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Diversity of Host Species and Strains of *Pneumocystis carinii* Is Based on rRNA Sequences

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We have amplified by PCR *Pneumocystis carinii* cytoplasmic small-subunit rRNA (variously referred to as 16S-like or 18S-like rRNA) genes from DNA extracted from bronchoalveolar lavage and induced sputum specimens from patients positive for *P. carinii* and from infected ferret lung tissue. The amplification products were cloned into pUC18, and individual clones were sequenced. Comparison of the determined sequences with each other and with published rat and partial human *P. carinii* small-subunit rRNA gene sequences reveals that, although all *P. carinii* small-subunit rRNAs are closely related (~96% identity), small-subunit rRNA genes isolated from different host species (human, rat, and ferret) exhibit distinctive patterns of sequence variation. Two types of sequences were isolated from the infected ferret lung tissue, one as a predominant species and the other as a minor species. There was 96% identity between the two types. In situ hybridization of the infected ferret lung tissue with oligonucleotide probes specific for each type revealed that there were two distinct strains of *P. carinii* present in the ferret lung tissue. Unlike the ferret *P. carinii* isolates, the small-subunit rRNA gene sequences from different human *P. carinii* isolates have greater than 99% identity and are distinct from all rat and ferret sequences so far inspected or reported in the literature. Southern blot hybridization analysis of PCR amplification products from several additional bronchoalveolar lavage or induced sputum specimens from *P. carinii*-infected patients, using a ^{32}P-labeled oligonucleotide probe specific for human *P. carinii*, also suggests that all of the human *P. carinii* isolates are identical. These findings indicate that human *P. carinii* isolates may represent a distinct species of *P. carinii* distinguishable from rat and ferret *P. carinii* on the basis of characterization of small-subunit rRNA gene sequences.

Pneumocystis carinii, an opportunistic lower respiratory tract pathogen, is a small unicellular organism that exists in two forms, trophozoite and cyst. *P. carinii* infection commonly results in pneumonia, which accounts for significant morbidity and mortality in AIDS and other immunocompromised patients (12, 24). Members of the genus *Pneumocystis* are known to infect a wide variety of mammalian hosts (9).

Formerly considered a protozoan, *P. carinii* recently has been reclassified as a fungus on the basis of DNA homology studies (31) and the reactivity of monoclonal antibodies raised against *P. carinii* to a variety of fungi (20). The 5S (38) and small-subunit (4, 33–34) rRNA sequences of rat-derived *P. carinii* are closely related to the rRNA sequences of fungi. The *P. carinii* mitochondrial DNA, coding for apocytochrome *b*, NADH dehydrogenase subunits 1, 2, 3, and 6, cytochrome oxidase subunit II, and the mitochondrial small-subunit rRNA, has 60% identity with fungal DNA but only 20% identity with DNA from protozoa (27). The sequence of the *P. carinii* mitochondrial large-subunit rRNA gene also is most closely related to that of ustomycetous red yeast fungi (36). Furthermore, Ypma-Wong et al. recently isolated the elongation factor 3 gene from *P. carinii*, which has so far been found only in fungi (39).

As early as 1974, the genus *Pneumocystis* was considered to contain more than one species (8). Although *P. carinii* organisms isolated from different hosts are indistinguishable morphologically and immunologically, DNA sequence studies indicate that at least some diversity exists between different host species. Most of the antibodies produced to *P. carinii* appear to react only with organisms obtained from the same host as those used in the immunization process (5–7, 11, 15, 37). Until recently, however, there was insufficient information to clearly differentiate species of *Pneumocystis*. Therefore, most reports used the name *P. carinii* for all *Pneumocystis* organisms. Recently, Gigliotti et al. reported species-specific variations (variations between different host species) in the *P. carinii* surface glycoprotein antigens (6). They showed that *P. carinii* surface glycoproteins from humans, ferrets, rats, and mice display patterns of both phenotypic and genotypic variation.

Analysis of a number of sequences from different genetic loci of *P. carinii* suggests that *P. carinii* isolates from different hosts are similar at some loci (4, 5, 10) but distinct at other loci (14, 16–18, 26). Kazuhiro et al. amplified 5S rRNA genes and sequenced the PCR products from 23 *P. carinii*-positive samples: 15 samples from mice, 1 from a rat, and 7 from human sources (10). All 23 5S rRNA sequences were identical, suggesting that there is homogeneity of the 5S rRNA sequences of *P. carinii* isolated from mice, rats, and humans. Edman et al., using in situ hybridization, demonstrated that a mixture of *P. carinii* probes designed to hybridize to the rat *P. carinii* small-subunit rRNA could hybridize to *P. carinii* in human lung tissue as well (4). On the basis of these results, they suggested that human and rat *P. carinii* organisms are closely related.

There are species-specific sequence differences in both the mitochondrial large-subunit 26S rRNA (14, 26, 35) and cytoplasmic rRNA genes (16–18) of *P. carinii*. Strain-specific variations (variations within a single species of host) also exist at some loci (2, 16). However, there is greater than 99% sequence identity in the 5′ fragment of the mitochondrial large-subunit rRNA among isolates of human *P. carinii* from diverse geographic regions (35). In the present study, we amplified by PCR *P. carinii* cytoplasmic small-subunit rRNA genes from

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DNA extracted from a variety of human and ferret tissues positive for *P. carinii*. The amplified *P. carinii* small-subunit rRNA genes were cloned into plasmid vectors and sequenced. The sequences were compared with each other and with published *P. carinii* small-subunit rDNA sequences. Sequence heterogeneity, by and large, is clustered in variable regions (13, 22) and permits discrimination among closely related organisms. Comparison of the determined sequences reveals that, overall, all *P. carinii* small-subunit rRNAs are highly related to one another. However, *P. carinii* rRNAs isolated from different host types exhibit distinctive species-specific patterns of sequence variation. In addition, there were strain-specific patterns of sequence variation in two strains of *P. carinii* isolated from a single ferret lung tissue specimen. Species-specific and strain-specific *P. carinii* oligonucleotide probes were designed, synthesized, and labeled with either ^{32}P or fluorescein. PCR-amplified rDNA products from clinical specimens and ferret lung tissues were analyzed by Southern blot technique, using ^{32}P-labeled probes. *P. carinii* cytoplasmic small-subunit rRNAs were analyzed directly from the samples by an in situ hybridization technique, using fluorescein-labeled probes.

