

DETECTION OF *ASPERGILLUS FUMIGATUS* BASED ON Asp fl GENE IN CLINICAL SPECIMENS

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ABSTRACT

Oligonucleotide primers were synthesised based on the gene sequence of an 18 kDa allergen/antigen of *A.fumigatus* isolated from a pathogenic strain. Polymerase chain reaction (PCR) was carried out using the forward and reverse primers and genomic DNA of *A.fumigatus*, *A.flavus* and *A.niger* as template. This resulted in a PCR product of 480 bp with only *A. fumigatus*. The absence of PCR product in *A. flavus* and *A. niger* with the primers of Asp fl facilitated use of these primers for detection of *A.fumigatus* in clinical specimens of patients. The results were compared with microscopy, culture and serology. Application of PCR test to clinical samples of aspergillosis patients is discussed.

KEY WORDS : *Aspergillus fumigatus*, microscopy, culture, PCR-diagnosis

INTRODUCTION

Aspergillus fumigatus is a ubiquitous filamentous fungus associated with a wide variety of clinical conditions ranging from allergic to invasive forms. Recent years have witnessed a steady increase in opportunistic infections including *Aspergillus* infections due to the increasing incidence of AIDS, tuberculosis and use of immunosuppressive cytotoxic drugs in malignancies and organ transplants etc. The life threatening condition of the patient warrants use of rapid diagnostic methods for early institution of therapy.

Currently available serodiagnostic methods for aspergillosis are based on

immunodiffusion and immunoelectrophoretic techniques which are time consuming and less sensitive. Enzyme Linked Immunosorbent Assay (ELISA) based tests are being used in limited laboratories with partially fractionated or purified antigens. The sensitivity and specificity of ELISA based tests have high diagnostic value for detection of specific antibodies in patients of allergic bronchopulmonary aspergillosis and aspergilloma. However the detection of the antibodies is not feasible in immunocompromised host in view of poor humoral responses in these patients. Detection of circulating antigens based on ELISA suffers from lack of sensitivity (1). In such cases, detection of the organism using molecular probes in clinical samples may be advantageous for screening as well as

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confirmation of its presence. PCR based tests are reported for detection of a number of pathogens for infectious diseases such as *Toxoplasma gondii*, HIV-1 in DNA of peripheral blood mononuclear cells, cytomegalovirus DNA and *Pneumocystis carinii* in clinical samples. (2-5). Present study aims at evaluation of utility of PCR test based on Asp fl gene for clinical applications.

MATERIALS AND METHODS

Materials : Pancreatin, chitinase, ethidium bromide, tween 20, tris (hydroxymethyl) aminomethane, sodium dodecylsulfate, agarose and mineral oil were purchased from Sigma Chemical Co, St. Louis, MO. PCR kit containing Taq polymerase, dNTPs and buffer was purchased from Perkin Elmer Cetus, Foster City, CA. All chemicals used in this study were of Excelar/GR grade.

Isolation of *A.fumigatus* from clinical specimens : Fresh morning sputa and bronchial aspirates from patients from the Department of Medical Mycology, Vallabh Bhai Patel Chest Institute, Delhi and Rajan Babu Tuberculosis Hospital, Delhi, were collected aseptically. The samples were processed for isolation of the fungus as reported earlier (6). Seven control specimens which were culture negative and seronegative to *A.fumigatus* were also processed in a similar manner.

Isolation of DNA from *A.fumigatus* in the clinical samples : DNA of *A.fumigatus* from clinical samples was isolated by the procedure described earlier (6).

Synthesis of PCR primers : Oligonucleotide primers were designed based on the DNA

sequence of the gene for Asp fl isolated from a pathogenic strain of *A.fumigatus*.

The sequence of primers US 1 and US 2 based on the sequence of 18kDa gene of *A.fumigatus* used for the detection of the fungus in clinical specimens is as given below :

US 1 (Forward) :

5' TGG ACA TGT ATA AAC CAG 3'

US 2 (Reverse) :

5' ATG AGA ACA CAG TCT CAA GTC 3'

Polymerase chain reaction : PCR mix was prepared in a clean dedicated area using all sterile pipet tips and microfuge tubes. Each PCR reaction mixture contained 10 µl of 10x reaction buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl), 10 µl 25 mM MgCl₂, 10 µl of 10 x dNTP stock (2.0 mM each of dATP, dCTP, dGTP and dTTP) and 3 µl of each primer (0.2 µM). Clinical DNA sample (25 µl) was added and the reaction volume was made upto 100 µl with water. Amplitaq DNA polymerase (2.5U) was added, mixed, spun briefly and overlaid with 100 µl of mineral oil. PCR was carried out in a Perkin Elmer Cetus, model No. 4800, thermal cycler. The reaction conditions were: melting temperature at 92°C for 1 min., annealing temperature at 50°C for 1.5 min. and primer extension temperature at 72°C for 1 min for 30 cycles.

