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Full Length Research Paper

Specific detection of *Aspergillus fumigatus* in sputum sample of pulmonary tuberculosis patients by two-step PCR

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The incidence of life-threatening systemic fungal infections has been increasing in recent years, and the increasing incidence has been correlated with increasing numbers of immunocompromised patients. We developed a two-step PCR assay that specifically amplifies a region of the 18S rRNA gene that is highly conserved in *Aspergillus fumigatus*. This assay allows direct and rapid detection of down to 10 fg of *Aspergillus fumigatus* DNA corresponding to 1 to 5 colony forming unit (CFU) per ml of sputum sample of pulmonary tuberculosis patients. The two primer pairs that produced PCR products with the highest sensitivity and species specificity were the *Aspergillus fumigatus* primers AFU7S and AFU7AS, which amplified a fragment of 405 bp, followed by AFU5S and AFU5AS, which produced an internal fragment of 236 bp. PCR has been shown to be a highly sensitive diagnostic tool for the detection of infectious fungi specimens. Our point to the considerable potential clinical value of this simple, specific, rapid, and inexpensive PCR assay is for improving the means of early diagnosis of systemic aspergillosis tuberculosis patients.

Key words: *Aspergillus fumigatus*, Polymerase chain reaction (PCR), pulmonary tuberculosis.

INTRODUCTION

The incidence of life-threatening systemic fungal infections has been increasing in recent years, and the increasing incidence has been correlated with increasing numbers of immunocompromised patients (Anaissie et al., 1989; Bodey et al., 1992; Beck-Sague, and Jarvis, 1993; Groll et al., 1996; Denning et al., 1998; Kami et al., 2000). Patients at the greatest risk are those with prolonged periods of pulmonary tuberculosis after intensive immunosuppressive chemotherapy, for example, during treatment for acute leukemia or after bone marrow transplantation. Particularly on the increase are invasive infections with *Aspergillus fumigatus,* resulting in high mortality rates or, if the patient survives, causing high levels of morbidity that often limit further antileukemic therapies. Antifungal prophylaxis is controversial and is not generally practiced (Arning et al., 1994; Cafferkey et al., 1994; Chandrasekar et al., 1995; Wald et al., 1997; Lin et al., 2001). Moreover, amphotericin B is toxic. In contrast to other infections, only limited conventional diagnostic tools with poor sensitivity and reliability are available for early detection of invasive aspergillosis (Walsh et al., 1991; Denning et al., 1994; Guiot et al., 1994; Hughes et al., 2002.) with the systemic infection frequently being diagnosed late or confirmed only at autopsy (Bodey et al., 1992; Pfaffenbach et al., 1994; Chandrasekar et al., 1995; Groll et al., 1996). The PCR test commonly used in the initial period of infection during pulmonary tuberculosis are often insufficient not only for accurate early diagnosis but also for monitoring of the subsequent course of invasive aspergillosis (Anaissie et al., 1989; Blum et al., 1994; Guiot et al., 1994; Logan et al., 1994; Obayashi et al., 1994; Hearn et al., 1995; Stynen et al., 1995; Verweij et al., 1995; Walsh et al., 1995a, b; Kappe et al., 1996; Caillot et al., 1997; Swanink et al., 1997; Lewin et al., 1999; Loeffler et al., 2000a; Kearns et al., 2001; Palladino et al., 2001). In view of the low specificity and sensitivity rates of these methods, the

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diagnosis of an invasive aspergillosis can be proven conclusively only by positive histology or culture results (Bart-Delabesse et al., 2001). However, establishing cultures from blood and bronchoalveolar lavage (BAL) samples is often unsuccessful due to the low yields of CFU, and in the case of immunocompromised high-risk patients who are febrile, pulmonary tuberculosis, neutropenic, thrombocytopenic, and often seriously ill, tissue biopsy specimens, in general, are not available.