MATERIALS AND METHODS

Human specimens. Human specimens from patients ranging in age from 26 to 63 years were submitted to *P. carinii* testing laboratories; 19 specimens were analyzed in the present study. Seventeen specimens (12 bronchoalveolar lavage [BAL] and 5 induced sputum specimens) were from Brigham and Women's Hospital, Boston, Mass. Two purified DNA preparations isolated from *P. carinii*-positive BAL samples (designated 71151 and 71161) were a gift from P. C. Hopewell, Division of Pulmonary and Critical Care, San Francisco General Hospital, San Francisco, Calif.

Of the 17 specimens from Brigham and Women's Hospital, 14 were *P. carinii* positive and 3 were *P. carinii* negative by standard staining methods. Of the 14 positive specimens, 4 (BAL) were from cancer patients, 2 (BAL) were from transplant patients, and 8 (5 BAL and 3 induced sputum) were from human immunodeficiency virus (HIV)-positive patients. Two induced sputum specimens from HIV-positive patients and one BAL specimen from a cancer patient were *P. carinii* negative. The BAL and induced sputum specimens had been stored for up to 1 year at −70°C. An aliquot (5 to 15 ml) was sent to our laboratory on dry ice. The samples were stored at −70°C until the day of processing (up to 2 weeks).

Ferret lung tissues. Lung tissues from ferrets infected with *P. carinii* and negative controls were provided by Dennis C. Stokes, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tenn. Lungs excised from the ferrets were sent to our laboratory immediately on dry ice. The lung tissues were kept at −70°C until the day of processing (up to 2 weeks).

DNA extraction. High-molecular-weight DNA was isolated from BAL or induced sputum samples from infected patients or from ferret lung tissue, as described below. Human-derived *P. carinii* organisms were concentrated from BAL samples by spinning the samples at 2,500 rpm for 10 min. Induced sputum samples were treated with 0.5% dithiothreitol for 10 min at 37°C prior to centrifugation. DNA was extracted from the cell pellets according to the method described by Shah et al. (29). Lung tissue (200 μg) from infected ferrets was homogenized under liquid nitrogen with mortar and pestle and transferred to a 15-ml tube. The concentrated cell pellets from BAL or induced sputum samples or homogenized ferret tissues were suspended in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl [pH 7.5]) to a final volume of 1 ml. Sarcosyl (Sigma, St. Louis, Mo.) was added to a final concentration of 1%, and proteinase K (Boehringer Mannheim, Indianapolis, Ind.) was added to a final concentration of 100 μg/ml. The mixture was incubated at 37°C for 45 min. RNase A (Sigma) was added to a final concentration of 100 μg/ml, and the mixture was further incubated at 37°C for 30 min. DNA was collected by ethanol precipitation following extraction with phenol-chloroform.

Amplification of small-subunit rRNA coding regions of *P. carinii*. *P. carinii* small-subunit rRNA coding regions were amplified by using primers A and B described by Medlin et al. (23). The sequences of the primers (5′-3′) were as follows:

Primer A, 5′-ccg aat tcg tcg aca ACC TGG TTG ATC CTG CCA GT-3′
Primer B, 5′-cccgggatccaagctTGATCCTTCTGCAGGTTCACCTAC-3′

Primer A contains a polylinker (lowercase letters) incorporating *Eco*RI and *Sal*I sites plus a conserved 20-nucleotide sequence (uppercase letters) which is complementary to the coding strand at the 5′ end of eukaryotic small-subunit rRNAs (23). Primer B contains a 24-nucleotide sequence complementary to the noncoding strand at the 3′ terminus of eukaryotic small-subunit rRNAs and has restriction sites for *Sma*I, *Bam*HI, *Hin*dIII, and *Pst*I. The small-subunit rRNA coding regions of *P. carinii* from infected lung tissue, BAL specimens, or induced sputum were amplified by PCR, using DNA prepared as described above (25, 28). Incubations were carried out in 0.5-ml Eppendorf tubes. Reaction mixtures contained 0.5 to 1.0 μg of DNA in 100 μl of 1× PCR buffer (10 mM Tris-HCl [pH 8.3]; 50 mM KCl; 2 mM $MgCl_2$; 100 μM [each] dATP, TTP, dCTP, and dGTP; 0.1% gelatin), 1 μM (each) amplification primers A and B, and 0.1 U of *Taq* DNA polymerase (BioLabs, Beverly, Mass.). The samples were covered with 50 μl of mineral oil to minimize evaporation. A three-temperature program was used for amplification: (i) primer annealing to the DNA template at 37°C for 2.5 min, (ii) primer extension at 68°C for 4 min, and (iii) denaturation at 87°C for 1.25 min. The sequential incubations were repeated for 29 cycles. After the 29th cycle, the samples were held for 10 min at 68°C to complete chain elongation and then stored at 10°C until further processing. Universal BL2 precautions were used to eliminate possible contamination of samples. Cross-contamination by aerosols was reduced by physical separation of laboratory areas used to prepare PCR reaction mixtures and to analyze PCR products and by using a combination of positive displacement pipetters and aerosol-resistant pipette tips.