Agarose gel electrophoresis: At the end of the reaction, 100 µl of chloroform was added, mixed and centrifuged briefly. Ten µl of the PCR product in the upper aqueous layer was subjected to electrophoresis in a 1% agarose gel containing 0.5-1 µg ethidium bromide per ml and visualized with UV light.

Immunodiffusion : Immunodiffusion was performed with the sera samples of patients using a well defined antigenic fraction of *A.fumigatus* according to the method of Ouchterlony's (8) as modified by Banerjee et. al. (9).

Enzyme linked immunosorbent assay: Sera of patients were also tested by indirect ELISA for specific IgE and IgG antibodies as described (9).

RESULTS

Detection of *A.fumigatus* in clinical samples by PCR : 19 clinical samples, out of 21 tested

were found to be positive by PCR. A few examples of the amplification of 480 bp product in clinical samples, after agarose gel electro-phoresis and visualisation by UV are shown in Fig.1 (lanes 3-6). Fig. 2 depicts the PCR product amplified from 1 pg of chromosomal DNA of *A.fumigatus* and PCR product from clinical samples. Lane 2 of Fig. 2 shows ~480 bp PCR product using the genomic DNA from the fungus; lane 3 shows the PCR product when sputum specimen of a patient of allergic bronchopulmonary aspergillosis (ABPA) was used as the source of *A.fumigatus* and lane 6 shows the PCR product when sputum of a patient having aspergilloma was used. Lanes 2, 3 and 4

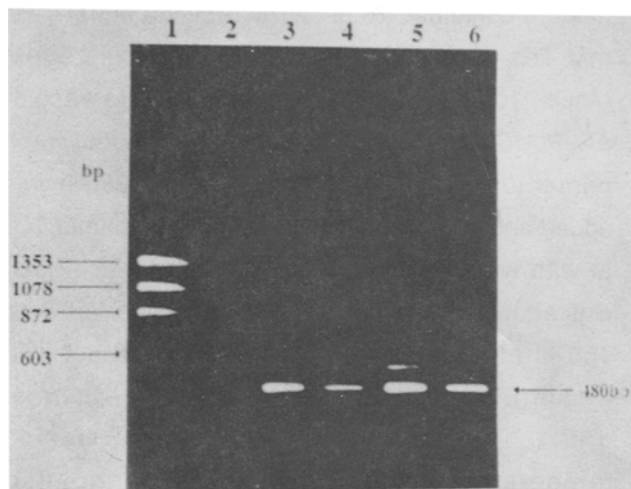


Fig. 1. Analysis of PCR products amplified from *A.fumigatus* in clinical samples on agarose gel.

Lane 1. ϕ X174/Hae III digest as base-pair marker. Lane 2. PCR using a sputum sample negative for culture and microscopy. Lanes 3 & 5. PCR product amplified from processed sputa of ABPA patients. Lanes 4 & 6. PCR product amplified from processed sputum and bronchial aspirate of aspergilloma patients.

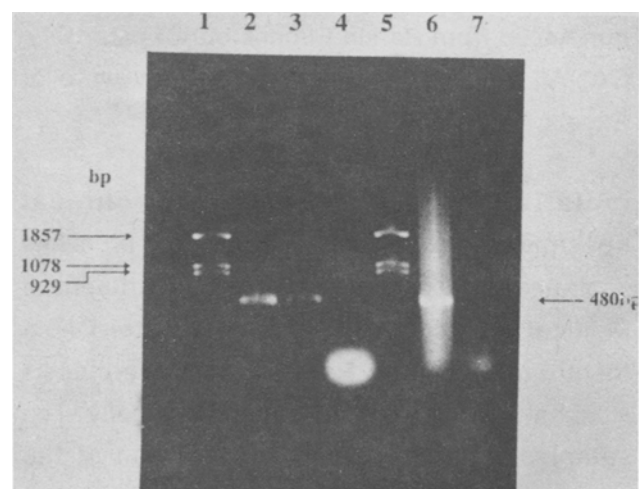


Fig. 2. Analysis of PCR amplified from genomic DNA extracted from *A.fumigatus* and from clinical samples on agarose gel.

