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Development of Type-Specific PCR for Typing *Pneumocystis carinii* f. sp. *hominis* Based on Nucleotide Sequence Variations of Internal Transcribed Spacer Regions of rRNA Genes

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The nucleotide sequence variations in the internal transcribed spacer region 1 (ITS1) and region 2 (ITS2) of rRNA genes were found to be useful for typing *Pneumocystis carinii* isolates that infect humans. Two types of ITS1 (A and B) and three types of ITS2 (a, b, and c) sequences have been found, and *P. carinii* isolates are classified based on sequence types of ITS1 and ITS2 as Ax or Bx (where x may be a, b, or c). Type determination has been achieved by sequencing the ITS regions or by reacting the ITS regions amplified by PCR with type-specific oligonucleotide (TSO) probes. However, TSO typing alone does not work on a specimen from an individual who is infected by more than one strain of *P. carinii* where different ITS1 types are present in the same specimen. In this study, type-specific PCR assays were developed to supplement TSO typing. Type-specific PCR primers were made so that they differ at their 3′ ends by the two nucleotides which distinguish type A from type B of ITS1 plus an additional "A" residue at the extreme 3′ ends of the primers. These two primers were paired separately with a general primer which anneals to a region downstream from ITS2 to specifically amplify Ax or Bx. The amplified products were then reacted separately with ITS2-specific probes 2-a, 2-b, and 2-c to identify their types.

Multiple strains of *Pneumocystis carinii* f. sp. *hominis* have been shown to exist. These strains differ in the nucleotide sequences of certain genetic loci (1, 3, 4, 6, 7, 10). One locus that has been shown to have nucleotide sequence variations is the internal transcribed spacer (ITS) region of the nuclear rRNA genes (6, 7). There are two ITS regions in the rRNA genes of *P. carinii*. 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. Two types of ITS1 (A and B) and three types of ITS2 (a, b, and c) sequences have been found (6), and *P. carinii* isolates are classified based on sequence types of ITS1 and ITS2 as Ax or Bx (where x may be a, b, or c). Combinations of ITS1 types with any of the ITS2 types may theoretically be found; therefore, a *P. carinii* isolate may belong to any of the following potential types: Aa, Ab, Ac, Ba, Bb, and Bc. To date, types Ac, Ba, Bb, and Bc have been found in infected patients (6).

Typing of *P. carinii* isolates based on ITS sequence variations has been achieved by determining the nucleotide sequences of the ITS regions amplified by PCR (6). This approach is quite time-consuming and laborious. To simplify the typing procedure, a method using oligonucleotide probes specific for each ITS type has been developed (7). These probes include ITS1 probes 1-A and 1-B and ITS2 probes 2-a, 2-b, and 2-c and are designed based on the unique sequence of each ITS type. This method is referred to as type-specific oligonucleotide (TSO) typing (7). Nested PCR is performed to amplify the entire ITS regions (both ITS1 and ITS2) of *P. carinii* from a specimen. The PCR products are then reacted with radiolabeled TSO probes. This method requires much less time to perform than sequencing, and many samples can be assayed at the same time. However, TSO typing is not effective on specimens containing multiple strains if these strains have different ITS1 types. For example, if a specimen contains both types Ac and Bb, the PCR product will react with probes 1-A, 1-B, 2-b, and 2-c. It is not certain whether the specimen contains types Ac and Bb or types Ab, Ac, Bb, and Bc.

TSO typing would work if the PCR products of the two different types, Ax and Bx, are separated and then reacted with TSO probes. Since the PCR products of Ax and Bx are almost identical in size, they cannot be separated by electrophoresis. There are also no appropriate restriction enzymes that specifically digest type Ax or Bx. To solve this problem, type-specific PCRs were developed to specifically amplify the Ax or Bx ITS region. Since ITS1 type A and type B differ by two base pairs at ITS1 nucleotide positions 76 and 77 (6), two type-specific primers were synthesized based on this sequence difference. The sequences of these two primers are 5′-GTGAATTTTTT TTTGTTTGGCGGA-3′ (ITS1A2) and 5′-GTGAATTTTTTT TTGTTTGGCGAG-3′ (ITS1B1). These two primers differ at the last two nucleotides.

