

IMMUNO-POLYMERASE CHAIN REACTION FOR DETECTION OF *ASPERGILLUS FUMIGATUS*

¹P.USHA SARMA, ¹NIVEDITA BIR, ¹ANUBHA PALIWAL AND ²PRASAD REDDY

¹Centre for Biochemical Technology, Mall Road, Delhi-110007, India ²DNA Technologies Group, Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, MD. 20899, USA.

ABSTRACT

A number of *Aspergillus* infections are caused by the opportunistic fungal pathogen *Aspergillus fumigatus* in humans especially under immunosuppressed conditions. Major forms of the disease include invasive aspergillosis, allergic bronchopulmonary aspergillosis and aspergilloma. A procedure that uses chitinase and microwave treatment is described for the extraction of genomic DNA of *Aspergillus* species from the sputum and bronchial aspirate of patients with established aspergillosis. Detection of *A.fumigatus* was compared by culture, microscopy, serology by ELISA, immunodiffusion, agarose gel electrophoresis of PCR products and colorimetric immuno-PCR. A colorimetric method for the detection of PCR product was developed based on immunoaffinity reactions. Out of the clinical samples tested from nineteen patients, fourteen were positive and five were negative by all the methods tested. It was established that at least 1 pg of DNA was extractable from the clinical samples sufficient to produce enough quantities of PCR product for detection on agarose gel or by immunoaffinity based color reaction. An absorbance value of 0.9 to 1.5 against 0.2 for negative control was obtained at 405 nm for colorimetric immuno-PCR. This method can be exploited for screening large number of clinical samples from immunocompromized as well as from suspected cases of aspergillosis.

KEY WORDS : *A. fumigatus*, PCR diagnosis, Colorimetric PCR

INTRODUCTION

Invasive infections caused by ubiquitous fungi of the genus *Aspergillus* are often encountered in immunocompromised patients with high degree of neutropenia. Pathogenic species of *Aspergillus*, particularly *Aspergillus fumigatus*, are the causative agents of a variety of pulmonary diseases in human including invasive aspergillosis, allergic bronchopulmonary aspergillosis and aspergilloma to mention a few (1). Invasive aspergillosis is difficult to diagnose and successful treatment depends on the early detection of the fungus (2). Current methods of diagnosis

include direct microscopy, culture and immunodiagnosis. Microscopy is simple and rapid but not sensitive. Culture is sensitive and more specific but takes several days for diagnosis. Immunodiagnosis is not useful for the detection of invasive aspergillosis because of the immunosuppressed state of patients (2). Therefore, nucleic acid based identification of *Aspergillus* species is prudent in the detection of the disease. Polymerase chain reaction (PCR) has replaced the conventional techniques for the rapid diagnosis of infectious agents in human diseases. DNA amplification methods enable genetic typing of the isolates. Random amplification of polymorphic DNA (RAPD) typing allows distinction between isolates of *A.fumigatus* (3).

Author for correspondence

Dr. P.Usha Sarma, at above address

The gene for major diagnostically relevant antigens/allergens has been cloned and sequenced from *A. fumigatus* of clinical origin (4-6). Cloning and sequencing of the gene from *A. fumigatus* isolated from different clinical specimens showed identical amino acid sequence (4-6). A 480 bp PCR product was detected in the sputa and bronchial aspirates of patients with aspergillosis diseases but not in the healthy controls. The method is rapid, dependable and was adopted to screen a large number of clinical samples using colorimetric PCR.

MATERIALS AND METHODS

Isolation of *A. fumigatus* from clinical specimens : Sputa and bronchial aspirates from patients were obtained from the Department of Medical Mycology, Vallabhbhai Patel Chest Institute, Delhi and Rajan Babu Tuberculosis Hospital, Delhi. Fresh morning sputum samples and bronchial aspirates were collected aseptically. The thick, viscous sputum specimens were digested with 1% pancreatin in phosphate buffer pH-7.5 and subsequent incubation at 37°C for one and a half hour. Bronchial aspirates were centrifuged at 2000 rpm for 10 minutes and the supernatant was discarded. Each specimen was divided into three parts, one part each for direct microscopy, culture and PCR. One part was subjected to direct microscopy to look for the presence of hyaline, septate hyphae characteristic of *Aspergillus* species. 0.5 ml from each specimen was used for inoculation on dextrose agar with and without chloramphenicol (Emmons modification) and incubated at 25°C and 37°C, one set each for 1-2 weeks. Seven control specimens, culture negative and seronegative to *A. fumigatus*, from healthy individuals were also processed similarly for controls.

