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SPECIFIC AMPLIFICATION OF *ASPERGILLUS FUMIGATUS* DNA BY POLYMERASE CHAIN REACTION

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ABSTRACT

Invasive aspergillosis (IA) is a life-threatening condition in immunocompromised patients. An early diagnosis is of great importance because early treatment may resolve this potentially fatal infection. Recently, the polymerase chain reaction (PCR) has been used successfully in detecting specific DNA of several pathogens. In this study, nested PCR was used to detect DNA specific for *Aspergillus* species isolated from bronchoalveolar lavage (BAL) fluid from patients with IA. In single PCR using the outer primers a specific 384-bp fragment was amplified. Similarly, by nested PCR with inner primers, a 357-bp fragment was amplified with DNA from *Aspergillus fumigatus* but not from the other microorganisms. The Southern blot hybridizations confirm the specificity of the PCR procedure for *A. fumigatus* using the cloned 374-bp PCR product probe. In conclusion, the nested PCR method appears to be quite rapid and specific.

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INTRODUCTION

A. fumigatus represents the most common airborne fungal pathogen. It commonly grows in damp environments such as soil, decaying vegetation, and organic debris, from which it releases high numbers of conidia into the atmosphere.[7]

In oncology patients with prolonged neutropenia, *Aspergillus sp.* infections represent an increasing problem.[10] *A. fumigatus* is by far the predominant species in the case of invasive disease, while *A. flavus* is isolated less frequently but more often causes extra-pulmonary invasive infections.[10] IA is a life-threatening condition in immunocompromised patients, particularly those with chemotherapy for hematologic malignancies or those receiving high doses of corticosteroids. An early diagnosis of aspergillosis is of great importance because early treatment may resolve this potentially fatal infection.[1] Unfortunately, the diagnosis of IA remains difficult and sometimes is confirmed only at autopsy. At present, a firm diagnosis is established by histological examination of a tissue sample obtained during an open lung biopsy or transbronchial lung biopsy as well as by detecting the causative pathogenic fungi in clinical samples. Sputum cultures are unfortunately unreliable.[6] Serological tests such as those involving the detection of antibodies for *Aspergillus sp.* are less helpful because of the poor antibody responses in immunosuppressed patients.[26] In addition, the methods used for detecting circulating *Aspergillus* antigens, such as radioimmunoassay, immunoblotting assay, enzyme immunoassay, and the latex agglutination test, have poor sensitivity.[16,17] Although a low level of (1-3)-β-D-glucan can be detected directly by the G test, the sensitivity of this test is also inadequate for early diagnosis of IA and chronic necrotizing aspergillosis.[13]

Recently, the PCR has been used successfully in detecting specific DNA of several pathogens.[14,21,24] For the diagnosis of IA, PCR has been used to detect DNA specific for *Aspergillus* species in BAL fluid from patients with IA.[4,12,15,19,20] In this study, nested PCR was used to detect DNA specific for *A. fumigatus* isolated from BAL fluid from patients with IA. The sensitivity of this technique is high and the results are available within a short period of time.

MATERIALS AND METHODS

Isolation of DNA from *A. fumigatus*

The two strains (1 and 2) of *A. fumigatus* isolated from BAL fluid from patients with IA were used in this study. DNA was isolated from *A. fumigatus* by an adapted method described by Aufauvre-Brown et al.[3] The modification is that after lysis by vortexing frozen with glass beads and resuspending in TNES buffer, the lysates were immediately immersed in 68°C shaking water bath. This step is important in maintaining maximal integrity of DNA.[5] The mixture was cooled to room temperature, and to improve the yield proteinase K (15-20 µg/mL lysate) was added and the mixture incubated for 1.5-2 hours at 37°C while shaking. One final modification involved resuspending the washed and dried pellet in TE (pH 8.0), instead of water containing 50 µg/mL of RNase A as described by Aufauvre-Brown et al.[3] On average 20 µg DNA was isolated from 3-4g wet weight of mycelia. DNA from the oomycete *Achlya ambisexualis* and the zygomycete *Entomophaga aulicae* were obtained from Dr. J. Silver (University of Toronto) and DNA from *Candida albicans* was provided by Shelly Brunt (Georgetown University).

