Application of DNA Amplification to Pneumocystosis: Presence of Serum Pneumocystis carinii DNA During Human and Experimentally Induced Pneumocystis carinii Pneumonia

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Summary

Pneumocystis carinii pneumonia is a leading cause of morbidity and mortality in patients with the acquired immunodeficiency syndrome (AIDS). Much remains unknown about the basic biology of P. carinii and studies of this infection have been hampered by the lack of cultivation methods. We developed a sensitive and specific assay for P. carinii by utilizing DNA amplification of the P. carinii dihydrofolate reductase (DHFR) gene. By this method, P. carinii DNA was detected in the lungs of rats with experimentally induced P. carinii pneumonia 2 wk before the onset of histopathological changes. DNA amplification analysis of serum demonstrated that by 10 wk of corticosteroid treatment, 12 of 12 (100%) infected rats had circulating DHFR DNA. P. carinii DHFR DNA also was detected in the serum of patients with AIDS and active P. carinii pneumonia (12 of 14 sera collected prospectively). Patients with advanced AIDS but without a history of P. carinii pneumonia were negative by this assay (0 of 6 sera examined). Serum polymerase chain reaction may facilitate investigations into the natural history and epidemiology of P. carinii infection, provide insight into the pathogenesis of parasite dissemination, and offer a useful, noninvasive diagnostic test for the detection of human pneumocystosis.

More than 60% of patients with AIDS develop P. carinii pneumonia at some point during their illness and many develop recurrent episodes (1, 2). Although Pneumocystis carinii was identified as a cause of pneumonia in immunocompromised patients over 30 yr ago, little is known about the natural history and transmissibility of P. carinii because of the lack of reliable isolation and culture techniques (3–5). Presently, the diagnosis of P. carinii pneumonia relies on the direct microscopic visualization of organisms present in respiratory specimens. This often requires invasive procedures such as fiberoptic bronchoscopy or occasionally, open lung biopsy.

DNA amplification by the PCR offers a sensitive and specific means of identifying diverse organisms which may be present in biological samples (6). In the case of P. carinii, amplification of ribosomal RNA genes has shown promise in increasing the sensitivity of sputum and bronchoalveolar lavage fluid examination (7). In the present study, we developed a sensitive DNA amplification assay for P. carinii to begin to address a variety of questions concerning the pathophysiology and natural history of P. carinii infection. We designed this assay to amplify the unique P. carinii dihydrofolate reductase (DHFR) gene (8). By combining PCR with Southern hybridization, as little as 10 fg DNA (or an average of one haploid genome) was detected.

This assay was applied to the study of experimentally induced rat pneumocystosis and to specimens obtained from human patients with P. carinii pneumonia. In the experimental rat model of pneumocystosis, DNA amplification detected P. carinii in infected lungs 2 wk before the onset of histopathological changes. P. carinii DHFR DNA also was present in human bronchoalveolar lavage fluid specimens obtained from patients with P. carinii pneumonia. It is significant that the serum of experimental animals as well as patients with P. carinii pneumonia contained readily detectable amounts of P. carinii DNA. Serum PCR may provide insight into several aspects of the natural history of P. carinii infection, such as parasite latency.

Abbreviations used in this paper: BAL, bronchoalveolar lavage; DHFR, dihydrofolate reductase.
and dissemination, and offer a noninvasive diagnostic test for human infection.

