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Typing of *Pneumocystis carinii* Strains with Type-Specific Oligonucleotide Probes Derived from Nucleotide Sequences of Internal Transcribed Spacers of rRNA Genes

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We have recently developed a method for typing *Pneumocystis carinii* strains that infect humans. The method takes advantage of nucleotide sequence variations in internal transcribed spacers (ITSs) of the rRNA genes of *P. carinii*. To date, two types of nucleotide sequences (designated types A and B) have been found in the ITS1 region, and three types of nucleotide sequences (designated types a, b, and c) have been found in the ITS2 region. Of the six potential combination types, we have detected four, designated types Ac, Bb, Ba, and Bc. To simplify typing, we have designed five oligonucleotide probes, probes 1-A, 1-B, 2-a, 2-b, and 2-c, which are specific to ITS1 type A and type B and ITS2 type a, type b, and type c, respectively, of *P. carinii* strains that infect humans. We also have designed an oligonucleotide which reacts specifically with *P. carinii* strains that infect rats. The ITS regions were amplified by PCR, and the PCR products were then probed with these type-specific oligonucleotide probes. Typing with the type-specific oligonucleotide probes was found to be effective with specimens containing only one type of *P. carinii*. These methods are rapid and simple to perform and will be useful for studying the epidemiology of *P. carinii* infections.

Pneumocystis carinii is a major cause of pneumonia in immunocompromised patients (3, 4, 9). Like those of most eukaryotic cells, the rRNA genes of *P. carinii* include those encoding 18S, 5.8S, and 26S rRNAs (2, 6, 10). These genes are located in a cluster but are not contiguous. The spaces located between these rRNA genes are referred to as internal transcribed spacers (ITSs). Two ITSs exist: ITS1 is located between the 18S and the 5.8S rRNA genes, and ITS2 is located between the 5.8S and the 26S rRNA genes.

We have recently discovered that nucleotide sequence variations exist in ITSs of the rRNA genes of *P. carinii* and that these nucleotide sequence variations can be used to type *P. carinii* strains that infect humans (referred to as human *P. carinii* hereafter). Two types of ITS1 sequences (designated A and B) and three types of ITS2 sequences (designated a, b, and c) were found (7). Therefore, there are six potential combination types of human *P. carinii*, and we have found four (types Ac, Ba, Bb, and Bc) to date. We also have found that the ITS sequences of *P. carinii* strains from rats (referred to as rat *P. carinii* hereafter) are distinct from those of human types (7).

The typing of *P. carinii* isolates described previously was achieved by determining the nucleotide sequences of ITS regions (7). Since DNA sequencing is quite time-consuming, we developed a DNA hybridization method for typing *P. carinii* isolates by using type-specific oligonucleotides (TSOs) designed on the basis of the ITS sequences that we have obtained previously.

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MATERIALS AND METHODS

Specimens. One *P. carinii*-infected rat lung and 25 clinical specimens were used for this study. The clinical specimens included 1 lung tissue specimen obtained by open lung biopsy and 24 bronchoalveolar specimens. They were portions of specimens sent to the Clinical Microbiology Laboratory, Indiana University Medical Center, for diagnostic tests. The open lung biopsy was from a 4-year old patient with acute lymphocytic leukemia, as described previously (5, 7). One bronchoalveolar specimen was from an AIDS patient with cytomegalovirus infection; the remaining 23 were from AIDS patients with *P. carinii* pneumonia.

Processing of specimens for PCR and PCR conditions. Procedures for the processing of specimens for PCR and the PCR method used in this study were as described previously (1, 7). The nested PCR method, designated Pc-ITS-PCR, amplified the ITS regions of the rRNA genes of *P. carinii*. Primers 1724F (AAGTTGGTCAAATTTGGTC) and ITS2R (CTCGGACGAGGATCCTCGCC) were used for the first step, and primers ITS1F (CGTAGGTGAACCTGCGGAAGGATC) and ITS2R1 (GTTCAGCGGGTGATCCTGCCTG) were used for the second step of the Pc-ITS-PCR.

Sequencing of PCR products. Most PCR products were sequenced directly. They were purified by using the Magic PCR Preps kit (Promega, Madison, Wis.) and then sequenced by using the *fmol* DNA sequence kit (Promega) according to the manufacturer's protocol. Those that could not be sequenced directly were cloned into the TA cloning vector pCRII (Invitrogen, San Diego, Calif.) and then sequenced by using the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) with appropriate primers. In these cases, at least three clones of the same PCR products were sequenced to verify the sequences, as described previously (7).