Oligonucleotide primers and PCR

The design of the oligonucleotides used in this study was similar to that used by Yamakami et al.[25] and was based on comparisons of the 18S rRNA genes of *Aspergillus sp.* and of other fungi in the GenBank database. The PCR was performed as nested PCR with two sets of primers. The outer primer set consisted of Asp. 5 (5′GATTAACGAACGAGACCTCGG 3′) and Asp. 8 (5′TGCCAACTCCCCTGAGCCAG 3′) amplifying a 384-bp sequence (Fig. 1). The inner primer set consisted of Asp. 1 (5′CGGCCCTTA AATAGCCCGGTC 3′) and Asp. 7 (5′CCTGAGCCAG TCCGAAGGCC 3′) amplifying a 357-bp sequence (Fig. 1). The PCR that was developed was an adaptation of a procedure described by Yamakami et al.[25]

The nested PCR products were electrophoresed on 2% agarose gel containing ethidium bromide, and the results photographed using a polaroid MP4 camera.

Cloning of an *A. fumigatus* PCR product

An *A. fumigatus* PCR product of approximately 374-bp was produced using the primer set Asp. 5 and Asp. 7. This product was cloned using TA cloning (Invitrogen) as described in the kit. The PCR product was ligated into the plasmid vector pCR™ II, containing Lac Z— a complementation fragment—for blue-white colour screening, and the ampicillin and kanamycin resistance genes for selection. The recombinant DNA molecules that were constructed were introduced into a bacterial host XLI Blue *E. coli*. Transformation of the bacterial cells was

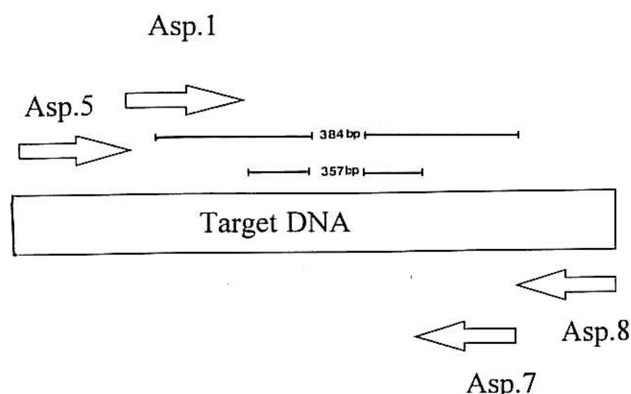

Fig. 1. Arrows refer to the primer sets used; primer Asp. 5 and Asp. 8 were the outer primers and primers Asp. 1 and Asp. 7 were the inner primers.

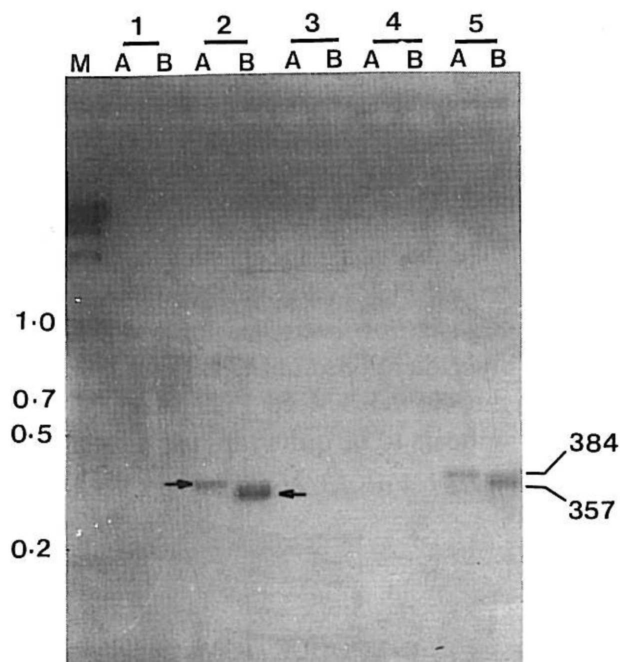

Fig. 2. Specificity of the PCR assay with DNA from various fungi. Lanes A, single PCR assay; Lanes B, nested PCR assay; Lanes 1-5, PCR products obtained with DNA from: 1, *Entomophaga aulicae*; 2, isolate 1 of *Aspergillus fumigatus*; 3, *Achlya ambisexualis*; 4, *Candida albicans*; 5, isolate 2 *Aspergillus fumigatus*; M, molecular size marker (λ DraI).

