A Single-Tube Nested PCR for *Pneumocystis carinii* f. sp. *hominis*

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A single-tube nested PCR which amplifies the internal transcribed spacer (ITS) regions of the rRNA genes of human *Pneumocystis carinii* was developed. The outer primers for the first PCR, which anneal to the 18S and the 26S rRNA genes of *P. carinii*, were made to have a midpoint temperature (T_m) of 74°C. The inner primers for the second PCR have a T_m of 56 to 58°C and are specific for human *P. carinii*; they anneal to an area close to the beginning of ITS1 and the junction of ITS2 and the 26S rRNA genes. The reaction mixture contained 2.5 pmol of the first-PCR primers and 25 pmol of the second-PCR primers. The first PCR was performed at an annealing temperature of 68°C, which did not allow the second-PCR primers to function. Since very small amounts (2.5 pmol) of the first-PCR primers were used, they were exhausted when the first PCR was completed. The single-tube nested PCR did not amplify *P. carinii* isolated from rats, mice, or ferrets. All 10 bronchoalveolar lavage (BAL) specimens from patients with *P. carinii* pneumonia were positive, whereas all 10 BAL specimens from patients with other diseases or patients infected with several commonly found fungi were negative by PCR.

Pneumocystis carinii is a major opportunistic infectious agent in immunocompromised patients, causing pneumonia which has a high mortality rate if the patient is not properly treated. Therefore, timely diagnosis of *P. carinii* pneumonia is critical for patient management. Currently, diagnosis of *P. carinii* pneumonia is usually made by morphological demonstration of organisms in bronchoalveolar lavage (BAL) fluid, induced sputum, or specimens obtained by open lung biopsy. Although morphologic diagnosis is rapid, it requires highly experienced personnel and good specimens. PCRs which amplify various regions of the *P. carinii* genome have been studied as an alternative method for detection of *P. carinii* (2, 4, 6–9).

We previously developed a PCR method, referred to as the Pc-ITS-PCR (5, 6), which amplifies the internal transcribed spacer (ITS) regions of the rRNA genes of *P. carinii*. It is a nested PCR with primers that anneal to the 18S and the 26S rRNA genes. Since the nucleotide sequences of these rRNA genes are well conserved, this Pc-ITS-PCR may also amplify genetic material of other fungi. Although the size of the Pc-ITS-PCR product is characteristic, confirmation that it is from *P. carinii* by hybridization with a probe specific to the ITS region of *P. carinii* is required. In addition, since Pc-ITS-PCR is a nested PCR, it is prone to contamination by products of previous PCRs. Although efforts to develop a single-step PCR to amplify this target have been made, the results have not been satisfactory. All the single-step PCR methods which we have tried failed to produce sufficient PCR products for subsequent studies.

In this study, we have redesigned the PCR primers so that they are specific to human *P. carinii* and have developed a single-tube nested Pc-ITS-PCR to minimize the chance of contamination. To perform a nested Pc-ITS-PCR in a single tube, primers for both the first and the second PCRs were mixed together during the initial setup and conditions were selected so that (i) when the first PCR was running, the primers for the second PCR did not anneal to the template to prime the

amplification reaction, and (ii) when the second PCR was running, the first-PCR primers were not available to prime the amplification reaction. To meet the first requirement, the first PCR was performed with a primer-annealing temperature much higher than the midpoint temperatures (T_m s) of the second-PCR primers. Under this condition, the second-PCR primers would not anneal to the template because the annealing temperature was too high. To prevent the first-PCR primers from interfering with the second PCR, very small amounts of the first-PCR primers were used so that they were almost completely used up when the first-PCR cycles were completed.

To achieve the goals described above, the first-PCR primers were made to have a high T_m and the second-PCR primers were made to have a T_m 16 to 18°C lower than that of the first-PCR primers. Upon inspection of the nucleotide sequence of the 18S rRNA gene of *P. carinii*, the sequence GAGGTCTTCGGACTGGTGATGGG, with a T_m of 74°C, was selected. This sequence is located 110 to 132 bases upstream from the first base of ITS1 (Fig. 1). This primer was designated FT1. Another primer (RT1) with the same T_m (74°C) was ACAACTCGGACGAGGATCCTCGC, which is located in the 26S rRNA gene, 128 to 150 bases downstream from the last base of ITS2 (Fig. 1).

In order to make the nested PCR specific for human *P. carinii*, the two inner primers for the second PCR were made specific to human *P. carinii*. The sequence ACAC(T/A)TCCC TAGTGTTTTAG, located at bases 17 to 36 of ITS1, was selected (Fig. 1). This primer, designated FT2, has a T_m of 56°C. The other primer, RT2 (CTGATTTGAGATTAAAAT TCTTG), was selected to pair with FT2. Primer RT2 has a T_m of 58°C and encompasses the last 8 bases of ITS2 and the first 16 bases of the 26S rRNA gene (Fig. 1). These two sequences were confirmed to be unique to human *P. carinii* by the BLAST (1) search.