Materials and Methods

**DNA Preparation.** *P. carinii* DNA was purified from *P. carinii* obtained from infected rats. Infection was established in female Sprague-Dawley rats (6-wk-old) which were immunosuppressed with twice weekly injections of hydrocortisone (25 mg) and administered a low protein (8%) diet (ICN Pharmaceuticals, Cleveland, OH) (9). Animal studies were conducted in accordance with established animal welfare guidelines. Infection in all treated animals was confirmed by Giemsa stain of touch prep obtained from whole lung tissue after 11 wk of corticosteroid treatment. Lung tissue also was examined histologically with hematoxylin and eosin, methenamine silver, and Gram-Weigert stains (5). Some sections also were studied by the avidin-biotin immunoperoxidase method for the 3F6 monoclonal anti-*P. carinii* antibody (10). *P. carinii* trophozoites were isolated from rat lungs as previously described (11) and purified by layering on iso-osmotic Percoll-sucrose gradients (initial density 1.05 g/ml) and centrifuging at 25,000 g for 25 min. Visible *P. carinii* organisms were resolved and judged to be >95% pure after histological staining. *P. carinii* genomic DNA was prepared by suspending organisms in 2 ml of 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA. SDS (0.2% final concentration) then was added, followed by ribonuclease (0.5 mg/ml). After incubation at 37° for 30 min, NaCl and protease K were added (final concentration 0.7 M and 1 mg/ml, respectively) and the solution was incubated at 37° for 1 h. DNA was extracted with phenol/chloroform and precipitated with ethanol.

**DNA Amplification and Detection.** The 5′ sense primer is: CTG CAA AAT CCT TGG ATC AT (nucleotides 324-344 of the DHFR coding region) and the 3′ antisense primer is: CTT TAG TAG CAA CCC AAG AT (nucleotides 577-597). For DNA amplification, 5 μl of template DNA was added to 90 μl of a reaction mixture containing 10 mM Tris buffer (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and 1 μM each of the oligonucleotide primers. Tag polymerase was present at a final concentration of 0.026 U/μl. After an initial 2-min denaturation step, each cycle consisted of 1 min denaturation at 92°, 1 min annealing at 55°, and 1 min polymerization at 72°. Samples were amplified for 45 cycles. 10 μl of reaction products were electrophoresed in 2% agarose gels containing 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, and 100 ng/ml of ethidium bromide. Standard protocols to minimize DNA contamination were followed, including inhibition of negative controls and handling DNA standards in a separate workplace (12). Positive specimens were extracted, amplified, electrophoresed, and hybridized at least twice by two operators working with independent reagents.

The sequence of the DHFR gene oligonucleotide probe is: 5′ GAT AGA ATT ATG GCT ACAATA (nucleotides 415-436). 32P-5′-endlabeling and Southern hybridization techniques followed standard protocols (13). Labeled oligonucleotide was separated from unincorporated γ-32P]ATP by passage over NucTrap push columns (Stratagene Inc., La Jolla, CA). ~30 μCi of probe (specific activity of 0.7 μCi/μm) was used in Southern hybridization after vacuum transfer of agarose gels to Nytran membranes (Schleicher & Schuell, Inc., Keene, NH) following recommended protocols (Pharmacia-LKB, Uppsala, Sweden). Unless otherwise indicated, gels were autoradiographed for 3 d at -80° with Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY) and DuPont Cronex intensifying screens (DuPont Instruments, Wilmington, DE).

**Tissue Preparation for PCR Analysis.** 1 g of lung tissue (wet weight) was added to 25 ml of PBS and homogenized completely in a homogenizer (Polytron FT3000; Brinkmann, Littau, Switzerland). The sample was centrifuged at 2,000 g for 10 min and the supernatant discarded. The pellet was then rinsed twice by adding PBS and centrifuging as above. Pelleted material was resuspended in 20 ml of buffer containing 10 mM Tris (pH 8.0), 20 mM EDTA, and 140 mM NaCl. 1 ml of 10% SDS was then added, followed by 2 mg of fresh protease K. The solution was digested overnight at 37°. The next day, the solution was extracted with phenol/chloroform, dialyzed against buffer containing 10 mM Tris (pH 8.0) and 1 mM EDTA, and precipitated and washed with ethanol. 150 ng of total DNA was used as template for PCR. To determine the yield of *P. carinii* DNA extracted from homogenized lung tissue, purified *P. carinii* DNA (1-100 pg) was added to control lung homogenate (*P. carinii* DNA negative by PCR), extracted, and amplified as described above. A 5-10-fold loss in *P. carinii* DNA template was observed to result from lung tissue extraction.