Cloning and sequencing of PCR products. The PCR-amplified DNA fragments were digested with restriction enzymes *Bam*HI, *Hin*dIII, and *Sal*I and force cloned into pUC18 at the *Hin*dIII and *Sal*I sites. Since there is no *Bam*HI site in the published rat *P. carinii* small-subunit rRNA coding region, *Bam*HI was used to digest the amplification products and promote the selective cloning of fungal small-subunit rRNA genes.

Sequences from human and ferret *P. carinii* small-subunit rRNA gene clones and gel-purified PCR products were determined by using Sequenase kits (U.S. Biochemicals). The sequences were compared with published small-subunit rRNA gene sequences of rat (4) and human (1) *P. carinii*. Genus-specific, species-specific, and strain-specific sequence regions were identified, and oligonucleotide probes (20 to 40 nucleotides in size) complementary to the rRNA were designed and synthesized (see Fig. 1). These probes were tested by Southern blot (see Fig. 2), dot blot, and in situ hybridization (see Fig. 3 and 4) for inclusivity and exclusivity.

Synthesis and labeling of synthetic oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems 380-B DNA synthesizer, using β-cyanoethyl phosphoramidite chemistry. The oligonucleotides were modified at their 5′ ends to contain a primary amine by addition of an aminopropyl-modified cytidine phosphoramidite. Deprotection of the phosphates and nucleotide bases and purification of the crude oligonucleotide mixtures by reverse-phase high-performance liquid chromatography were performed by standard methods.

Labeling of the amino-modified oligonucleotides was performed in the following manner. To a solution of 0.1 M Na_2CO_3 (pH 8.5) containing 200 μg of amino-modified oligonucleotide, 1.4 mg of fluorescein isothiocyanate or rhodamine X-isothiocyanate dissolved in dimethylformamide was added. Reactions were allowed to proceed in the dark at room temperature. Fluorescein labeling reactions were complete after 2.5 h. The rhodamine X-isothiocyanate reactions were complete after overnight incubation. Excess labeling reagent was removed by using Sephadex G-25 gel filtration columns. Fractions containing fluorescein-labeled oligonucleotide were dried and resuspended in sterile nuclease-free water (Milli Q water). Rhodamine X-labeled products were used as eluted from the gel filtration column because of difficulties in resuspending the lyophilized product.

Southern blot analysis. One-tenth of the 100-μl PCR amplification reaction mixture or 200 ng of a plasmid containing the small-subunit rRNA gene of *P. carinii* was digested with *Sal*I and *Hin*dIII, fractionated by electrophoresis on 1% agarose gels, and transferred to nitrocellulose membranes (21). Hybridizations were performed at 60°C for 14 to 16 h in a hybridization solution containing 0.9 M NaCl, 0.12 M Tris-HCl (pH 7.8), 6 mM EDTA, 0.1 M K_3PO_4, 0.1% sodium dodecyl sulfate (SDS), 0.1% pyrophosphate, 0.002% Ficoll, 0.02% bovine serum albumin, 0.002% polyvinylpyrrolidone, and ^{32}P-labeled oligonucleotide probe. The filters were washed three times for 15 min at 60°C in 0.03 M NaCl–0.004 M Tris-HCl (pH 7.8)–0.2 mM EDTA–0.1% SDS. The filters were exposed to X-ray film for 4 h at −70°C.

In situ hybridization. BAL and induced sputum from *P. carinii*-positive and -negative human specimens were concentrated to one-tenth of their original volume by centrifugation, and 50 μl of the resultant pellet was smeared on clean glass slides. Lung tissues were prepared by the touch-prep method. The smears or tissue touch-preps were air dried, fixed in either 75% methanol–25% glacial acetic acid or cold acetone for 10 min at room temperature, and then air dried again. A 100-μl portion of probe hybridization mixture containing 5× SET (750 mM NaCl, 100 mM Tris-HCl [pH 7.8], 5 mM EDTA), 0.2% bovine serum albumin (fraction V; Sigma), 10% dextran sulfate (Sigma), 10% sonicated denatured salmon sperm DNA, and 1.5 to 1.7 ng of either rhodamine X- or fluorescein-labeled oligonucleotide probe, or both, per μl was added to each slide. Slides were covered with coverslips and incubated in a humid chamber at 60°C for 3.5 to 18 h. Coverslips were removed by immersion in 2× SET at 60°C, and the slides were immediately washed three times in 0.2× SET at 60°C for 10 min. Slides were air dried and viewed immediately or stored in the dark at 4°C. Samples were mounted either in ACCU.MOUNT 60 mounting medium or in a buffered glycerol phosphate saline solution (9:1) and viewed under oil immersion with a ×100 objective on a Leitz photomicroscope with an epifluorescence condenser, a mercury lamp, and Leitz dual pass filter sets 513-608 (fluorescein)

and 513-674 (rhodamine X). Photomicrographs were taken with Kodachrome 400 ASA color film.

Some of the *P. carinii*-positive smear slides, stained with *P. carinii*-specific antibodies by using the Genetic Systems (Seattle, Wash.) protocol and reagents, were counterstained by in situ hybridization with the rhodamine X-labeled, "human species-specific" *P. carinii* probes.

RESULTS

PCR amplification. *P. carinii* small-subunit rRNA genes were PCR amplified from clinical samples (BAL and induced sputum) and also from infected ferret lung tissue, using rRNA-specific primers which amplify almost the entire small-subunit rRNA gene (23). Agarose gel analysis revealed that PCR resulted in one major band of approximately 1,800 bp for each of the PCR products (results not shown). The human small-subunit rRNA gene sequence contains a *Bam*HI restriction site, whereas the *P. carinii* sequence does not. Therefore, the PCR products were digested with *Hin*dIII, *Sal*I, and *Bam*HI to selectively clone the whole PCR fragments of *P. carinii* small-subunit rRNA genes into pUC18 at the *Hin*dIII and *Sal*I sites.