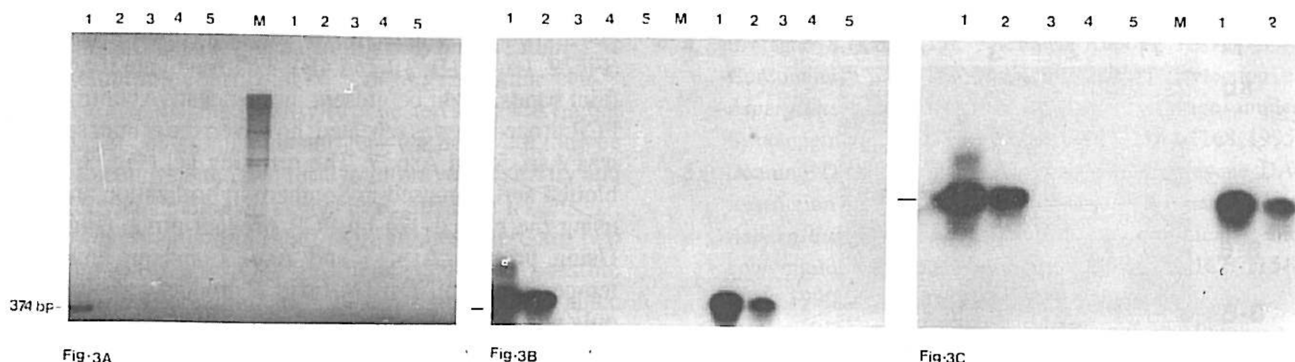

Fig. 3. PCR was performed as described in *Materials and Methods*. (A) Shown on the left side of the Marker (M) are the result of a single PCR with primers Asp. 5 and Asp. 7. Shown on the right of the Marker (M) are the result of the PCR using the same primers but in a different annealing temperature. (B) Southern blot hybridization of blot shown in (A) using the 374-bp PCR product as the probe. Sets using DNA from lanes: 1-2, isolate 1,2 of *Aspergillus fumigatus;* 3, *Achlya ambisexualis;* 4, *Candida albicans;* 5, *Entomophaga aulicae*. (C) The lower molecular weight region of the Southern blot is shown.

carried out as follows: 500 µL of OD_{600}=0.2-0.4 cells were incubated for two hours at 37°C in a shaking water bath. The cells were pelleted at 3000 rpm and the cell pellets resuspended in 1 mL of cold, filter-sterilized 50 mM $CaCl_2$ and stored on ice for 30 minutes. Recombinant plasmids were added to the prepared cells and the mixture incubated for one hour on ice. The mixture was heat shocked at 37°C for 90 seconds, and spread on to freshly poured plates of LB agar with ampicillin, isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal).The plates were then incubated at 37°C overnight.

Plasmid isolation and purification
Alkaline rapid isolation

Single white colonies growing on LB plus ampicillin plates were picked into 5 mL LB broth containing 100 µg/mL ampicillin and grown overnight while shaking at 37°C. A 1.5 mL Eppendorf tube was filled to the top with the culture and cells were spun down at 4000 rpm for 10 minutes. The cell pellets were resuspended by vortexing in 100 µL of 25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA, and placed on ice for 5 minutes. Subsequently 200 µL of 0.2 M NaOH, and 1% SDS was added, and the suspension mixed by vortexing and placed on ice for 5 minutes. Lastly 150 µL of 3M sodium acetate, pH 4.8 was added to the tube and kept on ice for 10 minutes and the mixture was centrifuged at 10000 rpm for 15 minutes. The supernatant was decanted into a fresh tube and the pellet discarded. One mL of cold 95% ethanol was added to the supernatant, the mixture mixed by inversion and placed on ice for 20 minutes. The precipitated DNA was centrifuged at 10000 rpm for 10 minutes. The pellet was washed with cold 70% ethanol and resuspended in TE, pH 8.0 (10 mM Tris, disodium EDTA). The plasmid was digested with restriction enzyme EcoRI to release the insert DNA and the vector. The RNA was then removed by treating with RNase. Briefly, after diluting resuspend pellets to 1/2 in distilled water RNase was added and incubated at room temperature for 30 minutes and then, the insert was separated electrophoretically on an agarose gel. Plasmid purification was performed as well using Qiagen as described in the manufacturer's protocol.