Human bronchoalveolar lavage (BAL) fluid was obtained from patients undergoing diagnostic evaluation for pulmonary disease. 2 ml of bronchoalveolar lavage fluid was centrifuged at 15,000 g for 10 min. The supernatant was discarded and the pellet was resuspended in 0.5 ml of buffer containing 10 mM Tris (pH 8.0) and 1 mM EDTA and recentrifuged. The supernatant was aspirated and 50 μl of H₂O was added to the pellet. This mixture was heated to 95° for 5 min. 5 μl was used for DNA amplification. To determine the yield of *P. carinii* DNA extracted from BAL specimens, purified *P. carinii* DNA (1-100 pg) was added to BAL pellets obtained after centrifugation of control BAL specimens (*P. carinii* DNA negative). Samples were then extracted and amplified as described above. The purification and extraction of DNA from BAL specimens was observed to result in a 5-10-fold loss in the ability to detect the DHFR template.

Whole blood samples were obtained from both uninfected rats (n = 20) and *P. carinii*-infected rats (n = 18). To minimize the possibility that blood could be contaminated with lung tissue containing *P. carinii*, blood was drawn in two ways. For rats undergoing serial blood samples, tail vein blood was obtained. For rats that were used both for analysis of lung tissue and blood, rats were sacrificed by CO₂. The chest cavity was opened immediately and blood samples obtained by cardiac puncture under direct visualization and then heated to 95°. Intra-assay negative controls were followed clinically for at least 3 mo after serum collection. Positive results were taken to ensure that serum was prepared expeditiously and stored frozen. DNA amplification analysis was performed in the following manner. To 20 μl of serum was added 25 μl of a solution of 10 mM Tris (pH 8.0), 5 mM EDTA, 0.25% SDS, and 0.25 mg/ml protease K. This preparation was incubated for 2 h at 56° and then heated to 95° for 10 min to inactivate protease. 45 μl of buffer containing 100 mM Tris (pH 8.3), 500 mM KCl, 25 mM MgCl₂, and 1% Tween 20 then was added. 5 μl were utilized for DNA amplification. Buffyc cells also were prepared from the whole blood of infected rats and digested with protease K and SDS as above before PCR analysis. Several positive sera were filtered through 0.22 μm microcone filters (Spin-X; Costar Corp., Cambridge, MA).
or subjected to DNase treatment before DNA amplification. For DNAse treatment, PCR positive serum (40 μl) was incubated with 0.4 U DNAse I (after addition of 1:10 volume of buffer containing 500 mM Tris [pH 7.5] and 100 mM MgCl₂) for 2 h at 37°. These conditions were sufficient to eliminate completely the template activity of 100 pg of P. carinii DNA which had been added to control serum beforehand. Treated serum was then incubated with proteinase K and SDS as described above, heated to 95° for 10 min to inactivate enzymes, and subjected to DNA amplification. To control for the possible carryover of DNAse into the PCR reaction, 100 pg of genomic P. carinii DNA was added to control (P. carinii DNA negative) serum after DNAse treatment and heat inactivation. Amplification of this sample was found to produce PCR products of the same intensity as samples without DNAse treatment. Sera also were examined for P. carinii by methenamine-silver staining and immunohistochemistry using 3F6 monoclonal anti-P. carinii antibody. For these studies, 0.1 ml of serum was concentrated onto glass slides by cytospin.

Results

A 273 bp portion of the DHFR gene which was 3’ to the intron was selected for DNA amplification. PCR conditions were tested empirically and optimized to attain maximum sensitivity. Amplification of P. carinii DNA with the DHFR primers was found to yield a single DNA band of a size consistent with that predicted for the 273-bp gene segment enclosed by the primer sequences. Cleavage of the amplified band with the restriction enzymes SnaI and Fnu4H1 resulted in DNA fragments which corresponded in size to those predicted by the position of these restriction sites within the amplified region. Amplification of genomic DNA from representative gram-negative (Escherichia coli) and gram-positive (Streptococcus pyogenes) bacteria, as well as the fungus Candida albicans, did not yield detectable PCR products (data not shown).