P. carinii small ribosomal subunit gene sequences were obtained from a total of 12 *P. carinii*-positive human patients and one *P. carinii*-positive ferret. The PCR amplification products from seven patients, three cancer (BAL) and four HIV-positive (three BAL and one induced sputum specimen) patients, were cloned into pUC18 as described in Materials and Methods and sequenced. PCR amplification products from the remaining five patients (one BAL specimen from a transplant patient and two induced sputum specimens and one BAL specimen from HIV-positive patients) were gel purified and partially sequenced without an intervening cloning step.

Four human- and two ferret-derived *P. carinii* small-subunit rDNA clones were sequenced entirely (corresponding to clones HUM1, HUM2, HUM3, HUM4, FER1, and FER2 in Fig. 1), while several other clones were partially sequenced through the variable regions. Two human-specific *P. carinii* clones, HUM1 and HUM2, were derived from a pool of four *P. carinii*-positive BAL DNA samples, isolated from three cancer patients and one HIV-positive patient. Clone HUM3 represents a clone of a PCR amplification product from a single (unpooled) *P. carinii*-positive human induced sputum DNA sample, isolated from an HIV-positive patient. Clones HUM4 and HUM5 represent clones of PCR amplification products from *P. carinii*-positive DNA samples 71151 and 71161, respectively, isolated from two HIV-positive patients. Clones HUM1, HUM2, and HUM3 were from *P. carinii*-positive patients in New England, and clones HUM4 and HUM5 were from patients in California. Two types of *P. carinii* small-subunit rRNA gene clones (FER1 and FER2 [Fig. 1]) were isolated from the PCR amplification products of a single ferret lung specimen. Clones FER1 and FER2 were fully sequenced. Eight additional clones were partially sequenced. The sequences were compared with each other and also with the published rat (1, 4) and human (1) *P. carinii* small-subunit rRNA sequences.

There was less than 1% difference (2 to 7 bases; Fig. 1) in the human *P. carinii* small-subunit rRNA gene sequences in a comparison of the four fully sequenced clones, 10 of the 11 partially sequenced clones, and the five partially sequenced PCR amplification products. HUM5 (Fig. 1), a representative clone of the PCR amplification products from DNA sample 71161, was the only clone with 6 extra nucleotides at position 691 (Fig. 1). However, the rest of the sequence was identical to the other human *P. carinii* sequences. Two types of small-subunit rRNA genes were isolated from the infected ferret lung tissue DNA, one as a predominant species (9 of 10 clones) and the other as a minor species (1 of 10 clones). Clone FER1 represents the predominant species of small-subunit rDNA gene and FER2 represents the minor species. There was greater than 3% variation between the two genes.

P. carinii small-subunit rRNA genes isolated from different host types exhibited distinctive patterns of sequence variation (Fig. 1). Overall, there was 3 to 4% sequence variation among human, rat, and ferret *P. carinii* small-subunit rRNA genes. Several oligonucleotide probes complementary to the hypervariable regions of the human and ferret small-subunit rRNA genes were designed and tested in the dot blot format (data not shown). Genus (all *P. carinii* species)-, species (i.e., human and ferret specific)-, strain-, and broader-specificity (i.e., fungal)-specific oligonucleotide probes were identified.

A human *P. carinii* species-specific probe (probe 1485), a ferret *P. carinii* strain-specific probe (probe 1493), a *P. carinii* genus-specific probe (probe 1486), and a fungus-specific probe (probe 1159) were further tested by Southern blot analysis against ferret and human small-subunit rRNA gene clones and PCR-amplified products from *P. carinii*-infected human samples to verify their host specificity. PCR amplification products from *Candida albicans*-positive and normal (uninfected) human DNAs, isolated from BAL and sputum samples with primers A and B, were run as controls. Figure 2 shows the hybridization results. Probe 1485, designed to be specific to human *P. carinii*, hybridizes only to the *P. carinii*-infected human samples and not to the ferret *P. carinii* rDNA clones, *C. albicans*-positive DNA, or normal human DNA (Fig. 2a). Probe 1493, designed to be specific to the predominant ferret rRNA species (FER1), hybridizes to two of three ferret *P. carinii* rDNA gene clones (Fig. 2b). Probe 1486 hybridizes to all *P. carinii*-positive samples but not to normal human DNA or *C. albicans* DNA (Fig. 2c). Probe 1159 hybridizes to all *P. carinii*-positive samples and also to the *C.-albicans*-positive sample but not to the control human sample (Fig. 2d). Dot blot hybridization demonstrated that *P. carinii*-specific probes 1485, 1493, 1495, and 1486 (Fig. 1) did not hybridize to RNAs from a broadly representative collection of fungal species (~100 isolates); uninfected rat, ferret, or human lung tissue; and common upper and lower respiratory tract bacteria such as *Bacillus*, *Citrobacter*, *Enterobacter*, *Hafnia*, *Haemophilus*, *Klebsiella*, *Listeria*, *Micrococcus*, *Neisseria*, *Mycobacterium*, *Pseudomonas*, and *Staphylococcus* species (data not shown). Probe 1159 has broader specificity and hybridizes to all *P. carinii* and fungal isolates tested but does not hybridize to any other common respiratory bacterial flora (data not shown).