Only 2.5 pmol each of the first-PCR primers, FT1 and RT1, was used, whereas 25 pmol each of the second-PCR primers, FT2 and RT2, was used. After many trials of various conditions with 50 ng of DNA, which had been isolated from BAL fluid containing *P. carinii*, as the template, the following conditions were found to be optimal. The PCR was performed in a total volume of 40 μ l containing PCR buffer, 1 U of *Taq* polymerase,

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FIG. 1. Regions amplified by the single-tube nested Pc-ITS-PCR. Primers FT1 and RT1 were used for the first PCR, which amplifies a 774-bp fragment containing both ITS1 and ITS2. The second PCR was performed with inner primers FT2 and RT2 and produced a 492-bp fragment.

3 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, and primers FT1, RT1, FT2, and RT2 at the above-mentioned concentrations. The entire reaction mixture was heated at 94°C for 2 min, followed by 5 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min and 30 cycles of 94°C for 20 s, 68°C for 20 s, and 72°C for 1 min. This was the first PCR.

The first PCR was immediately followed by the second PCR, which included 5 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min and 30 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 1 min. The major differences between the two steps were in the amounts of the primers and in the annealing temperatures. The amount of the first-PCR primers (2.5 pmol) was 10 times lower than that of the second-PCR primers, and the annealing temperature for the first-PCR primers (68°C) was 12°C higher than that for the second-PCR primers. Since the T_m s of the second-PCR primers (56°C for FT2 and 58°C for RT2) are much lower than the annealing temperature (68°C) for the first PCR, the second-PCR primers will not anneal to the templates during the first PCR to prime the reactions. The following experiment was performed to determine whether primers for the first PCR were exhausted when the first PCR was completed. The first-PCR primers, FT1 and RT1, were labeled with ^{32}P at their 5' ends, and 2.5 pmol of each primer was used for PCR under the first-PCR conditions described above. A 10- μ l aliquot of the PCR product mixtures was then electrophoresed on a 1.2% agarose gel. The gel was dried and exposed to X-ray film. The autoradiogram of the gel showed a band of 774 bp, which is the first-PCR product, and a very light shadow of the primer band. The lane which contained only the labeled primer showed an extremely heavy band; this sample was a 10- μ l aliquot of the control reaction mixture, which contained all ingredients except the template DNA. These results indicate that the 2.5 pmol of primers was almost completely used up after the first PCR.

After the PCR conditions were established, they were applied to clinical specimens. Twenty BAL specimens from 20 patients were used, including 10 specimens with *P. carinii*, 2 with *Histoplasma capsulatum*, 2 with *Cryptococcus neoformans*, 2 with *Aspergillus fumigatus*, and 4 with unknown etiology. These BAL specimens were obtained from the Clinical Microbiology Laboratory, Indiana University Hospital, after all diagnostic procedures had been completed. They were examined for microorganisms as described previously (6). Briefly, cells and microorganisms in BAL fluids were pelleted by centrifugation. The sediments were spread onto slides and stained with Giemsa stain, Diff-Quick, methenamine-silver nitrate, and modified acid-fast stain. The slides were examined under a microscope, and the *P. carinii* organism load in each specimen was indicated as few (average, 1 clump of *P. carinii* organisms in 30 fields at a magnification of $\times 1,000$), moderate (average, 1 clump of organisms in 10 to 20 fields at a magnification of

FIG. 2. Specimens amplified under second-PCR conditions (A), by the regular two-step nested PCR (B), and by the single-tube nested Pc-ITS-PCR (C). PCR products of various specimens, indicated by number, and those of positive (+) and negative (-) controls were electrophoresed on a 1% agarose gel along with ϕ 174/HaeIII DNA size markers (M). (D) Southern hybridization of the blot shown in panel C.

$\times 1,000$), or heavy (average, 5 to 10 clumps of organisms in 1 field at a magnification of $\times 1,000$). Among the 10 BAL specimens with *P. carinii*, specimens 14, 62, and 91 were found to have a heavy organism load. Specimens 29, 40, and 62 contained moderate amounts of *P. carinii* organisms, and specimens 33, 46, 57, 92, and 93 were shown to contain few organisms.