For development of the type-specific PCR methods, four different recombinant plasmids were used, each of which contained one type of ITS sequence, Ac, Ba, Bb, or Bc, as described previously (6). Nested PCRs were performed to amplify the ITS region. The first PCR was performed with primers 1724F and ITS2R (Fig. 1) on plasmid samples of known types, including types Ac, Ba, Bb, Bc, Ac plus Ba, Ac plus Bb, Ac plus Bc, Ba plus Bb, Ba plus Bc, and Bb plus Bc. PCR was performed in a reaction mixture containing PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM $MgCl_2$, 0.001% gelatin), 0.2 mM (each) PCR primer, 0.2 mM (each) deoxynucleoside

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FIG. 1. Locations and sequences of primers for type-specific PCR. Primer sequences used for type-specific PCRs are boxed with solid lines and are labeled with names above or below the sequences. Primer 1724F, which was used previously as a primer for the first-step nested PCR for TSO typing, is boxed with dotted lines. The locations of the areas amplified with each set of primers are indicated with nucleotide numbers above the sequences.

triphosphate, and 2.5 U of *Taq* DNA polymerase in a total volume of 50 μl. Small aliquots (~2 μl) of the first PCR products were then used as templates for the second PCR. Primers ITS1A2 and ITS1B1 were used with primer ITS2R1 in separate reactions in an attempt to amplify types Ax and Bx, respectively. Various Mg^{2+} concentrations in the reaction mixture, ranging from 0.5 to 5.0 mM with an increment of 0.5 mM in different reactions, were used. The temperatures for denaturation and DNA synthesis were set at 94 and 72°C, respectively. Various primer annealing temperatures were tried, including 42, 45, 48, 51, 54, 57, 60, 63, 66, 69, and 72°C. The lengths of time for denaturation, primer annealing, and DNA synthesis varied by 15-s increments from 30 s to 2 min.

Several sets of conditions which allowed primers ITS1B1 and ITS2R1 to specifically amplify Bx were found. Unfortunately, none of the various conditions that were tried allowed specific amplification of Ax with primers ITS1A2 and ITS2R1. The above-mentioned primers, ITS1A2 and ITS1B1, differ from each other by only 2 nucleotides at their 3′ ends. It was described by Cha et al. (2) that addition of a mismatched base at the extreme 3′ end of the primers can improve the specificity of allele-specific PCR. This technique was referred to as the mismatch amplification mutation assay (2). To determine whether this technique would work for type-specific PCR of human *P. carinii*, an additional A residue was added to the 3′ ends of both ITS1A2 and ITS2B1. The new primers were designated ITS1A3 (5′-GTGAATTTTTTTTTGTTTGGCGGAA-3′) and ITS2B2 (5′-GTGAATTTTTTTTTGTTTGGCGAGA-3′). In this manner, the two primers differ at their 3′ ends by three bases. The A residue of the extreme 3′ ends of both primers is a mismatched base which is forced to pair with the C residue on the native templates.

Since ITS1A3 has a T_m of 66°C, it was paired with primer ITS2R2 which has a T_m of 60°C. Primer ITS1B2 was paired with ITS2R1 because their T_ms are similar (ITS1B2, 68°C and ITS2R1, 72°C). The length of primer 1724F was increased from 19 to 26 nucleotides so that its T_m was the same as that of ITS2R (68°C), and the new primer was named 1724F2. Primer set 1724F2-ITS2R was used for the first-step PCR, and primer sets ITS1A3-ITS2R2 and ITS1B2-ITS2R1 were used for the second-step PCR to specifically amplify type Ax or Bx. Many PCR conditions as described above were tried. The following were found to be the most effective: the first-step PCR was 40 cycles of 94°C for 1 min, 47°C for 1 min, and 72°C for 3 min, with a Mg^{2+} concentration of 3.0 mM. The second step PCR was 35 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min, with a Mg^{2+} concentration of 1.5 mM for both primer sets (1A3-2R2 and 1B2-2R1). Under these conditions,