Many authors have reported detection of *Aspergillus* nucleic acids by PCR for improved diagnosis of invasive aspergillosis. Target sequences, either panfungal ribosomal DNA (rDNA) (Einsele et al., 1997; Hebart et al., 2000; Hendolin et al., 2000; Lass-Florl et al., 2001; Loeffler et al., 2000; Loeffler et al., 1998) or *Aspergillus*-specific mitochondrial

DNA (mtDNA) (Bretagne et al., 1998; Costa et al., 2002; Jones et al., 1998) or rDNA sequences (Buchheidt et al., 2002; Buchheidt et al., 2001; Kami et al., 2001; Skladny et al., 1999) have been amplified from BAL fluids (Skladny et al., 1999; Buchheidt et al., 2002) serum (Bretagne et al., 2002; Costa et al.,2002), or whole blood (Buchheidt et al.,2002; Hebart et al., 2000; Einsele et al., 1997; Lass-Florl et al., 2001). The diagnosis of mycotic infections in pulmonary tuberculosis patients by molecular methods such as Southern blot analysis has been performed successfully with lung. More sensitive and rapid detection assays have been established by use of the PCR method, particularly following the identification and sequencing of multicopy gene templates in a range of fungi and other organisms. PCR assays for the detection of fungal nucleic acids may be the optimal diagnostic approach because they are potentially more sensitive than current culture-based methods and may be designed to encompass the desired range of genera and specimen types. Previous studies evaluating PCR-mediated detection of *Aspergillus* species showed significantly improved sensitivity but involved assays with different methods and objectives, partly to optimize culture assays (Burg et al., 1989; Makimura et al., 1994; Hayden et al., 2001.) and partly for typing in epidemiological studies (Aufavre et al., 1992; Girardin et al., 1994; Birch et al., 1995; Groll et al., 1996). Therefore, the results of different groups are not consistent or comparable. By using PCR primers specific for the multicopy 18S rRNA gene, very small amounts (down to 1 pg) of genomic DNA from *A. fumigatus* have been detected, the sensitivity being increased to 100 fg by (Spreadbury et al., 1993).

Melchers et al. (1994) first described a PCR assay for the detection of DNA from an *Aspergillus* sp. in immunocompromised patients with primers based on the coding sequence of the 18S rRNA gene which is highly conserved and which is amplified some hundredfold in the *Aspergillus* genome. PCR products were obtained from BAL samples of immunocompromised while no amp- licons were obtained from immunocompetent individuals. However, Yamakami et al. (1996) first described the use of a two-step PCR to detect *Aspergillus* spp. in blood with increased specificity and sensitivity, but that study was performed with only a small number of patients. Einsele et al. (1997) described a PCR assay with subsequent Southern blot analysis, which allowed the detection of fungal pathogens (including *Aspergillus* spp.) in blood samples.

In order to achieve an improved, specific, and rapid means of detection of *Aspergillus fumigatus* in clinical specimens, we developed a two-step PCR assay for sputum samples. Two optimal pairs of oligonucleotide primers derived from sequences of the 18S rRNA gene, which are specific for *A. fumigatus*. The assay was evaluated for its sensitivity and specificity *in vitro* and was used to analyze clinical samples of immunocompromised patients.

MATERIALS AND METHOD

Clinical samples and isolation

A. fumigatus test strains were isolated from the sputum samples of pulmonary tuberculosis patients from the civil hospital of Washim, Washim District, Maharashtra state, India. Sputum samples (2 - 4 ml) were obtained under sterile conditions. The samples were inoculated in appropriate sterile Czapek Dox Agar plates at 25°C for 3 to 4 days. The cell density of the fungal suspensions (conidia) was determined microscopically by counting the cell number in a Neubauer cell chamber.