Design of type-specific probes. TSO probes were designed on the basis of the ITS sequences described previously (7). Two oligonucleotides based on the sequence difference between ITS1 types A and B were synthesized. Oligonucleotide 5′-TTGTTTGGCGGAGCTGG-3′ (Fig. 1, positions 65 to 84, double-underlined sequence) was used as the type A-specific probe, and oligonucleotide 5′-TTGTTTGGCGAGGAGCTGG-3′ was the type B-specific probe (the two underlined nucleotides are the bases which differ in the two probes). A similar approach was taken to design type-specific probes for ITS2 types. The sequence 5′-AAAGCTTTTATAGATAC-3′ was chosen as the type a-specific probe, and the sequence 5′-AAAGCTTTTATATAGATAC-3′ was used as the type b-specific probe (Fig. 2, positions 151 to 169, double-underlined sequence). For the type c-specific probe, the sequence 5′-TATTTAGAAATAATTATATGC-3′ (Fig. 2, positions 48 to 75) was used because it differs from those of types a and b at 9 bases in this area. Another oligonucleotide, with the sequence 5′-TTGTCAAGAACTAGTTTATC-3′, located in the ITS1 region (Fig. 3, positions 8 to 40, double-underlined sequence), was used as the rat *P. carinii*-specific probe. This

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Type  A  GAAAACTCAG CTTTAAACAC TTCCCTAGTG TTTTAGCATT TTTCAAACAT   50
Type  B  .....T.... ...-...... .......... .......... ..........

Type  A  CTGTGAATTT TTTTTT-GTT TGGCG--GAG CTGGCTTTTT TGCTTGCCTC  100
Type  B  .......... ......T... .....AG... .......... ..........

Type  A  GCCAAAGGTG TTTATTTTTA AAATTTTAAA TTGAATTTCA GTTTAGAATT  150
Type  B  .......... .......... .......... .......... ..........

Type  A  TTTTAA                                                 156
Type  B  ......
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FIG. 1. Nucleotide sequences of type A and B ITS1 regions of human *P. carinii*. The sequences above and below the double line (positions 65 to 84) were used to make the type A- and B-specific oligonucleotide probes, respectively. Identical sequences are represented with periods, missing bases are represented with dashes, and sequence differences are as indicated.

sequence was chosen because it had only 50% homology with that of human *P. carinii* within the corresponding region. All of the type-specific probe sequences were determined to be unique and specific to *P. carinii* by comparing them with sequences in GenBank.

Dot blot hybridizations. Fifty microliters of each PCR product was mixed with 1,200 μl of 0.4 N NaOH to denature the DNA. This 1,250-μl volume was then divided into six aliquots (approximately 200 μl per aliquot). These aliquots were applied onto a Nytran membrane (Schleicher & Schuell, Keene, N.H.) by using a dot blot apparatus. The membrane was air dried, baked for 2 h at 80°C in a vacuum oven, and cut into six identical strips. Each strip of the membrane contained four sample spots of PCR products from rat *P. carinii* and human *P. carinii* types Ac, Bb, and Ba. The strips were labelled R, 1-A, 1-B, 2-a, 2-b, and 2-c, respectively. The membrane strips were sealed separately in plastic bags and prehybridized with a prehybridization solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 0.05% sodium PP_i, and 100 μg of denatured calf thymus DNA per ml. After an overnight incubation at 37°C, the prehybridization solutions in the bags were replaced with hybridization solutions containing different type-specific probes; these probes were 5′ labelled with ^{32}P. The hybridization was allowed to proceed at 37°C overnight. The membrane strips were then removed from the bags and washed with 2× SSC–0.1% SDS at various temperatures according to the melting temperature of each probe. The washing temperatures for the R, 1-A, 1-B, 2-a, 2-b, and 2-c probes were 50, 50, 55, 37, 42, and 42°C, respectively. After being washed, the membrane strips were wrapped in plastic film and used to expose an X-ray film.

RESULTS

Determination of probe specificity. To determine the specificities of the TSO probes, they were reacted with PCR products of known types. These PCR products were generated by using recombinant plasmids containing ITS regions of various types as templates, as follows: pHPC1, type Ac; pHPC3, type Ba; pHPC2, type Bb; pRPC, rat type. The results of these experiments are shown in Fig. 4. The R probe reacted only with the rat-type ITS. Probe 1-A reacted strongly with type Ac and slightly with types Bb and Ba, probably because of insufficient washing of the filter. Probe 1-B reacted with types Bb and Ba, and probe 2-a reacted only with type Ba. Probe 2-b reacted strongly with type Bb and only slightly with type Ba. These results suggest that these probes are type specific.