The BAL specimens were processed for PCR as described previously (3), and 50 ng of DNA from each specimen was used for each PCR. To ensure that the second-PCR primers did not work under the first-PCR conditions, a first PCR was done with 25 pmol each of the second-set primers, FT2 and RT2, on each of the specimens. No PCR products were produced from any of the specimens. When the PCRs were done under the second-PCR conditions, 6 of the 10 specimens (no. 14, 29, 40, 46, 62, and 91) from *P. carinii* pneumonia patients produced a visible band of the right size (Fig. 2A). The band intensity of specimen 14 was the strongest. Specimen 91 also produced a relatively strong band, and specimens 29, 40, 46, and 62 produced weaker bands. These six specimens were shown to have a moderate or heavy organism load.

A two-step nested PCR was then performed on all specimens. The first PCR was performed under the first-PCR conditions with primers FT1 and RT1 described above. Two microliters of the first-PCR product was then used as the template for the second PCR with primers FT2 and RT2 under the second-PCR conditions described above. A reaction with DNA from a BAL specimen confirmed to contain *P. carinii* was used as the positive control, and a reaction with no template DNA was used as the negative control. All 10 BAL specimens from *P. carinii* pneumonia patients and the DNA used for the positive control produced a band of 492 bp (Fig. 2B). The 10 BAL specimens from patients with other diseases produced no PCR products. The 20 BAL specimens were then subjected to the single-tube nested Pc-ITS-PCR under the conditions described above. All 10 specimens from *P. carinii* pneumonia

patients produced a visible band (Fig. 2C), and the 10 BAL specimens from the other patients were negative. To ensure that the amplified products were of *P. carinii*, a Southern hybridization was performed with the DNA fragment containing the ITS regions as the probe as described previously (6). Two bands from each sample hybridized with the probe (Fig. 2D). The upper band is 774 bp, which is the size of the first-PCR product; this band is not visible on the autograph of the gel (Fig. 2C). The lower band is 492 bp, which is the size of the second-PCR product. The hybridization signal of the lower band is much stronger than that of the upper band.

To ensure that this single-tube nested Pc-ITS-PCR is specific for human *P. carinii*, the PCR was applied to *P. carinii* isolated from other animals, including rats, mice, and ferrets. Only human *P. carinii* yielded a positive Pc-ITS-PCR result, but all samples were positive by the mitochondrial rRNA gene PCR with primers pAZ102-E and pAZ102-H (9). Several other fungi, including *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Blastomyces dermatitidis*, were also examined by the single-tube nested Pc-ITS-PCR. The fungal DNA was isolated as described by White et al. (10). No PCR products were generated from DNA from any of these fungi. To ensure that the fungal DNAs were amplifiable, they were amplified with primers NS3 and NS6, which have been shown to amplify DNA from all fungi (10), and a 600-bp band was produced from all the fungal specimens. These results indicate that the single-tube nested Pc-ITS-PCR is specific for *P. carinii*.

A major difference in the intensities of bands produced by the regular two-step and the single-tube nested Pc-ITS-PCRs was observed. The intensities of bands produced by the two-step PCR on different specimens were all the same, whereas those from the single-tube nested Pc-ITS-PCR were different from each other (Fig. 2). This difference in intensity is relatively consistent with the organism load in each specimen. Specimens 14, 62, and 91, which contained a heavy organism load, each produced a very intense band. Specimens 29, 40, and 62 contained moderate amounts of *P. carinii* organisms and generated a less intense band, with the exception of specimen 40, which produced a very strong band. The specimens (no. 33, 46, 57, 92, and 93) that contained few organisms produced a weaker band. These results indicate that the endpoint of this single-tube nested PCR is still within the semiquantitative range of PCR. This semiquantitative result may prove to be valuable in patient management because it may relate to the severity of the disease or to the effectiveness of therapy.

Although the single-step nested PCR did not produce bands as intense as those produced by the two-step nested PCR (Fig. 2), it had sufficient sensitivity to detect *P. carinii* in clinical specimens. Among the specimens that we have examined in this study by the single-tube nested PCR, none was positive by

morphological examination but negative by the single-tube Pc-ITS-PCR. To determine the sensitivity of the single-tube nested PCR, it was run on a series of samples containing a known number of organisms. A smear of a 5- μ l aliquot of BAL fluid on a slide was stained, and the number of organisms in the smear was determined. Aliquots containing 250, 50, 10, 2, and 0 *P. carinii* organisms were made and subjected to the single-tube nested PCR. Positive PCR results were generated by samples containing 250, 50, 10, and 2 organisms, and the intensity of the PCR product band correlated with the number of organisms in the sample. The sample containing no organisms was negative. This result indicates that the single-tube PCR has a sensitivity of approximately two *P. carinii* organisms. Based on the results of this study, we consider the single-tube nested Pc-ITS-PCR better than the regular two-step Pc-ITS-PCR for diagnosis of *P. carinii* pneumonia because it has sufficient sensitivity and is simpler to perform than the two-step nested PCR.

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