Isolation of DNA from *A. fumigatus* in the clinical samples : The remainder of the specimen was centrifuged at 3500xg for 20 min and the pellets were suspended in 0.2ml of 0.1 M phosphate buffer pH-6.8. (N/15 Sorenson's phosphate buffer). Chitinase (0.5U) was added to each tube and incubated for 1 h at 25°C. Tubes were then heated three times in a microwave oven for 3 sec. cycles each (7). Debris from the chitinase treated sputa and bronchial aspirate was pelleted by centrifugation at 12,000 x g for 10 min. The supernatant was siphoned off and the pellet containing

fungal cells was emulsified by vigorous vortexing in 100 µl of chloroform. Distilled water (50µl) was added and emulsified with the chloroform phase. After centrifugation at 12,000 x g for 10 min. the upper aqueous phase was collected. The samples containing the chromosomal DNA were stored at -20°C with chloroform, if not used immediately, and were recentrifuged prior to use.

Isolation of genomic DNA from *Aspergillus* species : *A. fumigatus* and *A. flavus* strains were grown in asparagine broth. Mycelia were harvested after three days, filtered and washed several times with sterile distilled water. Mycelial mat was made into a paste by grinding in a mortar under liquid nitrogen. Fungal cells were suspended in 0.1 M phosphate buffer, pH 6.8 (1 gm wet weight/mL), and treated with 0.5 U chitinase per mL of cell suspension of 1 h at 25°C. Equal volume of lysis buffer (50mM Tris-Cl, was added to the cell suspension and cells were treated in a microwave as described above. DNA was precipitated with ethanol after phenol: chloroform extraction as described (8).

Synthesis of PCR primers : Oligonucleotide primers for the 5' and 3' ends of the gene were designed based on the DNA sequence of the gene from *A. fumigatus*. (9) The oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer.

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 10 x reaction buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl), 10µl of 25mM MgCl₂, 10 µl of 10 x dNTP stock (2.0 mM each of dATP, dCTP, dGTP and 1.3 mM dTTP), 10 µl of 0.07 mM digoxigenin-11-dUTP, 17 µl of 0.3 mM biotinylated -11-dUTP, 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 (0.5 µl-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 microliters 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.

Colorimetric detection of PCR product : Each well of microtitre plate was coated with 100 µl of streptavidin (10 µg/ml in 50 mM carbonate buffer, pH 9.6) for 1 h at 37°C. Wells were rinsed twice in Tris buffered saline, pH 7.5 containing 0.5% (V/V) Tween-20 (TBS-T20). Ten microliters of PCR product was diluted to 100 µl in TBS-T20 and added to each well. The plate was incubated for 30 min at 37°C. The biotinylated PCR product was captured in the streptavidin-coated microtitre well. The wells were rinsed twice with TBS-T20 and 100 µl of antidigoxigenin alkaline phosphatase conjugate diluted to 1:5000 in TBS-T20 was added to each well. After 30 min of incubation at room temperature, wells were washed thoroughly with TBS-T20 to remove the unbound conjugate. One hundred microliters of paranitrophenylphosphate (PNPP) in 50 mM carbonate buffer, pH 9.6, containing 1 mM each of MgCl₂ and CaCl₂ was added to each well and the plate was incubated at room temperature for 30 min. The optical density of the reaction product was read at 405 nm.

Immunodiffusion : Immunodiffusion was performed with the sera samples of all the patients using a well defined antigen fraction of *A.fumigatus* according to the method of Ouchterlony (10) as modified by Banerjee et al (11).

ELISA : Sera of patients were tested by IN-DIRECT ELISA for specific IgE and IgG antibodies to *A.fumigatus* antigen as described (12).