Application of TSO probes to specimens. The TSO probes were then used to type *P. carinii* isolates from clinical specimens. PCRs were performed to amplify the ITS regions of *P. carinii* isolates from 1 infected rat lung, 1 biopsy, and 24 bronchoalveolar specimens. These PCR products and control samples were each divided into six aliquots, and each aliquot was probed with a TSO probe. The positive controls included one each of the PCR products of *P. carinii* strains of the rat, Ac, Bb, and Ba types amplified from the recombinant plasmids mentioned above. Two negative controls were included. One was a PCR without template DNA, and the other was the PCR product of one (specimen SZ) of the 25 clinical specimens, from a patient who had cytomegalovirus pneumonia.

The control specimens of known types (types Ac, Bb, Ba, and rat) were typed correctly by the TSO probes (Fig. 5). The PCR products of the *P. carinii* isolates from specimens 6, 38, and T-4 reacted with TSO probes 1-A and 2-c and were determined to be type Ac. Those from specimens 13, 22, 36, and 102 reacted with TSO probes 1-B and 2-b and were identified to be type Bb. The PCR products of the *P. carinii* isolates from specimens 7, 7A, 95, and 96 hybridized with TSO probes 1-B and 2-a; therefore, they were determined to be type Ba. Specimen 94 was a *P. carinii*-infected rat lung; its PCR products reacted only with the rat TSO probe, indicating that the specimen contained rat *P. carinii*. PCR products from five specimens (1A, 2A, 3A, 4A, and 5A) reacted with the rat TSO probe and with probes 1-B and 2-a, suggesting that these five speci-

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Type  a  TTAAGTTCCT TTTTTCAAGC AGAAAAAAGG GGATTGGGCT TTGCAAATAT    50
Type  b  .......... .......... .......... .......... .......... 
Type  c  .......... .......... .......... .......... .........-

Type  a  AATTAGAA-T AAAATATTTA TATGCATGCT AGTCTGAAAT TCAAAAGTAG  100
Type  b  ........-. .......... .......... .......... ..........
Type  c  --......A. ..-----... .......... .........G ..........

Type  a  CTTTTTTTCT TTGCC-TAGT GTCGTAAAAA TTCGCTGGGA AAGAAGGAAA  150
Type  b  .......... .....C.... .......... .......... ..........
Type  c  .......... .....-.... .......... .......... ..........

Type  a  AAAGCTTTT- -ATAGATACA AGAATTT                          177
Type  b  .........A T......... .......
Type  c  ..........- -...A..... .......
```

FIG. 2. Nucleotide sequences of type a, b, and c ITS2 regions of human *P. carinii*. The sequence below the double line at positions 48 to 75 was used to produce the type c-specific oligonucleotide probe. The sequences above and below the double line at positions 151 to 169 were used to make the type a- and b-specific oligonucleotide probes, respectively. Symbols are as described in the legend to Fig. 1.

```
Rat Pc  ATGAAA-TGT TGTCAAG--- AA-------- CTAGTTTATC TGGTTCTT-G     50
Hu Pc   --....A.-- --...-.CTT ..ACACTTCC .....----- --...-..A.

Rat Pc  ACATTTTCAT CATAACA-CT TGTGAACATT AAAGAT---T TGCTTTGAC-     100
Hu Pc   -...C..--. ..-....T.. -.....--.. ---TT.TTT. ..-....G.G

Rat Pc  AGGA--TGGG AG-----TAA GCTTTCGTCC T-GTC--AGA GGT-TTTCAA     150
Hu Pc   ....GC...- --CTTTT.T- .....--.-. .C.-.CA.-. ...G...--.

Rat Pc  TT---AAAAC TTTTT---TG GTG--TTTC- GGTTAAAAAT AT--AATTTT     200
Hu Pc   ..TTT....- -....AAA.- -..AA....A .-..-----. -.AG......