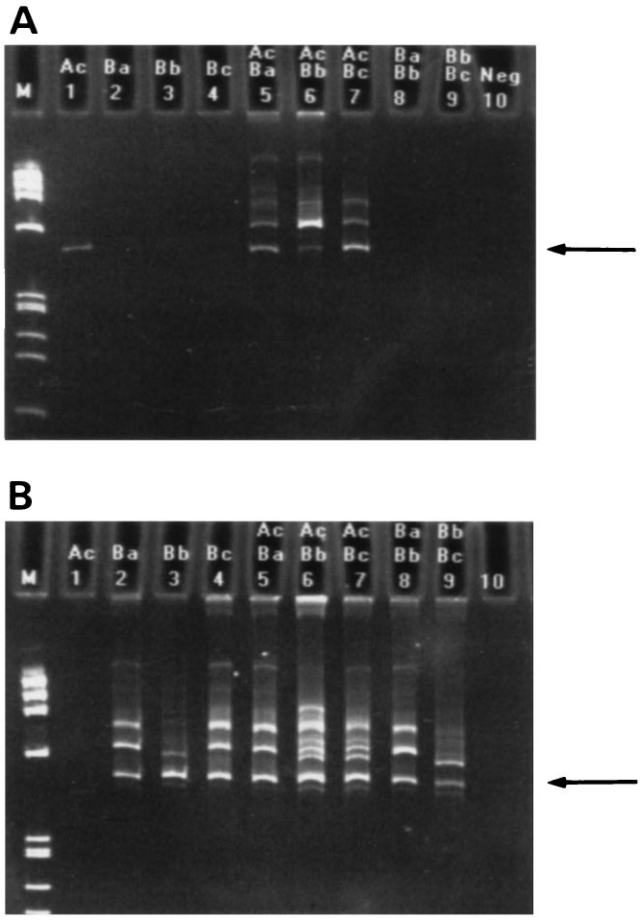

FIG. 2. Results of type-specific PCR. (A) Type A-specific PCR. (B) Type B-specific PCR. The lanes containing size markers are labeled as M. Other lanes are labeled with numbers. Lane 10 of both panels contained the negative control which was PCR performed with no template DNA. The types of ITS sequences present in each sample are as indicated. The arrow indicates the band of expected size of type-specific PCR products.

the first-step PCR amplified a fragment of approximately 690 base pairs and the second-step PCR amplified a fragment of approximately 470 base pairs. As shown in Fig. 2A, type A-specific PCR with the primer set ITS1A3-ITS2R2 only amplified specimens containing Ax. No bands are seen in lanes 2, 3, 4, 8, and 9 that contained type-specific PCR products amplified from samples with no Ax type. Multiple bands are present in lanes 1, 5, 6, and 7 that contained types Ac, Ac plus Ba, Ac plus Bb, and Ac plus Bc, respectively. The lowest band in these lanes is the one with the expected size.

Similarly, type B-specific PCR with the primer set ITS1B2-ITS2R1 generated positive results only from samples containing Bx (Fig. 2B). No band is observed in lane 1 that contained the reaction mix of type-B specific PCR on a sample containing type Ac (Fig. 2B). Bands are seen in lanes 2 to 9 that contained PCR products of samples containing types Ba, Bb, Bc, Ac plus Ba, Ac plus Bb, Ac plus Bc, Ba plus Bb, and Bb plus Bc, respectively (Fig. 2B).

In this study, a negative type-specific PCR generated no products, so no bands were seen when the reaction mixtures were run on the gel. However, almost all positive type-specific PCRs produced multiple bands in addition to the specific band (Fig. 2). Although numerous PCR conditions were tried, a set of conditions which would result in production of only the

TABLE 1. Typing of *P. carinii* isolates by type-specific probes and type-specific PCRs