Materials : Pancreatin, chitinase, ethidium bromide, tween 20, tris (hydroxymethyl) aminomethane, sodium dodecylsulfate, mineral oil, Bio-11-UTP 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. Streptavidin, dig-11-dUTP, antidigoxigenin, alkaline phosphatase were obtained from Boehringer Mannheim, Specific oligonucleotide primers were synthesized based on the nucleotide sequence of the gene for the 16.9 kDa virulence factor/antigen of *A.fumigatus*. All other chemicals used in this study were Excelar/GR grade.

RESULTS

Culture and Microscopy of Clinical Samples :

Out of nineteen clinical samples tested by microscopy and culture, eleven were found to be positive. The other four samples were found to be culture and microscopy negative (Table 1). Sera of the same patients were tested by both immunodiffusion and ELISA. Consistent with culture and microscopy, identification of *Aspergillus* species by immunodiffusion and ELISA techniques were positive for culture positive specimens and negative for culture negative specimens.

Detection of *A.fumigatus* by PCR. : Polymerase chain reaction of *A.fumigatus* genomic DNA (9). It is worthwhile noting that a similar amplification using *A.flavus* genomic DNA did not produce any PCR product. This observation formed the basis for PCR based rapid diagnosis of a variety of diseases caused by *A.fumigatus*.

Detection of *A.fumigatus* in clinical samples by PCR : Fifteen clinical samples, out of the nineteen tested, that were positive by culture and microscopy also tested positive by PCR (9). Amplification of one of the negative specimens by culture and microscopy failed to yield the 480 bp product (9).

Colorimetric immuno-PCR : Analysis and handling of a large number of PCR products by agarose gel electrophoresis is laborious. We have instead chosen to analyze a large number of PCR products by immuno-screening in a microtitre plate. In the amplification protocol, biotinylated and digoxigenin labeled dUTP was included along with dNTPs to obtain biotinylated and digoxigenin labeled DNA fragment. The labeled DNA fragment was bound to streptavidin coated wells and coupled to the antidigoxigenin alkaline phosphatase conjugate and the PCR product was quantitated by reaction with paranitrophenylphosphate and the absorbance was measured at 405nm. The absorbance for PCR positive clinical samples from patients with aspergillus infection was in the range of 0.9 to 1.5 whereas the absorbance for PCR negative clinical samples from normal control humans was about 0.2

DISCUSSION

Polymerase chain reaction has been proven to be an invaluable tool for the diagnosis of infectious diseases in the field of clinical micro-

Table 1 The results of different diagnostic methods to identify *A fumigatus* infection.