Rat Pc  T-AA
Hu Pc   .T..
```

FIG. 3. Comparison of nucleotide sequences of rat and human (type B) *P. carinii* ITS1 regions. The sequence above the double line at positions 8 to 40 (5′-TTGTCAAGAACTAGTTTATC-3′) was used to produce the rat type-specific oligonucleotide probe. Abbreviations: Pc, *P. carinii*; Hu, human.

mens contained a rat *P. carinii* strain and a type Ba human *P. carinii* strain. All of these TSO typing results agreed completely with those of typing by sequencing (Table 1).

The PCR products of specimen 12 reacted with the R, 1-B, 2-a, and 2-c probes, suggesting that the specimen contained a rat *P. carinii* type and two human *P. carinii* types (Ba and Bc); however, the specimen was determined to contain a rat type and only one human type (Ba) by sequencing. This may be due to the possibility that the recombinant clones picked for sequencing did not represent all *P. carinii* types present in the sample. The PCR products of specimens 34 and 35 reacted with probes 1-A, 1-B, 2-b, and 2-c. According to these results, these two samples could contain type Ab, Ac, Bb, and Bc *P. carinii*. However, specimen 34 was determined to contain types Ac and Bb, and specimen 35 was determined to contain types Bb and Bc, by sequencing. It is unknown whether these two samples actually contain four or two types of *P. carinii*. The situation for specimens T-2 and 37 was similar to that for specimens 34 and 35. Both T-2 and 37 were determined to have types Ac and Ba by sequencing but could have types Aa, Ac, Ba, and Bc, since their PCR products hybridized with probes 1-A, 1-B, 2-a, and 2-c. Specimens 6A, T-1, and T-3 had more complicated results. The PCR products of these three samples hybridized with probes 1-A, 1-B, 2-a, 2-b, and 2-c; therefore, they all could have *P. carinii* of types Aa, Ab, Ac, Ba, Bb, and Bc. However, sample 6A was determined to have types Ba and Bc, sample T-1 was shown to have type Ba, and sample T-3 was determined to contain type Bb by sequencing.

DISCUSSION

The PCR method used in this study has been shown previously to be specific for *P. carinii* (1, 8). In this study, the TSO probes, R, 1-A, 1-B, 2-a, 2-b, and 2-c, were shown to be specific by their reactions with specific types of *P. carinii*. The R probe had only 50% homology with the corresponding region of human *P. carinii* and reacted only with rat *P. carinii*. It had no cross-reaction with human *P. carinii* under the hybridization conditions used in this study. Although the 1-A and 1-B probes differ from each other by only 2 nucleotides, they were able to distinguish type A and B ITS1 regions, respectively (Fig. 4). Similarly, the 2-a and 2-b probes were able to distinguish type a and b ITS2 regions, respectively, although they also differ by only 2 bases. The 2-c probe differs from the 2-a or 2-b probe by 9 bases and was very specific to type c ITS2. No nonspecific hybridizations were observed.

The effectiveness of the TSO probes in typing *P. carinii* isolates was demonstrated by comparing the TSO typing results with those of typing by sequencing. The *P. carinii* isolates in all specimens containing only one type of *P. carinii* were typed exactly the same by both methods (Table 1). The ability to type *P. carinii* isolates by use of TSO probes would simplify typing tremendously. Before the development of the TSO typing method, typing of *P. carinii* isolates was done by DNA sequencing. Most DNA sequences in this study were achieved by direct sequencing of PCR products. However, some PCR products could not be sequenced directly. They had to be cloned and then sequenced. This process included cloning, screening of recombinant clones, and sequencing of the inserts. Because of the possibility of cloning nonspecific fragments, many recombinant clones had to be sequenced, and each clone had to be sequenced several times to ensure the accuracy of sequences. The entire process required at least a month to complete. With TSO typing, results were obtained within 3 days.