In situ hybridization. *P. carinii*-infected human specimens (BAL, infected tissue, and sputum) were analyzed by in situ hybridization, using fluorescently labeled probes. As is evident in Fig. 3, the human *P. carinii*-specific probe, probe 1485 (rhodamine X labeled), hybridizes strongly only to *P. carinii* and not to human rRNA (indicated by bright red fluorescence). These results clearly indicate that *P. carinii* can be identified in a clinical sample by in situ hybridization to rRNA. Specificity of the rhodamine X-labeled probe for *P. carinii* was confirmed by first staining slides with fluorescein-labeled, anti-*P. carinii* monoclonal antibodies (indicated by bright green fluorescence; Fig. 3) and then counterstaining by in situ hybridization with the rhodamine X-labeled probe. From the lung tissue of one infected ferret, two different *P. carinii* small-subunit rRNA genes were isolated. In situ hybridization, using probes specific for each type (probe 1493, predominant type, rhodamine X labeled, indicated by bright red fluorescence; and probe 1495, fluorescein labeled, indicated by bright green fluorescence), clearly indicated the copresence of two different strains of *P. carinii* (Fig. 4).

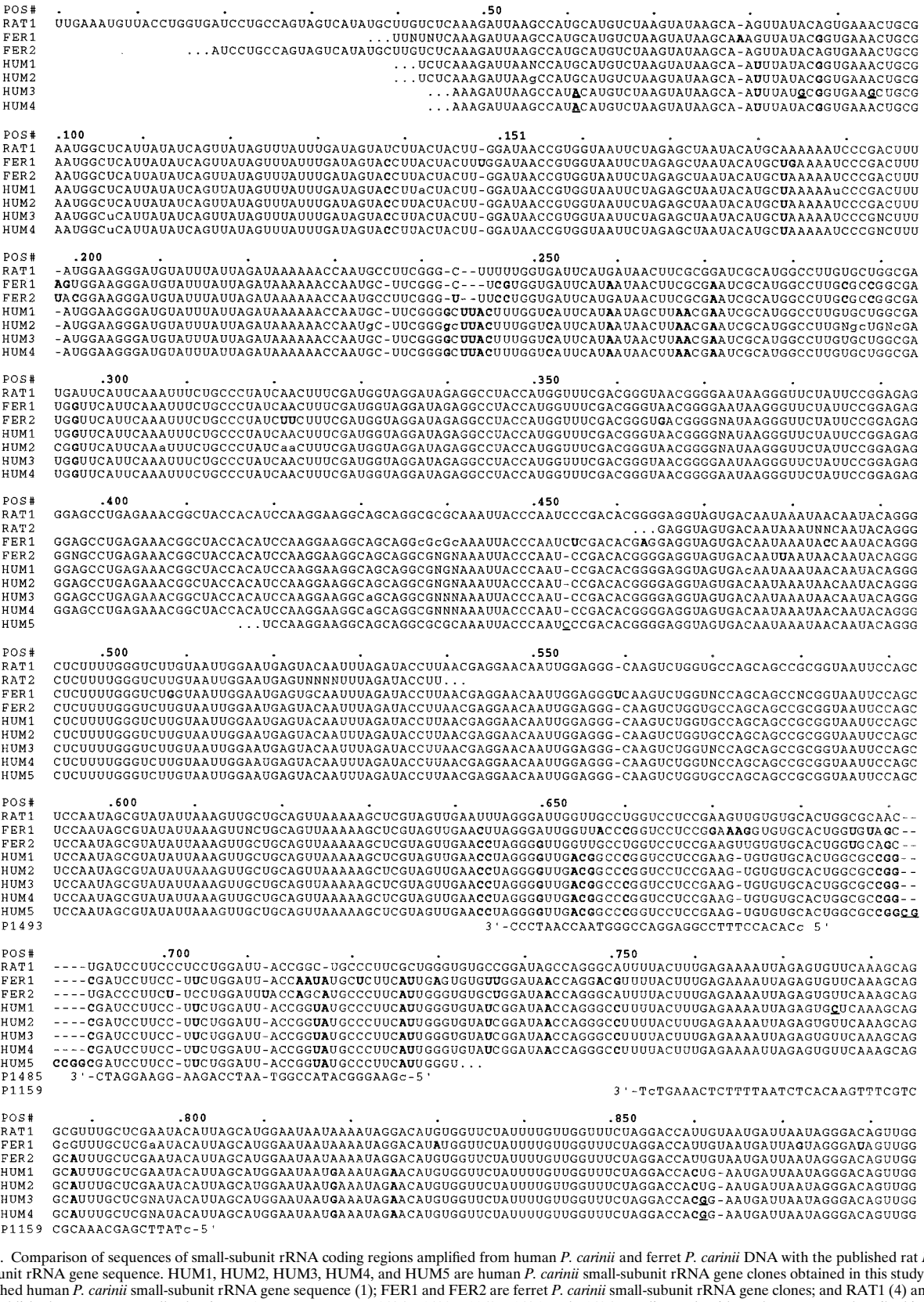

FIG. 1. Comparison of sequences of small-subunit rRNA coding regions amplified from human *P. carinii* and ferret *P. carinii* DNA with the published rat *P. carinii* small-subunit rRNA gene sequence. HUM1, HUM2, HUM3, HUM4, and HUM5 are human *P. carinii* small-subunit rRNA gene clones obtained in this study; HUM6 is a published human *P. carinii* small-subunit rRNA gene sequence (1); FER1 and FER2 are ferret *P. carinii* small-subunit rRNA gene clones; and RAT1 (4) and RAT2 (33) are published rat *P. carinii* small-subunit rRNA gene sequences. P1485, P1493, P1159, P1486, and P1495 are oligonucleotide probe sequences described in the text. Nucleotide differences between the RAT1 sequence and others are shown in boldface type. Nucleotide differences among human *P. carinii* sequences HUM1 through HUM6 are underlined.