Sl. No.	Clinical sample	Microscopy	Culture	ELISA absorbance at 490nm for specific IgG and IgE antibodies (average of duplicates)		Immuno Diffusion test	Detection of PCR Product on Agarose	Immuno PCR Absorbance at 405nm (average of duplicates)
ABPA								
1.	Sputm	+ ve	+ ve	1.530(+)	0.604(+)	+ ve	+ ve	1.410
2.	sputum	+ ve	+ ve	1.864(+)	0.539(+)	+ ve	+ ve	1.420
3.	sputum	+ ve	+ ve	1.339(+)	0.620(+)	+ ve	+ ve	1.432
4.	sputum	+ ve	+ ve	1.353(+)	0.539(+)	NA	+ ve	1.336
5.	sputum	- ve	- ve	0.996(+)	0.535(+)	+ ve	+ ve	1.439
6.	B.A.	+ ve	+ ve	0.637(+)	0.630(+)	+ ve	+ ve	1.502
7.	Sputum	+ve	+ ve	1.928 (+)	0.499 (+)	+ve	+ve	1.509
8.	B.A.	-ve	+ve	1.895 (+)	0.617 (+)	+ve	+ve	0.909
9.	sputum	+ ve	+ ve	1.038(+)	0.536(+)	+ ve	+ ve	0.932
ASPERGILLOMA								
10.	B.A.	+ ve	+ ve	0.922(+)	0.464(+)	NA	+ ve	1.309
11.	sputum	+ ve	+ ve	1.607(+)	0.406(+)	+ ve	+ ve	1.535
12.	sputum	+ ve	+ ve	0.928(+)	0.450(+)	NA	+ ve	1.504
13.	sputum	+ ve	+ ve	1.445(+)	0.399(+)	+ ve	+ ve	1.525
14.	sputum	+ ve	+ ve	0.985(+)	0.435(+)	+ ve	+ ve	0.869
15.	sputum	- ve	- ve	1.513(+)	0.419(+)	+ ve	+ ve	0.888
IMMUNOCOMPROMISED PATIENTS								
16.	BAL	- ve	+ ve	0.252(-)	0.110(-)	- ve	+ ve	1.232
17.	BAL	- ve	- ve	0.111(-)	0.080(-)	NA	+ ve	0.978
18.	BAL	- ve	+ ve	0.222(-)	0.151(-)	NA	+ ve	1.839
19.	BAL	- ve	- ve	0.188(-)	0.103(-)	NA	+ ve	0.910
OTHER ALLERGIC DISORDER								
20.	sputum	- ve	- ve	0.232(-)	0.090(-)	NA	- ve	0.315
21.	sputum	- ve	- ve	0.202(-)	0.050(-)	- ve	- ve	0.213
NORMAL CONTROLS								
1.	sputum	- ve	- ve	0.090(-)	0.035(-)	- ve	- ve	0.242
2.	sputum	- ve	- ve	0.151(-)	0.056(-)	- ve	- ve	0.251
3.	sputum	- ve	- ve	0.211(-)	0.031(-)	- ve	- ve	0.212
4.	sputum	- ve	- ve	0.099(-)	0.042(-)	- ve	- ve	0.222
5.	sputum	- ve	- ve	0.165(-)	0.039(-)	- ve	- ve	0.205
6.	sputum	- ve	- ve	0.142(-)	0.091(-)	- ve	- ve	0.255
7.	sputum	- ve	- ve	0.210(-)	0.073(-)	NA	- ve	0.210
8.	sputum	- ve	- ve	0.102(-)	0.081(-)	NA	- ve	0.203
9.	sputum	- ve	- ve	0.198(-)	0.065(-)	NA	- ve	0.238
10.	sputum	- ve	- ve	0.221(-)	0.092(-)	NA	- ve	0.258

Cut off absorbance value : IgG<0.350; IgE<0.200; NA : not applicable

biology. PCR based diagnostic methods have been developed for the detection of *Mycobacterium tuberculosis* (13-16), *Legionella pneumophila*, *Aspergillus* species (17, 18), and a large number of other pathogens (19, 20). Many of the procedures for isolating DNA prior to PCR involve numerous steps like protease digestion, use of the chaotrophic-silica method, mechanical disruption of cells, phenol extraction, DNA precipitation and RNase treatment (9). Extraction of DNA from *A.fumigatus* is a difficult procedure because of its chitin rich cell wall. Extraction of DNA of *A.fumigatus* from clinical samples is even more tedious because of the inhibitors found in sputum samples. The present study describes the processing of sputum samples, rapid isolation of DNA from *Aspergillus* by chitinase and microwave treatment (7) and development of colorimetric PCR method for the detection of *A.fumigatus* in the clinical samples. The method is rapid, sensitive and needs only few hours to obtain results.

The protocol for clinical sample preparation offers significant advantage over the current procedures of sample treatment. The DNA extraction procedure described here is simple and efficient. It is performed in a short period, avoids mechanical disruption of cells and the use of phenol extraction. Apart from the microwave oven no special equipment is needed. This protocol may be adopted for organisms other than *Aspergillus* species.

The sensitivity and specificity of PCR methods are reported to be far greater than those of the culture method for detection of tuberculosis (21). Detection of *A.fumigatus* by PCR has previously been shown to be more sensitive than microscopy and, of course, much faster than culture technique. In the present study, detection of *A.fumigatus* in the clinical samples from patients with *Aspergillus* infections was compared by microscopy, culture, serology by ELISA, immunodiffusion, and PCR amplification. With the exception of time involved for the detection of *A.fumigatus* by these methods, the results agreed in all the cases (Table 1). The primers used in this study are highly specific. Amplification of 1 pg of genomic DNA from a laboratory strain of

A.fumigatus gave the PCR product suggesting that at least 1 pg of DNA was isolated from the clinical samples. About 25% of the clinical samples from patients were negative for *A.fumigatus* by all the methods of testing (Table-1). Infection in these patients might have been caused by other species of *Aspergillus*.