DNA isolation

DNA was isolated from larger scale (25 ml) cultures. Mycelium were separated by filtration and washed three times in cold buffer (20mM sodium citrate pH 5.8 in 1 M sorbitol), ground in liquid nitrogen and resuspended in lyses buffer (50 mM Tris-HCl (pH 8), 50 mM EDTA, 3% sodium dodecyl sulfate, and 1% β- mercaptoethanol). The suspension was then incubated at 65ºC for 1 h. After the cell debris had been pelleted, the lysate was extracted once with phenol-chloroform (1:1) and once with a chloroform-isoamyl alcohol (24:1). The DNA was precipitated by the addition of 0.2 volume of 3 M potassium acetate and 0.5 volume of isopropanol; after centrifugation, the DNA pellets were washed with 70% ethanol and resupended in buffer (10 mMTris-HCl pH-8, 1mM EDTA).

The purification of fungal DNA was performed by conventional extraction (Sambrook et al., 1989). The DNA was precipitated by the addition of 0.7 volume of isopropanol, pelleted, and washed once with 70% ethanol and air-dried. The DNA concentration was assessed by spectrophotometer at 260 and 280 nm.

Oligonucleotide primers, primer sequences, and PCR assay

The alignment of the three DNA sequences was performed with the program Gene works (Intelligentsias, Inc.) by using standard algorithms. Primers were designed to have sequences homologous to those of *A. fumigatus* but not to include the human 18S rRNA gene or the 16S rRNA genes of *Candida* spp. or other pathogenic microorganisms. Therefore, selection of the primer sequences was based on a close check for sequences with matching homologies in current DNA databases (GenBank, release June 1998) with a DNA alignment program (Blast). By using a nested, two-step PCR technique, 4 different primers (Table 1) were tested, and the optimum two pairs (primers AFU5S and AFU5AS and primers AFU7S and AFU7AS) were chosen for all subsequent PCR assays (Figure 1).

Table 1. Primer sequence and location.

Primer	DNA sequence (5'-3')	Location	Species specificity
AFU5S	AGG GCC AGC GAG TAC ATC ACC TTG	1436-1459	*A. fumigatus*
AFU5AS	GG G (AG)GT CGT TGC CAA C(CT)C (CT)CC TGA	1648-1771	*A. fumigatus*
AFU7S	CGG CCC TTA AAT AGC CCG	1296-1313	*A. fumigatus*
AFU7AS	GA CCG GGT TTG ACC AAC TTT	1681-1700	*A. fumigatus*

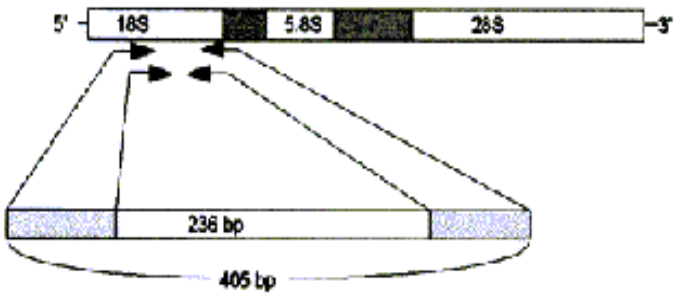

Figure 1. Locations of primer pairs AFU5S-AFU5AS and AFU7S-AFU7AS used in the two-step PCR to detect *Aspergillus* DNA. The primers are derived from the 18S rRNA gene of *Aspergillus* spp. The first PCR step (with AFU7S-AFU7AS) results in amplification of a 405-bp fragment, and the second step with AFU5S-AFU5AS) amplifies an internal fragment of 236 bp.

