, we recommend that PCR with these kinds of specimens be carried out both neat and with dilutions of 1 to 20, with only the diluted sample being spiked with the internal control target (thus, a total of three reactions per clinical specimen).

Although the sensitivity of the one-tube nested PCR was slightly higher than that of IFA (94.8 compared to 93.8%), PCR missed five IFA-positive specimens. In our study a positive IFA result was scored when even a single typical cyst or trophozoite was detected. Other laboratories consider a specimen positive only if at least two typical cysts are identified (6). By this criterion, one BAL sample and four sputum samples that are now scored positive (two of them positive by PCR) would be classified as having equivocal IFA results (Table 2), thereby further increasing the superiority of the amplification assay. The detection of trophozoites only (in two sputum samples; Table 2) by IFA needs a very experienced microscopist. Of these two samples, only one proved to be positive by PCR.

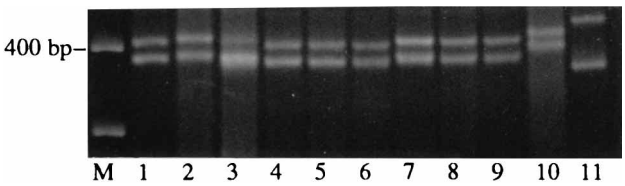

FIG. 1. SSCP analysis of the *P. carinii*-specific PCR fragments (265 bp). Lane M, 100-bp marker; lanes 1 to 10, diagnostic PCR fragments from different patients; lane 11, diagnostic PCR fragment from rat *P. carinii*; lane 10, variant pattern of sample 2262.

This might be due to lysis of the fragile trophozoites during storage in the refrigerator (up to 1 week) before processing for DNA amplification.

The sensitivity of the one-tube nested PCR was slightly higher than that of IFA (92.5 versus 90.6%; Table 3) with the sputum samples, which are preferred because they can be obtained by noninvasive means, as well as with BAL specimens (100 versus 92.3%) obtained from HIV-seropositive patients. In contrast, with specimens from HIV-negative patients (only positive BAL specimens, no positive sputum specimens), IFA was superior to PCR (sensitivities, 100 versus 92.9%). Three false-positive PCR results in this particular specimen group led to a positive predictive value of only 81.3%.

Aliquots of the amplification products that were not purified but that were supplemented with monoinoic salts were directly used in a simple SSCP analysis, which can be performed at room temperature. Results were unequivocal because the PCR did not yield nonspecific products (as resolved on agarose gels) that could be confused with the diagnostic band or that interfered with SSCP analysis. SSCP analysis distinguished rat-derived from human-derived *P. carinii* isolates. It also revealed that the amplicons from two samples were different from all other amplicons. Analysis of clinical data for these two patients did not reveal any distinctive findings. Whether these differences might reflect strain variation that is often detected at other genetic loci among *P. carinii* isolates from humans (7, 23) must be further elucidated. Lu and colleagues (7) analyzed the entire sequences of *P. carinii* 18S rRNA (which also is the target of our assay) and found no differences among isolates from 15 patients. Considerable variability was observed in the amplified regions of the sequences of the two known rat-derived *P. carinii* sequevars, which differed at 9 positions in the 265-bp sequence (14). SSCP analysis could be expected to discriminate the amplicons of these sequevars and also probably those from *P. carinii* isolates from other host animals (the mouse, ferret, and rabbit) that were already found to differ in their antigenic properties (for a review, see reference 20). Hence, the one-tube nested PCR described here, in combination with a simple SSCP analysis, might be useful for detecting and typing *P. carinii* isolates from environmental samples.

In conclusion, our complete and reliable protocol for the detection of *P. carinii* in respiratory specimens combines a short procedure time with technical ease and is therefore better suited than previous methods for implementation in a diagnostic laboratory. Furthermore, Evans and colleagues (5) concluded that nested PCR is less expensive than IFA when the costs of both materials and capital costs are included. With decreasing prevalences of PCP and therefore increasing numbers of negative samples for which more time is needed when the IFA detection method is used, our PCR assay with its good predictive values is a valuable and effective diagnostic tool.

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