To establish the utility of P. carinii DHFR DNA amplification in pneumocystosis, we first studied the corticosteroid-treated rat, an experimental model of P. carinii pneumonia (5, 9). At intervals, animals were killed and whole lung tissue was processed for DNA amplification. At 4 wk of corticosteroid treatment, rats developed pulmonary pneumocystosis as determined both by characteristic histopathological changes (foamy, eosinophilic alveolar exudates) and by direct visualization of P. carinii. Fig. 1 shows the results of DNA amplification analysis of rat lungs.

Figure 1. DNA amplification analysis of rat lungs. (Top) Ethidium bromide stained gel of PCR products obtained after DNA amplification of indicated samples. (123 bp) Multimeric DNA standards. (STDs) 10 μl of DNA products obtained after amplification of purified P. carinii genomic DNA (lanes a-d). (Control) Results obtained from analysis of three representative control, untreated rats which were housed separately from the corticosteroid-treated group (out of five studied) (lanes e-g). (Corticosteroid-treated) PCR analysis of lungs obtained from 11 rats after the indicated duration of corticosteroid treatment (lanes h–r). (Bottom) Southern hybridization of same gel with 32P-labeled oligonucleotide probe. Exposure was for three d at -80° with Kodak X-OMAT film and an intensifying screen.
of DHFR DNA amplification of DNA isolated from whole lung tissue in control and corticosteroid-treated rats. Ethidium bromide staining clearly demonstrated an amplified DNA band of predicted size in lung homogenates that were analyzed at 6 wk of corticosteroid treatment. Southern hybridization of this gel (with a 32P-labeled internal oligonucleotide probe) confirmed that the amplified product was *P. carinii* DHFR DNA. Inspection of the intensities of *P. carinii* DNA standards showed that after three days of autoradiography, the combination of DNA amplification with Southern hybridization produced a 100-fold greater sensitivity in detection than did DNA amplification alone. After 6 d of autoradiography, faint signals specific for *P. carinii* DNA were visualized as early as 2 wk after the initiation of corticosteroid treatment. Under these conditions, as little as 10 fg of *P. carinii* DNA generally could be detected. Assuming a minimum haploid genome size of 7,000 kb (14), this would correspond approximately to one organism. Neither histopathological changes, nor direct visualization of organisms by Gram-Wiegert, methenamine silver, or immunoperoxidase stains with 3F6 mAb suggested the presence of active infection at this time. Lungs obtained from five control rats (untreated and housed separately) were negative when analyzed by DNA amplification and Southern hybridization. The addition of known quantities of *P. carinii* DNA (1-100 pg) to control, PCR-negative lung homogenates resulted in a 5-10-fold loss in *P. carinii* DNA after amplifications of extracted material.

We next applied DHFR DNA amplification to an analysis of other biological samples. Serum samples from rats with pulmonary pneumocystosis were amplified and analyzed by gel electrophoresis and Southern hybridization. Fig. 2 shows the results obtained from nine representative animals which were killed at the indicated times. Sera from three animals, two at 10 wk of corticosteroid treatment, and one at 6 wk of treatment, demonstrated DHFR DNA at levels which ranged from 0.1 to 1 pg per μl of serum. In a second series of animals, amplified DHFR DNA signals were observed to be present in the serum of all 10 rats whose blood was sampled from tail veins at 8, 9, and 10 wk of corticosteroid treatment. Blood specimens sampled at 2 and 4 wk were uniformly negative (0/10). Control, untreated rats showed no detectable levels of serum DHFR DNA (n = 20, age range 3-16 mo). Analysis of whole blood or buffy coats obtained from *P. carinii*-infected rats also showed positive DHFR DNA signals. The PCR signals obtained from these specimens, however, showed greater variability than those obtained from serum. This appeared to be due to variable yields of *P. carinii* DNA obtained from extracted blood cells, as well as the presence of substances that interfered with the DNA amplification reaction (data not shown).

Because of the exact sequence homology between the rat and the human-derived *P. carinii* DHFR gene (8), we reasoned that the DHFR amplification assay might readily detect *P. carinii* in human specimens. Fig. 3 shows the PCR analysis of 11 bronchoalveolar lavage fluid samples obtained from patients who underwent fiberoptic bronchoscopy for a variety of clinical indications. *P. carinii* DHFR DNA was readily demonstrated in samples obtained from three patients with histopathologically proven *P. carinii* pneumonia. It was not detected in samples obtained from eight patients without pneumocystosis.