Southern hybridization of *A. fumigatus* genomic DNA with the cloned PCR products

DNA (5 µg) from *A. fumigatus* was digested with 20U each of EcoRI, HindIII, XbaI and EcoRv per µg of DNA at 37°C for six hours. The samples were precipitated ovenight at -20°C with 1/10 volume 3M sodium acetate, pH 7.0 and 2-2.5 volume 95% ethanol, collected by centrifugation and resuspended in sterile distilled water. The DNA fragments were separated on 1% agarose gel and transferred to nitrocellulose using conventional methods.[18] The blot was prehybridized at 50°C for 4-5 hours with a solution containing 50% formamide (V/V), 5×SSC, 5×Denhardts at 37°C, 40mM $NaPO_4$ and denatured sheared salmon sperm DNA. Southern hybridization using the PCR product as a probe was carried out under the following conditions (50% formamide (V/V), 5×SSC, 5×Denhardts, 40mM $NaPO_4$ and denatured sheared salmon sperm DNA at 50°C for 17-20 hours) and the final washes carried out in 0.1×SSC (15 mM NaCl, 1.5mM sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) at 55°C for 20 minutes while shaking. The probe used was the PCR product obtained, using the primers (Asp. 5, Asp. 7), and *A. fumigatus* DNA as target. The PCR product of approximately 374-bp was obtained and cloned, using TA cloning (Stratagene). The clone containing the PCR product was digested with enzyme EcoRI, and the plasmid and insert DNAs separated on 1% agarose gel using the freeze thaw technique.[18] The DNA

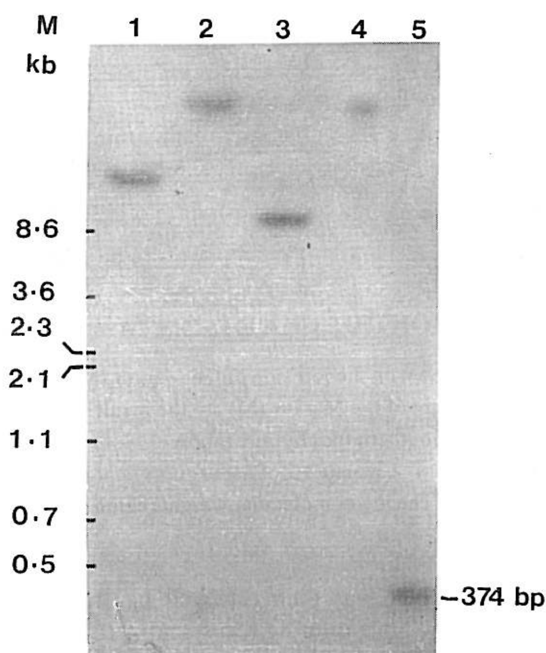

Fig. 4. Southern blot analysis of DNA from *Aspergillus fumigatus*. Genomic DNA from *Aspergillus fumigatus* was digested with the restriction enzymes EcoRI (lane 1), HindIII (lane 2), XbaI (lane 3), and EcoRv (lane 4). The cloned PCR product was used as a positive control (lane 5). The blot was hybridized with the PCR product as described in *Materials and Methods*. The molecular sizes (in kilobases) of the marker DNA are noted to the left of the figure.

was labelled with [α-^{32}P]-dCTP using the random primer oligolabelling method. Radioactive blots were exposed to Kodak XAR film at -70°C for 4 hours in the presence of an intensifying screen.

The Southern blot patterns of the DNA digested with the different enzyme were compared. Band positions were identified by comparison with DNA size markers consisting of bacteriophage Lambda DNA digested with the enzyme DraI and the probe itself as a positive control.