As described above, TSO typing failed to produce conclusive results for specimens containing more than one type of *P. carinii*. Although no such samples were found in this study, TSO typing would work on those containing more than one type of *P. carinii* if the isolates had the same ITS1 type. For example, a specimen that contains type Aa and Ab *P. carinii* will generate PCR products that will react with probes 1-A, 2-a, and 2-b but not with probes 1-B and 2-c. Similarly, the PCR products will react with probes 1-B, 2-a, and 2-b if the specimen contains type Ba and Bb *P. carinii*. In this study, five specimens were found to contain both a rat *P. carinii* strain and a type Ba human *P. carinii* strain. Since the ITS1 sequence of rat *P. carinii* was distinct from those of human types, these samples were typed identically by both the TSO and sequencing methods. However, TSO typing will not work if a specimen contains more than one type of human *P. carinii* with different ITS1 types. For example, if a sample reacts with the 1-A, 1-B, 2-a, and 2-b probes, it is not known whether this specimen

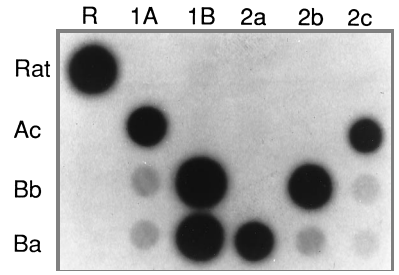

FIG. 4. Reactions of various ITS types with type-specific probes. Recombinant plasmids containing ITS regions of rat and human (types Ac, Bb, and Ba) *P. carinii* were probed with labelled R, 1-A, 1-B, 2-a, 2-b, and 2-c TSO probes.

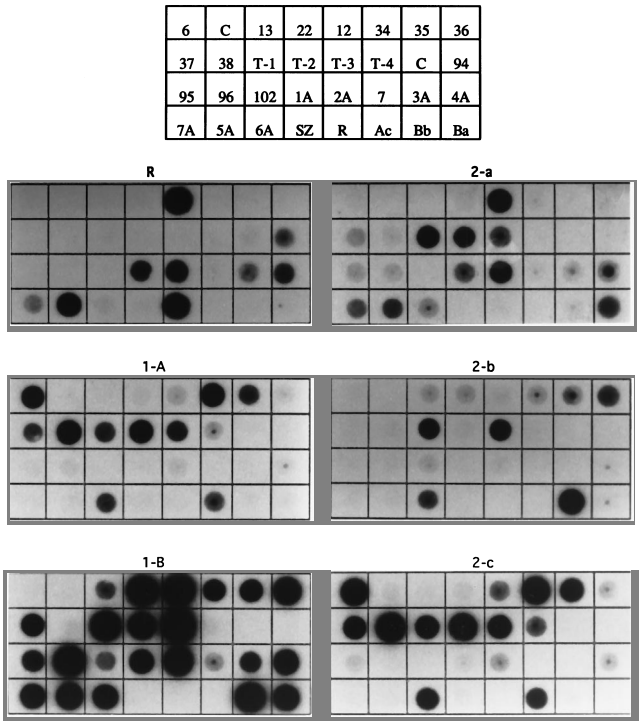

FIG. 5. Reactions of ITS PCR products from specimens containing *P. carinii*. The locations of samples on each membrane are indicated on the grid at the top.

contains types Aa and Ba; Aa and Bb; Ab and Ba; Ab and Bb; Aa, Ab, Ba, and Bb; or other combinations.

For specimens that contain *P. carinii* isolates with different ITS1 types, typing will have to be done by cloning and then sequencing, since direct sequencing of PCR products will not work. Typing by cloning and sequencing for these samples may also be problematic. This was seen with specimen 12 (Table 1), which was determined to contain a rat type and two human types (Ba and Bc) by TSO typing but was determined to contain a rat type and only one human type (Ba) by sequencing. This was an example of the problem with typing by sequencing if the recombinant clones picked do not represent all organism types present in a sample. In order to type these specimens, a different typing method will have to be developed. If the specimens contain *P. carinii* with both type A and B ITS1 sequences, the Ax and Bx (where x may be a, b, or c) types must be separated to allow typing by use of TSO probes. Since the sizes of Ax and Bx are almost identical, it is difficult to separate these types by size. In addition, it is impossible to distinguish them by restriction enzyme analysis, since the nucleotide sequences that are different in the two types do not contain any restriction sites. We are developing a type-specific PCR to specifically amplify Ax or Bx.