We next examined patient sera for the presence of *P. carinii* DHFR DNA. Fig. 4 shows a representative autoradiograph of this analysis and Table 1 summarizes the results obtained from different patient groups. The initial application of this
assay to previously stored sera obtained from patients with
*P. carinii* pneumonia revealed evidence of *P. carinii* DNA in
two of six samples. We then collected 14 samples prospec-
tively from acutely infected patients and processed the sera
in a standardized fashion. Serumsamples were collected within
48 h of diagnosis and the majority were drawn at the time
of initial clinical presentation, before the start of therapy, and
before any invasive diagnostic procedure. Of these specimens,
12 of 14 (86%) were observed to have *P. carinii* DHFR DNA
as detected by PCR. Sera from six patients with advanced
AIDS (CD4 lymphocyte count <50 mm$^3$), but without a
history of *P. carinii* pneumonia were negative by this assay.
Sera obtained from nine healthy laboratory workers also
showed no evidence of *P. carinii* DNA. Based on the *P. carinii*
DNA standard dilution curve (lanes o-q) and the recovery
of *P. carinii* DNA after serum extraction (lane m), we esti-
mate that positivesera contained from 5-25 DHFR templates
per $\mu l$.

To investigate the physical nature of serum DHFR DNA,
serum from experimentally infected rats (Fig. 2 B, lanes g
and h) and humans (Fig. 4, lane 1) was filtered through a
0.22 $\mu$ filter and the filtrate amplified by PCR. This resulted
in a disappearance of the DHFR band, suggesting that cel-
lar or particulate material was the source of the *P. carinii*
template DNA. When *P. carinii* DNA was added to control
serum, filtration did not affect the ability of DNA ampli-
fication to detect it, indicating that *P. carinii* DNA had not
been removed by nonspecific binding to the micropore filter
(Fig. 2 A, lanes e and f). Although these experiments sug-
gest that whole parasites might be the source of DHFR tem-
plate, direct microscopic examination of serum (100 $\mu l$) or
whole blood (10 $\mu l$) by either methenamine silver staining
or immunohistochemistry with anti- *P. carinii* mAb revealed
no demonstrable organisms. To further address the nature
of serum DHFR template, PCR positive patient sera were
incubated with DNAse under conditions in which ex-
genously added *P. carinii* DNA is completely hydrolyzed.
As shown in Fig. 4, DNAse digestion completely eliminated
the PCR positive signal present in patient sera. Taken to-
gether, these results suggest that the serum DHFR template
is associated with filterable material that is sensitive to DNAse.
Template DNA may represent damaged organisms that are
sensitive to DNAse or high-molecular weight, protein-DNA
aggregates released from phagocytic cells.

### Discussion

*P. carinii* pneumonia is a leading cause of morbidity and
mortality among patients with AIDS (1, 2). The lack of reli-
able cultivation methods has hampered investigations into
the basic biology and epidemiology of *P. carinii* and the diag-
nosis of infection relies solely on the direct visualization of
organisms in respiratory secretions or lung biopsy material
(3, 5).

DNA amplification of the DHFR gene of *P. carinii* offers
a highly sensitive and specific technique for the detection of
*P. carinii*. Recent studies utilizing primers for *P. carinii* ribosomal
RNA genes have demonstrated the utility of this technique
in increasing the sensitivity of the detection of *P. carinii* in
bronchoalveolar lavage fluid (7). For our studies, we selected
to amplify the *P. carinii* DHFR gene. This gene has no se-
quence homology with any known prokaryotic or euarya-
totic DHFR genes (8). DNA amplification of crude lung
homogenates known to contain *P. carinii* resulted in a single
DNA product of predicted size. Sequence identity with
authentic DHFR DNA was confirmed by restriction enzyme
mapping and Southern hybridization with internal oligonu-

### Table 1. Summary of DNA Amplification Analysis of
Human Serum for P. carinii DHFR DNA

| Patient Category                      | Positive sera/ Total sera |
|--------------------------------------|--------------------------|
| AIDS, active *P. carinii* pneumonia  | 12/14                    |
| Advanced AIDS, no history of *P. carinii* pneumonia | 0/6                      |
| Normal controls                      | 0/9                      |
cleotide probe. By combining DNA amplification with Southern hybridization, as little as 10 fg of genomic *P. carinii* DNA was detected, an amount corresponding, on average, to one *P. carinii* haploid genome.