RESULTS

Specificity of nested PCR for *A. fumigatus* DNA

DNA samples from two different isolates of *A. fumigatus* were tested to see whether the primers amplified the same DNA products in all cases. In single PCR using only the outer primers, a specific 384-bp fragment was amplified with DNAs from the two *A. fumigatus* strains tested (Fig. 2 lanes 2A and 5A) but not with DNA from the other micro-organisms, i.e. *Achlya ambisexualis* (Fig. 2, lane 3A), *Entomophaga aulicae* (Fig. 2, lane 1A) and *Candida albicans* (Fig. 2, lane 4A). Similarly, by nested PCR with the inner primers, a 357-bp fragment was amplified with the DNA from both *A. fumigatus* strains (Fig. 2, lane 2B and 5B) but not with DNA from the other micro-organisms (Fig. 2, Lanes 1B, 3B and 4B). To investigate if possible faint bands might be present in the negative controls, the PCR procedure was repeated; however, the primer set used was Asp. 5 and Asp. 7. The resulting gel (Fig. 3A) was blotted and then used in Southern hybridization analyses using the cloned 374-bp PCR product probe (Fig. 3B). Using primers Asp. 5 and Asp. 7 and an annealing temperature of 50°C a 374-bp PCR product was observed, only when *A. fumigatus* DNA was used as the target (Fig. 3A, lane 1 and 2), Although a 374-bp band in Fig. 3 A, lane 2 was present, it did not photograph well. When the PCR reaction was carried out at 65°C a similar result was obtained. When the gel shown in Fig. 3A was blotted and probed with the cloned 374-bp Aspergillus PCR product, bands were seen only in the lanes containing the *Aspergillus* DNA PCR product (Fig. 3B, lane 1 and 2). The lower molecular weight region of the Southern blot is shown in Fig. 3C. As shown in these figures (3B and 3C) no other molecular weight bands were observed. The Southern blot hybridization therefore confirmed the specificity of the PCR procedure for *A. fumigatus* DNA (Fig. 3B).

In order to confirm that the sequence represented in the 374-bp was indeed present in the *A. fumigatus* genome and was not an artifact of PCR, southern blot analysis of genomic DNA from *A. fumigatus* was used. As shown in Fig. 4. the aspergillus genome contains a DNA sequence that hybridizes with the 374-bp PCR product.

DISCUSSION

IA is one of the major clinical concerns in immunocompromised patients, because it is difficult to detect, even in the case of extensive systemic infections, and treatment appears to be successful only when started in an early stage of disease. While prevention of infection remains the ultimate goal, identifying those individuals at greatest risk by rapid diagnosis would provide an alternative, since these are most likely to benefit from early treatment.[12] Although the recovery of *Aspergillus* sp. from BAL fluid is highly indicative of IA, the diagnostic yield from culture of BAL fluid samples is only 30%.[2] Recently, several investigators reported the use of PCR to detect *A. fumigatus* and *A. flavus* in high risk patients.[15,19,20,25] In this preliminary study we have demonstrated the usefulness of PCR for detecting DNA specific for *Aspergillus*. Several other investigators have reported the use of PCR in detecting DNA specific for *Aspergillus sp.* in BAL fluids [4,12,15,19,20] and serum[25] or urine samples.[2] In this study, we developed novel primers to detect DNA specific for *Aspergillus sp.* by nested PCR. Southern blot hybridization using the PCR product probe was helpful in confirming the specificity of the PCR procedure for *A. fumigatus* DNA.

A recent study by Verweig et al[23] demonstrated that the

sandwich enzyme-linked immunosorbent assay (ELISA), which detects galactomannan antigen in serum samples, is also useful for the early diagnosis of IA. Yamakami et al.[26] demonstrated that the sensitivity of nested PCR was higher than that of galactomannan antigen We believe it may be useful to combine the two tests to improve sensitivity and specificity since the two assays have different targets.

However, as is true in all PCR studies, there are two potential problems. One problem relates to possible environmental contamination of the sample DNA since *Aspergillus sp.* are commonly present in the environment. However in the studies presented here, none of the control DNAs used were positive, indicating that environmental contamination does not appear to be a problem when standard microbiological procedures are followed as recommended by Kwok and Higuchi.[9] A second limitation is that PCR cannot yet be easily performed in every microbiological laboratory, since trained personnel and experience with the PCR procedure are necessary. However, this will likely change in the future.

Given the specificity and rapidity of PCR, this diagnostic method could have a major impact in reducing the morbidity and mortality of patients undergoing treatment for cancer and for other hematological malignancies, by allowing the early institution of therapy on a more selective basis than is possible at present. For this reason, we believe that diagnostic tests using PCR warrant widespread further evaluation using a large population of clinical samples.

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