Lanes 1 & 5. Bst N1 digest of pBR322 as base-pair marker. Lane 2. 480 bp product using 1 pg of genomic DNA extracted from *A.fumigatus*. Lane 3. PCR product using sputum of a patient of ABPA. Lanes 4 & 7. Similarly treated sputum samples from control individuals (only unreacted primers are seen). Lane 6. PCR product when sputum of an aspergilloma patient was used.

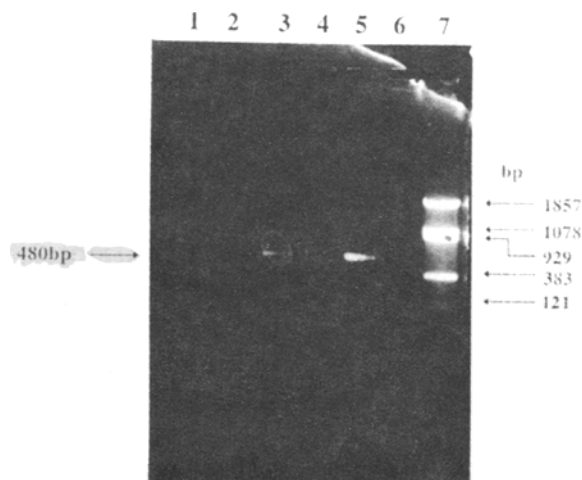


Fig. 3. Agarose gel electrophoresis of PCR product using BAL samples.

Lanes 1 & 6. Similarly treated sputum and BAL from control individual (only primers are seen)., Lanes 3, 4 & 5. PCR product when BAL samples were used., Lane 7. Bst N1 digest of pBR322 as base-pair marker.

of the Fig. 3 shows the ~480 bp PCR products from the bronchoalveolar lavage fluid of patients of invasive pulmonary aspergillosis. Amplification of samples from healthy controls and patients suffering from non *Aspergillus* disorders failed to yield the ~480 bp product as shown in Figs. 2 and 3 respectively. In fifteen out of the nineteen patients clinically suspected of aspergillosis, *A.fumigatus* could be isolated in culture, though microscopically hyphae characteristic of the fungus could be observed only in twelve patients. Serologically, precipitins could be detected against *A.fumigatus* in twelve out of the thirteen patients. Using ELISA, IgG antibodies against the

A.fumigatus antigens could be detected in only fifteen patients and antibodies could not be detected in the case of all immunocompromised patients or patients suspected of invasive aspergillosis on basis of clinical and radiological findings.

DISCUSSION

Polymerase chain reaction has proven to be an invaluable tool for the diagnosis of infectious diseases in the field of clinical microbiology. PCR based diagnostic methods have been developed for the detection of *Mycobacterium tuberculosis* (10-13) and *Legionella*. However, these methods have not reached clinical laboratories so far due to the high cost involved in performing the test. Extraction of DNA from the pathogen in clinical samples is a tedious procedure and one of the limiting factors for clinical applications of PCR. The presence of inhibitors to Taq polymerase and use of suboptimal extraction procedures pose potentially serious problems as false negative results can occur. *A. fumigatus* has a hard chitin rich cell wall and extraction of DNA requires the disruption of cell wall. Current method is rapid and needs only few hours to obtain the results.

The sensitivity and specificity of PCR methods over conventional culture techniques has already been shown for detection of tuberculosis (14). In the present study PCR test could be applied successfully for clinical samples such as bronchial aspirates, sputum and bronchoalveolar lavage samples. The PCR test based on Asp fl gene is observed to be useful in comparison to other techniques routinely employed especially in case of immuno-compromised

patients. All the controls and patients with non *Aspergillus* disorders did not show a PCR product. This result was well correlated with culture and ELISA techniques. The primers used in this study appear to be specific as the 480 bp PCR product was seen with only *A. fumigatus* and not with other *Aspergillus* species (7). Amplification of 1 pg of genomic DNA from a laboratory strain of *A. fumigatus* suggests that sufficient quantity of DNA could be isolated from the clinical specimens for PCR tests. Current study indicates the feasibility of PCR based detection of *A.fumigatus* in clinical samples of aspergillosis

patients. It may be advantageous to use this test in screening immunocompromised patients and in transplant wards etc.

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