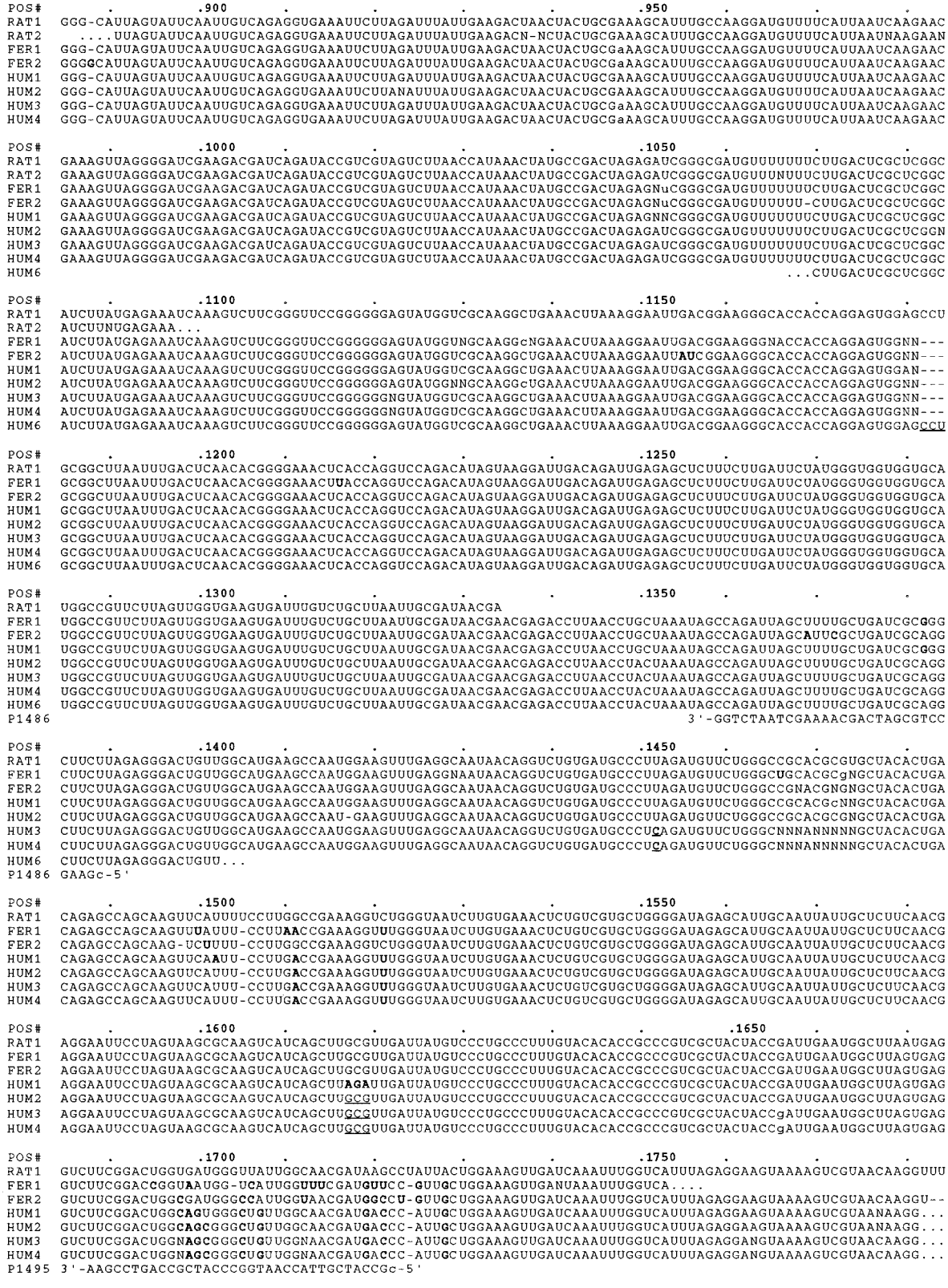

FIG. 1—*Continued.*

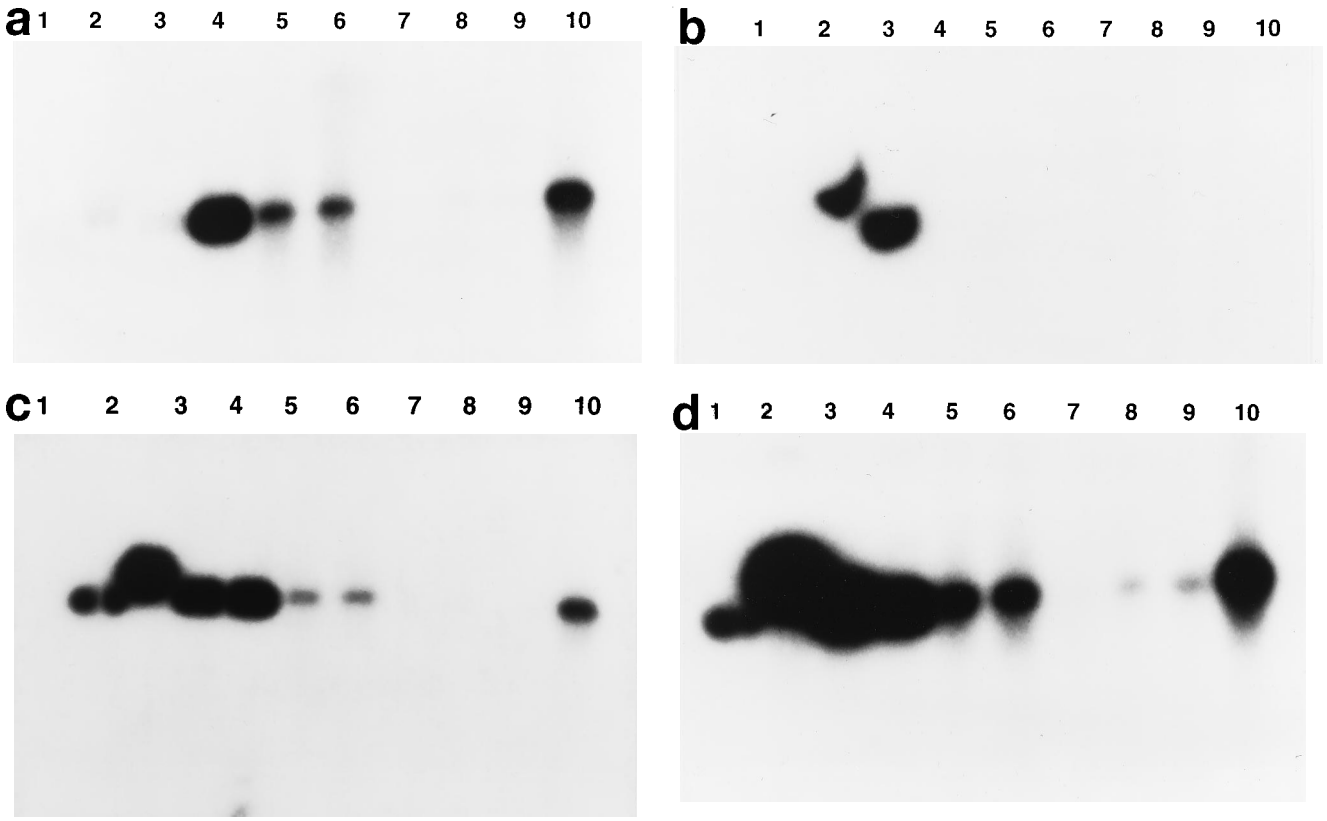

FIG. 2. Southern blot analysis of *P. carinii* probes. (a) Southern blot probed with ^{32}P-labeled human *P. carinii* species-specific probe 1485. (b) Second Southern blot probed with ^{32}P-labeled ferret *P. carinii* strain-specific probe 1493. (c) Blot a, washed and reprobed with ^{32}P-labeled *P. carinii* genus-specific probe 1486. (d) Blot b, washed and reprobed with ^{32}P-labeled fungus-specific probe 1159. Lanes 1 to 4 are *P. carinii* rRNA gene-containing plasmids digested with *Hin*dIII and *Sal*I: lanes 1, 2, and 3 are ferret *P. carinii* clones PCF1, PCF2, and PCF3, respectively; lane 4 is human *P. carinii* clone PCH3. Lanes 5 to 10 are PCR amplification products from clinical samples: lane 5 is from an HIV-positive, *P. carinii*-positive human BAL sample; lane 6 is from a *P. carinii*-positive human BAL sample from a transplant patient; lane 7 is from a *P. carinii*-negative BAL sample from a cancer patient; lanes 8 and 9 are from HIV-positive, *C. albicans*-positive, *P. carinii*-negative induced sputum samples; and lane 10 is from a *P. carinii*-positive human BAL sample from a cancer patient.

DISCUSSION

In the present study, colocalization of targets by in situ hybridization of rRNA-specific oligonucleotide probes and *P. carinii*-specific monoclonal antibodies confirmed that the cloned genes from the PCR amplification product were derived from *P. carinii* (Fig. 3). These results demonstrate that the two synthetic oligonucleotide primers A and B, originally described for in vitro amplification of eukaryotic small-subunit rRNA genes of *Skeletonema costatum* and *Kluyveromyces lactis* (23), also can be used to PCR amplify small-subunit rRNA genes of human and ferret *P. carinii*. In addition, the results in Fig. 3 show that *P. carinii* can be directly identified in clinical specimens by in situ hybridization with rhodamine X- or fluorescein (results not shown)-labeled *P. carinii*-specific probes targeted to rRNA. Preliminary studies with the human species-specific *P. carinii* probes indicate that *P. carinii* (all stages, i.e., cysts, trophozoites, spores, and intracystic bodies) can be easily identified in any clinical samples by in situ hybridization. The samples do not require any special preparation prior to in situ hybridization. Since rRNA is distributed throughout the cytoplasm, useful morphological information is obtained at the same time (3).