Specimen no(s).	ITS sequence type by reaction with TSO probes	Result with type A specific assay[a]				Result with type B specific assay[a]				Type determination
		PCR	Probes			PCR	Probes			
			2-a	2-b	2-c		2-a	2-b	2-c	
1	A, c	+	−	−	+	−	ND	ND	ND	Ac
2	B, a	−	ND	ND	ND	+	+	−	−	Ba
3	B, b	−	ND	ND	ND	+	−	+	−	Bb
4	B, c	−	ND	ND	ND	+	−	−	+	Bc
5–12	A, B, a, c	+	−	−	+	+	+	−	−	Ac and Ba
13–14	A, B, b, c	+	−	−	+	+	−	+	−	Ac and Bb
15–17	B, a, c	−	ND	ND	ND	+	+	−	+	Ba and Bc

[a] +, positive reaction; −, negative reaction; ND, not done.

specific band remains to be discovered. The presence of multiple bands in positive type-specific PCR reactions did not affect the subsequent typing step since only the lowest band reacted with ITS2-specific probes on a Southern blot (data not shown).

Having established the type-specific PCR techniques, we applied the techniques to specimens of bronchoalveolar lavage (BAL) fluids that were known to contain one or more types of *P. carinii* (Table 1). The BAL specimens were obtained from the Clinical Microbiology Laboratory, Department of Pathology and Laboratory Medicine, Indiana University Medical Center. The samples used for this study were aliquots of the BAL fluids remaining after all diagnostic procedures were completed. Seventeen BAL fluids, designated specimens 1 to 17, were used. The organisms in BAL fluids were pelleted by centrifugation. The pellets were suspended in appropriate amounts of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). DNA for use as templates for PCR was then isolated from the organisms by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation as described previously (4).

Specimens 1, 2, 3, and 4 were known to contain types Ac, Ba, Bb, and Bc, respectively, and specimens 15 to 17 were determined to contain Ba plus Bc. These specimens were examined by type-specific PCR assays to serve as controls. Specimens 1, 5 to 12, and 13 and 14 were positive for type A-specific PCR, and specimens 2 to 17 were positive for type B-specific PCR. The type-specific PCR products were then reacted with ITS2 type-specific probes 2-a, 2-b, and 2-c that were labeled at their 5′ ends with [γ-^{32}P]ATP by T4 polynucleotide kinase as described previously (7). All the type A-specific PCR products of specimens 1, 5 to 12, and 13 and 14 reacted with probe 2-c, indicating that these specimens contained type Ac. Type B-specific PCR products from specimens 2, 5 to 12, and 15 to 17 reacted with probe 2-a; those from specimens 3, 13, and 14 reacted with probe 2-b; and those from specimens 4 and 15 to 17 reacted with probe 2-c. These results indicate that specimens 2, 5 to 12, and 15 to 17 contained type Ba; 3, 13, and 14 contained type Bb; and 4 and 15 to 17 contained type Bc (Table 1).

Since *P. carinii* isolates in specimens 5 to 12 and 13 and 14 were not typed conclusively with TSO typing, other experiments were performed to confirm the results obtained by type-specific PCR assays. One specimen each (specimens 5 and 13) from these two groups was selected. The PCR products of these two specimens were cloned into the TA-cloning vector pCRII as described previously (6). Two hundred colonies of potential recombinants from each specimen were picked with toothpicks and inoculated onto each of five separate sets of agar plates as dots at identical positions. After an overnight incubation at 37°C, the colonies on each plate were transferred to a piece of Whatman no. 46 filter paper. The filters were then processed for colony hybridization as described previously (5). Each of the five filters containing the same colonies was hybridized separately with type-specific probes 1-A, 1-B, 2-a, 2-b, and 2-c (7). Colonies from specimen 5 were found to contain either type Ac or Ba; no other types were found. Similarly, colonies from specimen 13 were found to contain either type Ac or Bb. These data suggest that the typing results obtained from type-specific PCR followed by TSO typing are accurate.