The TSO typing method described in this paper is for ITS types that have been identified. As more *P. carinii* isolates are examined, new types may be discovered and new TSO probes may be developed. Although TSO typing had limitations with specimens containing mixed types, these specimens were not the majority; only 30% of the specimens examined in this study contained mixed types. The ability to type *P. carinii* isolates will enable study of the prevalences of different types in different geographical locations and correlation of various types with clinical symptoms. Typing will also allow study of potential differences in virulence and in drug resistance of different *P. carinii* strains. A very important application of typing is to determine whether a relapse is due to reactivation of the original strain or to reinfection by a second strain.

TABLE 1. Summary of results of typing by TSO probes

Specimen	Reaction with TSO probe:						Type(s) determined by:	
	R	1-A	1-B	2-a	2-b	2-c	TSO probes	Sequencing
No template DNA[a]	−	−	−	−	−	−		
SZ[a]	−	−	−	−	−	−		
I (Ac)[b]	−	+	−	−	−	+	Ac	Ac
6	−	+	−	−	−	+	Ac	Ac
T-4	−	+	−	−	−	+	Ac	Ac
38	−	+	−	−	−	+	Ac	Ac
II (Bb)[b]	−	−	+	−	+	−	Bb	Bb
13	−	−	+	−	+	−	Bb	Bb
22	−	−	+	−	+	−	Bb	Bb
36	−	−	+	−	+	−	Bb	Bb
102	−	−	+	−	+	−	Bb	Bb
III (Ba)[b]	−	−	+	+	−	−	Ba	Ba
95	−	−	+	+	−	−	Ba	Ba
96	−	−	+	+	−	−	Ba	Ba
7	−	−	+	+	−	−	Ba	Ba
7A	−	−	+	+	−	−	Ba	Ba
R[b]	+	−	−	−	−	−	Rat	Rat
94	+	−	−	−	−	−	Rat	Rat
1A	+	−	+	+	−	−	Rat, Ba	Rat, Ba
2A	+	−	+	+	−	−	Rat, Ba	Rat, Ba
3A	+	−	+	+	−	−	Rat, Ba	Rat, Ba
4A	+	−	+	+	−	−	Rat, Ba	Rat, Ba
5A	+	−	+	+	−	−	Rat, Ba	Rat, Ba
12	+	−	+	+	−	+	NC[c]	Rat, Ba
34	−	+	+	−	+	+	NC	Ac, Bb
35	−	+	+	−	+	+	NC	Bb, Bc
T-2	−	+	+	+	−	+	NC	Ac, Ba
37	−	+	+	+	−	+	NC	Ac, Ba
6A	−	+	+	+	+	+	NC	Ba, Bc
T-1	−	+	+	+	+	+	NC	Ba
T-3	−	+	+	+	+	+	NC	Bb

[a] Negative control.
[b] Positive control.
[c] NC, not conclusive.

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REFERENCES

1. **Atzori, C., J. J. Lu, M. S. Bartlett, G. Orlando, S. F. Queener, J. W. Smith, A. Cargnel, and C. H. Lee.** Diagnosis of *Pneumocystis carinii* pneumonia in AIDS patients using polymerase chain reactions on serum specimens. J. Infect. Dis., in press.
2. **Edman, J. C., J. A. Kovacs, H. Masur, D. V. Santi, H. J. Elwood, and M. L. Sogin.** 1988. Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the fungi. Nature (London) **334:**519–522.
3. **Hawley, P. H., J. J. Ronco, S. A. Gillemi, J. Quieffin, J. A. Russell, L. M. Lawson, M. T. Schechter, and J. S. G. Montaner.** 1994. Decreasing frequency but worsening mortality of acute respiratory failure secondary to AIDS-related *Pneumocystis carinii* pneumonia. Chest **106:**1456–1459.
4. **Hughes, W. T.** 1977. *Pneumocystis carinii* pneumonia. N. Engl. J. Med. **297:** 1381–1383.
5. **Lee, C. H., J. J. Lu, M. S. Bartlett, M. M. Durkin, T. H. Liu, J. Wang, B. Jiang, and J. W. Smith.** 1993. Nucleotide sequence variation in *Pneumocystis carinii* strains that infect humans. J. Clin. Microbiol. **31:**754–757.
6. **Liu, Y., M. Rocourt, S. Pan, C. Liu, and M. J. Leibowitz.** 1992. Sequence and variability of the 5.8S and 26S rRNA genes of *Pneumocystis carinii*. Nucleic Acids Res. **20:**3763–3772.
7. **Lu, J. J., M. S. Bartlett, M. M. Shaw, S. F. Queener, J. W. Smith, M.**

Ortiz-Rivera, M. J. Leibowitz, and C. H. Lee. 1994. Typing of *Pneumocystis carinii* strains that infect humans based on nucleotide sequence variations of internal transcribed spacers of rRNA genes. J. Clin. Microbiol. **32:**2904–2912.

8. **Lu, J. J., C. H. Chen, M. S. Bartlett, J. W. Smith, and C. H. Lee.** 1995. Comparison of six different PCR methods for detection of *Pneumocystis carinii*. J. Clin. Microbiol. **33:**2785–2788.
9. **Sepkowitz, K. A.** 1993. *Pneumocystis carinii* pneumonia in patients without AIDS. Clin. Infect. Dis. **17:**S416–S422.
10. **Stringer, S. L., J. R. Stringer, M. A. Blase, P. D. Walzer, and M. T. Cushion.** 1989. *Pneumocystis carinii*: sequence from ribosomal RNA implies a close relationship with fungi. Exp. Parasitol. **68:**450–461.