Reaction and conditions of (PCR)

Per 25 µl PCR mixtures, approximately 50 to 150 ng of total DNA was used as the template. The standard PCR mixture contained 0.5 U of *Taq* DNA polymerase, 6.25 nmol of the deoxynucleoside triphosphates, 10 pmol of primer (first step, primer AFU7S-AFU7AS; second step, primer AFU5S-AFU5AS). In preliminary studies the optimum reaction conditions were established by testing different DNA, primer, enzyme, and deoxynucleoside triphosphate concentrations as well as a range of cycling conditions. PCR was performed in a thermal cycler (Perkin-Elmer Cetus), as follows: for the first PCR, 2 min at 94°C and then 35 cycles of 40 s at 94°C, 1 min at 60°C, and 1 min at 72°C with a terminal step of 5 min at 72°C and then the mixture was held at 4°C; for the second PCR, 2 min at 94°C and then 45 cycles of 40 s at 94°C, 1 min at 60°C, and 1 min at 72°C, with a terminal step of 5 min at 72°C, and then the mixture was held at 4°C. For the second PCR, 10 µl of the first-round PCR product was used. The PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized with Gel-doc instrument. Control samples included all the constituents in the reaction mixture except genomic DNA. As negative PCR controls, DNA samples of *Phoma*, were used as templates.

RESULT AND DISCUSSION

The alignment of the 18S rRNA genes of *A. fumigatus*, humans, and *Candida* sp. revealed several regions of significant divergence, which were the basis for selection of the primers. In order to establish a PCR assay specific for several *Aspergillus* species of clinical importance the 18S rRNA gene (rDNA) sequence of *A. fumigatus* (GenBank accession no. AB008401) was aligned with the human 18S rDNA sequence (GenBank accession no. M10098) and the 16S rDNA sequence of *C. albicans* (GenBank accession no. X53497), another ubiquitous microorganism of major clinical importance. Four different primers, comprising 7 sequences upstream and 8 sequences downstream from various divergent regions of the *Aspergillus* species gene, which showed the least homology with the human, or *Candida* genes, were selected for the evaluation and the optimization of the PCR assay.

The two primer pairs that produced PCR products with the highest sensitivity and species specificity were the *A. fumigatus* primers AFU7S and AFU7AS, which amplified a fragment of 405 bp, followed by AFU5S and AFU5AS, which produced an internal fragment of 236 bp (Figures 2 and 3). PCR products could be obtained only from DNA of cultures of *A. fumigatus*, whereas the PCRs with the other *Phoma* test strains were negative. By monitoring the clinical course of this pulmonary tuberculosis patient, in whom a small lung infiltration was detected by chest X ray, we could neither prove nor exclude invasive aspergillosis. PCR has been shown to be a highly sensitive diagnostic tool for the detection of infectious fungi in diverse specimens.

Spradbury et al. (1993) initiated pioneer work in this direction after multicopy gene targets, such as the 28S rRNA gene, had been identified and sequenced. The first PCR assay for the detection of *Aspergillus* DNA in immunocompromised patients described (Melchers et al., 1994) used primers derived from the multicopy 18S rRNA gene, a gene of numerous microorganisms that has now been sequenced. More recent reports described PCR techniques with primers designed to target conserved 18S rRNA sequences common to a variety of fungal pathogens (Einsele et al. 1997; van Burik et al., 1998). However, additional Southern blot analyses with longer probes were also necessary to achieve the detection sensitivity for the identification of the specific fungal species or genera present. Nested PCR assays improved the detection sensitivity. In the first description of such a two-step PCR for the detection of *A. fumigatus* in serum samples of patients, Yamakami et al. (1996) reported that a PCR with two sets of 18S rRNA primers had considerably improved sensitivity compared to that of a PCR assay with a single set of primers. This could be further