In the present study, two types of ferret *P. carinii* rRNA genes were amplified by PCR amplification, using primers A and B. FER1 was the predominant sequence (9 of 10 clones had the FER1 sequence) and FER2 was a minor sequence. In situ hybridization with fluorescein-labeled probes specific for each type confirmed the PCR results (Fig. 4). The in situ results clearly demonstrated that there were two different strains of *P. carinii* present in the ferret lung tissue and that the two rRNA genes were not present in the same *P. carinii* organism.

Although *P. carinii* isolates from different hosts are closely related (4, 5, 10), there are species-specific differences at the DNA level (6, 14, 16–18, 30, 32). Pulsed-field gel electrophoresis has been used to demonstrate that rat and human *P. carinii* chromosomes are similar but distinct (32). There is species-specific genotypic, as well as phenotypic, variation in the surface glycoprotein antigens of *P. carinii* isolated from humans, ferrets, rats, and mice (6). These results also are consistent with immunological studies in which species-specific *P. carinii* antigenic determinants have been identified by using monoclonal (7, 11) or polyclonal (5, 15, 37) antibodies. There is almost a 27% sequence variation in the 5′ end of the large-subunit mitochondrial rRNA among *P. carinii* organisms isolated from rats, humans, mice, ferrets, foals, rabbits, and horses (14, 26, 30, 35). Furthermore, there are sequence differences between the rat and the human *P. carinii* small-subunit (18S-like) and 26S rRNAs and in the intron located between nucleotides 1921 and 1922 of the 26S rRNA genes (16–18). The comparison of different host species-specific *P. carinii* small-subunit rRNA sequences (Fig. 1) and Southern blot analysis of PCR products

FIG. 3. In situ hybridization of a BAL sample: probed with (left) *P. carinii*-specific monoclonal antibodies, labeled with fluorescein (indicated by bright green fluorescence), and (right) counterstained with human *P. carinii* probe 1485, labeled with rhodamine X (indicated by bright red fluorescence).

with host species-specific ^{32}P-labeled *P. carinii* probes (Fig. 2) in the present study reveals that, although small-subunit rRNA genes of *P. carinii* isolated from different hosts are closely related to each other (less than 4% variation), there are species-specific patterns of sequence variation in *P. carinii* rRNAs. In addition, about 10 other *P. carinii*-positive clinical specimens from the New England region (including a small number from Cornell, N.Y.) also were all positive for *P. carinii* by in situ hybridization with human species-specific *P. carinii* probe 1485 (unpublished data).