The type-specific PCR developed in this study is one form of allele-specific PCR which is based on the principle that mispaired bases are not extended as efficiently as perfectly paired bases. Therefore, primer that matches with the template sequence is preferentially amplified by PCR. Usually, allele-specific primers are made so that they differ in nucleotide sequence at their 3′ ends according to the nucleotide sequence of specific alleles (2, 8, 9, 11). However, the type-specific primers, which differed at the last two bases at the 3′ ends, did not work for *P. carinii* isolates examined in this study. One possibility is that the type-specific primers have nine T residues in a row which may render the primers less specific. Therefore, a two-nucleotide difference at the 3′ ends of primers is not sufficient to confer specificity. This problem was solved by using the technique referred to as the mismatch amplification mutation assay (2). A mismatched base is added to the 3′ end of the type-specific primers. This is thought to cause *Taq* polymerase to slow down the extension reaction so that the type-specific primers would be more discriminatory and specific (2). In this study, only an A residue was used as the 3′ end mismatched base since Cha et al. have shown that this residue worked the best for the type-specific allele PCR used to detect mutant alleles of c-H-*ras* (2). The possibility that other residues may work equally well or better for the ITS1-specific PCR remains to be investigated.

With the establishment of the type-specific PCR, we propose the following typing scheme for *P. carinii* f. sp. *hominis* (Fig. 3). Nested PCR is performed on a specimen with primers 1724F2 and ITS2R for the first-step PCR; this is followed by the second-step PCR with primers ITS1F and ITS2R1. The nested PCR products are then reacted with type-specific probes 1-A, 1-B, 2-a, 2-b, and 2-c. This process will type single or multiple strains that have the same ITS1 type. Specimens that contain multiple strains with different ITS1 types, i.e., those that generate PCR products that react with both 1-A and 1-B probes, are further typed by type-specific PCRs. Both type A- and type B-specific PCRs are then performed. Aliquots of the first-step PCR products mentioned above are used as the template for the type-specific PCR. Type A-specific PCR is performed with primers ITS1A3 and ITS2R2, and type B-specific PCR is per-

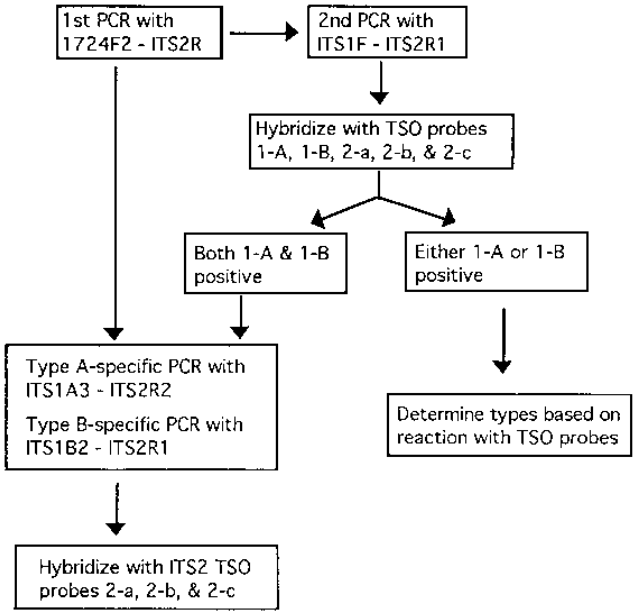

FIG. 3. Typing scheme for *P. carinii* strains. Nested PCR is performed to amplify the ITS region for typing. The first-step PCR is performed with primers 1724F2 and ITS2R, and the second-step PCR is performed with primers ITS1F and ITS2R1. The nested PCR products are then hybridized with TSO probes 1-A, 1-B, 2-a, 2-b, and 2-c. If the products hybridize with either 1-A or 1-B, type determination is achieved based on their reaction with the TSO probes. If the PCR products hybridize with both 1-A and 1-B probes, the first PCR products are subjected to type-specific PCR with primers ITS1A3 and ITS2R2 to amplify Ax and with primers ITSB2 and ITS2R1 to amplify Bx. The type-specific PCR products are then hybridized with ITS2 TSO probes 2-a, 2-b, and 2-c.

formed with primers ITS1B2 and ITS2R1. The type-specific PCR products are then reacted separately with probes 2-a, 2-b, and 2-c to determine the types. It is not recommended to perform type-specific PCRs on all specimens in the first step of the typing procedures since only approximately 30% of the specimens contain multiple strains of *P. carinii* (6). Although the methods developed in this study allow typing only of isolates that belong to types already identified, new type-specific PCRs can be developed by using the same principle as additional types of *P. carinii* are identified.

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