The utility of this assay was confirmed in the corticosteroid-treated rat model of pneumocystosis and allowed for the detection of *P. carinii* DNA in infected rat lungs 2 wk before the onset of histological changes. DNA amplification analysis of lung tissue obtained from control, untreated rats showed no evidence of *P. carinii* DNA. Based on our DNA yield and standard dilution curves for DHFR DNA amplification, we estimate that the control (uninfected) rat lung contains <3,000 organisms per gram of lung tissue (wet weight). Although *P. carinii* pneumonia generally is considered to result from the reactivation of a latent infection, this analysis of rat lung suggests that the carriage of organisms is low. Serum analysis revealed that by 10 wk of treatment, 12 of 12 (100%) of corticosteroid-treated rats demonstrated *P. carinii* DNA in their serum, representing ~2–25 templates per μl.

The PCR assay for *P. carinii* DHFR DNA is equally applicable to human pneumocystosis and detects *P. carinii* both in bronchoalveolar lavage fluid and in the serum of AIDS patients with active *P. carinii* pneumonia. In the sera analyzed for this study, there appears to be a high correlation between active *P. carinii* pneumonia and the presence of *P. carinii* DNA in the serum (12 of 14 specimens analyzed). In six patients with advanced AIDS, but without a history of *P. carinii* pneumonia, no positive PCR signals could be detected. These results present the possibility that serum PCR for *P. carinii* may be useful as a diagnostic procedure in some patients with suspected *P. carinii* infection. This would be of particular value because current diagnosis of *P. carinii* pneumonia frequently requires an invasive procedure such as bronchoscopy to obtain respiratory specimens for direct microscopic examination. We have yet to determine how long the serum DHFR DNA signal persists in infected patients. Examination of serial serum specimens, however, may offer prognostic information about the severity of infection and parasite burden, as well as efficacy of antibiotic prophylaxis and treatment.

The precise nature of the *P. carinii* DNA which is detected by PCR has not been elucidated in the present study. Although the DHFR template could be removed by serum filtration through a 0.22 μ filter, efforts to demonstrate intact organisms in serum by histochemical or immunostaining were unsuccessful. Treatment of *P. carinii* DNA-positive serum with DNase resulted in a loss of PCR signal, suggesting that the serum DHFR template consists of either free DNA or organisms that had been rendered sensitive to DNase treatment. It is possible that *P. carinii* template represents high molecular weight DNA complexes originating from parasites damaged by phagocytosis or serum storage. The reported occurrence of extrapulmonary pneumocystosis (15), however, suggests that in some patients, *P. carinii* may have a blood-borne phase. Evidence by DNA amplification that *P. carinii* may be blood-borne offers important information about the natural history of this infection and would readily explain the pathogenesis of extrapulmonary pneumocystosis.

An important, unresolved question in the pathophysiology of pneumocystosis concerns the transmissibility of this infection. For example, it has been noted that at an early age a majority of individuals possess serological evidence of *P. carinii* infection (16, 17). This has lent support to the concept that clinical pneumocystosis results from the reactivation of latent infection (3, 5, 17), as occurs in other infections associated with T cell immunodeficiency such as tuberculosis, histoplasmosis, and cryptococcosis. Alternatively, the occurrence of *P. carinii* point epidemics (18, 19), as well as the failure to demonstrate organisms in autopsy studies, suggests that the asymptomatic carriage of *P. carinii* in fact may be quite low (20, 21). DNA amplification analysis of serum, sputum, bronchoalveolar lavage fluid, and autopsied lung tissue may help to clarify this central issue concerning *P. carinii* infection (22). Better insight into the pathophysiology and epidemiology of this infection will offer more effective strategies in preventing this frequently fatal opportunistic infection.

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