Molecular studies suggest that strain-specific genetic diversity exists (at some loci) among isolates of *P. carinii* infecting a

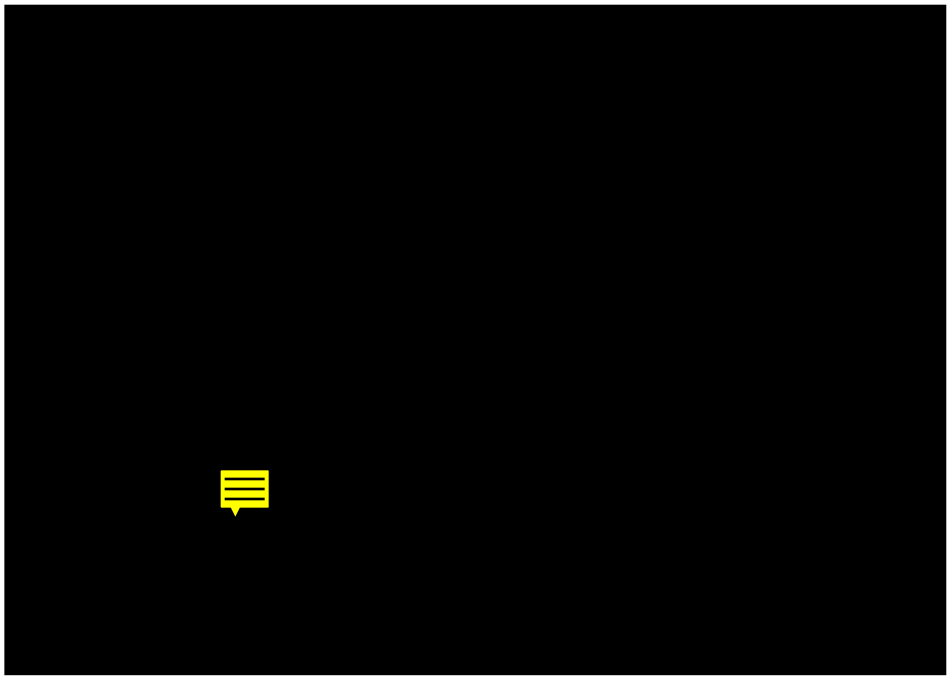

FIG. 4. In situ hybridization of *P. carinii*-infected ferret lung tissue: probed with ferret *P. carinii* probes 1493 (rhodamine X labeled) and 1495 (fluorescein labeled). Hybridization of the abundant species to probe 1493 is indicated by bright red fluorescence, and hybridization of minor species to probe 1495 is indicated by green fluorescence.

particular mammalian host (2, 19). Recently, it has been shown by pulsed-field gel electrophoresis that *P. carinii* isolates from different rats have different chromosomal patterns (19) and that a single rat can be coinfected with two variant strains of *P. carinii* (2). In infected ferret lung tissue, two types of *P. carinii* strains were present, as discussed earlier (Fig. 4). In contrast, we found that there were only minor differences (less than 1% variation) in the sequences between human *P. carinii* rRNA genes from 11 of 12 patients (one clone had a 6-base insertion at position 691). A human *P. carinii* partial small-subunit rRNA gene sequence from a human *P. carinii* isolate reported by Borensztein et al. (1) (HUM6; positions 1070 to 1402 in Fig. 1) also has greater than 99% identity to all of the human *P. carinii* rRNA sequences described here through the same region. The sequences of the 5.8S rRNA genes, partial 26S rRNA genes (93 bases), and the intron located between nucleotides 1921 and 1922 of the 26S rRNA gene (numbered according to the system for 23S rRNA of *Escherichia coli*) also are identical in all of the human isolates of *P. carinii* sequenced so far (16–18).

Further evidence that human *P. carinii* is a coherent species comes from the sequence data of the 5′ fragment of the small mitochondrial rRNA. There was greater than 99% identity among the 52 isolates sequenced: 30 by Wakefield et al. (35), 11 by Sinclair et al. (30), and 11 by Lee et al. (14). There is only a single report describing two different *P. carinii* mitochondrial rRNA gene sequences from one infected patient. One was a typical human *P. carinii* mitochondrial rRNA sequence, while the other was identical to that of rat *P. carinii* mitochondrial rRNA (18). Interestingly, in the same patient only one *P. carinii* cytoplasmic rRNA sequence was isolated (18), the sequence of which was identical to the "consensus" sequence of human *P. carinii* cytoplasmic small-subunit rRNA. In the present study, none of the human *P. carinii* rRNA gene clones had sequences similar to that of rat or ferret *P. carinii* small-subunit rRNA.

The present study suggests that human *P. carinii* may be a taxonomically coherent species at the rRNA sequence level. Hybridization surveys of additional geographically diverse *P. carinii* isolates, using the genus-specific, species-specific, and strain-specific probes identified here, would be useful in extending this conclusion to include broader geographic representation. On the basis of information available to date, and the results presented in this study, we conclude that human *P. carinii* is a coherent grouping at the species level and is genetically distinct from rat and ferret *P. carinii.*

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