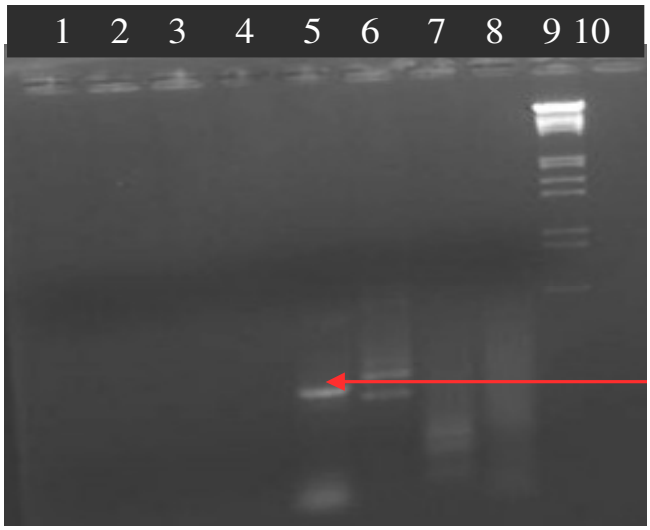

Figure 2. Determination of the sensitivity of the two-step PCR assay with purified *A. fumigatus* DNA (50 ng). The *Aspergillus* template DNA was clearly detectable by ethidium bromide staining of an agarose gel. Only DNA extracted from *A. fumigatus* (lane 5) was used in a single PCR amplification of the 236-bp fragment with the second primer pair. Negative reagent control amplification without addition of DNA (lane 1 and 2) as well as purified DNA from Phoma, DNA resulted in no bands (lane 3, 4). The Lambda DNA EcoR1/Hind III was used as molecular size marker (lane 9). Diluted sample of DNA in (lane 6, 7 and 8) in second step PCR product.

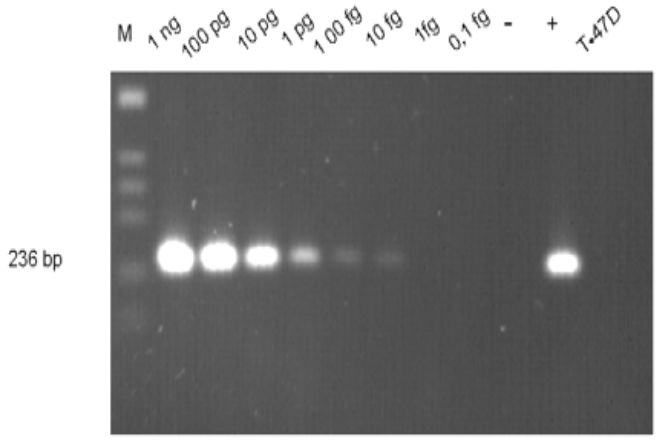

Figure 3. Determination of the sensitivity of the two-step PCR assay with purified *A. fumigatus* DNA (50 ng). The single derived from 10 fg of *Aspergillus* templet DNA was clearly detectable by ethidium bromide staining of agarose gel. As a positive control, only DNA extracted from *A. fumigatus* (10pg) was used in a single PCR amplification of 236-bp fragment with the second primer pair (lane +) Anegative control amplification without addition of DNA lane (-) as well as purified DNA from a human cell line (T47D) resulted in no bands. The 123-bp ladder (Gibco BRL) was used as molecular size marker (lane M).

improved by subsequent Southern blot hybridization. These data also highlighted for the first time the use of PCR for the detection of *Aspergillus* DNA in blood samples, which can be obtained by a less noninvasive procedure that is associated with fewer risks than the procedure used to collect BAL specimens, which cannot be performed repeatedly with immunocompromised patients.

We also used a two-step PCR procedure with carefully selected primers to increase the sensitivity and specificity for *A. fumigatus* detection. The multicopy 18S rRNA gene is highly conserved in all species but includes variable regions that are conserved among most *Aspergillus* species. On the basis of comparisons with sequences in a data bank, we chose oligonucleotides from the variable regions that specifically matched only the 18S rRNA gene of *A. fumigatus* to avoid problems arising from co amplification of human or bacterial DNA or contamination with other fungus-derived DNA. Two pairs of nested primers were chosen for use in all subsequent assays. PCR products were obtained only from DNA from *A. fumigatus*.

Conclusion

A specific two-step PCR assay for the rapid detection of the full range of human pathogenic *Aspergillus* species in sputum samples was established, facilitating improved early diagnosis and better monitoring of systemic *A. fumigatus* infection in pulmonary tuberculosis patients during therapy in high-risk patients.

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