PCR products are generally detected by agarose gel electrophoresis and ethidium bromide stain. This technique is not convenient for screening a large number of clinical samples. Colorimetric detection of the PCR products (12) in a microtitre plate would be more convenient and efficient to perform. In this study we developed a colorimetric PCR assay for the detection *A.fumigatus*. The method is based on labeling the PCR product with biotin and digoxigenin and binding the PCR product to streptavidin coated microtitre wells. The bound digoxigenin labeled DNA was coupled to antidigoxigenin alkaline phosphatase and the color was developed with paranitrophenyl phosphate. The colorimetric detection of PCR products was found to be at least as sensitive and specific as agarose gel electrophoresis and is useful for analysing a large number of samples. Since the samples are analyzed in a microtitre plate, 96 samples can be analyzed at a time. Color intensity can be measured with the help of ELISA reader and the quantitation of DNA may be possible. Positive samples can also be identified by the presence of color in the wells against control. This test may be used for screening large number of samples.

ACKNOWLEDGEMENTS

This work was supported in part by grants from Department of Biotechnology and University Grants Commission, Government of India. The authors are grateful to Prof. H.S. Randhawa of VPCI, Delhi for providing cultures and clinical samples. We thank Prof. K.Muralidhar, Department of Zoology, University of Delhi for his valuable suggestions. Thanks are also due to Mr.Joel Hoskins, Centre for Advance Research in Biotechnology, Rockville, MD for the synthesis of oligonucleotides.

REFERENCES

1. Bardana E.J.Jr. (1980). The clinical spectrum of aspergillosis. Part 2. Classification and description of saprophytic, allergic, and invasive variants of human disease. *CRC Crit.Clin.Lab.Sci.* 13, 85-159.
2. Young, R.C. and Bennett, J.E. (1971). Invasive aspergillosis: absence of detectable antibody response. *Am.Rev.Respir.Dis.* 104, 710-716.
3. Aufauvre-Brown, A., Cohen, J. and Holden, D. (1992). Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus* *J.Clin.Microbiol.* 30, 2991-2993.
4. Arruda, L.K., Mann, B.J. and Chapman, M.D. (1992). Selective expression of major allergen, and cytotoxin, Asp 1, in *Aspergillus fumigatus*. *J.immunology.* 149, 3354-3359.
5. Moser, M., Cramer, R., Menz, G., Schneider, T., Dudler, T., Virchow Sr. C., Gmachl, M., Blaser, K. and Suter, M. (1992). Cloning and expression of recombinant *Aspergillus fumigatus* allergen 1/a (r Aspl/a) with IgE binding and type I skin activity. *J. Immunol* 149, (2) 454-460.
6. Sarma, P.U. and Reddy, P. Unpublished data.
7. Bir.N., Paliwal, A., Muralidhar, K., Reddy, P. and Sarma, P.U. (1995). A rapid method for the isolation of genomic DNA from *Aspergillus fumigatus*. *Preparative Biochem.* 25, 171-181.
8. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Vol. 1, p. 1.21
9. Sarma, P.U., Bir, N., Paliwal, A. and Reddy, P. (1998) Detection of *A. fumigatus* by immuno-PCR from clinical samples. (Communicated).
10. Ouchterlony, D.(1953) Antigen-antibody reaction in gels: Types of reaction in coordinated system of diffusion. *Acta Pathol. Microbiol. Scand.* 31, 231-237.
11. Banerjee, B., Madan, T., Sharma, G.L., Prasad, K., Nath, I. and Sarma, P.U. (1995). Characterization of 45 kDa glycoprotein antigen of *A.fumigatus*. *Serodiagnosis and immunotherapy. Infectious Diseases.* 7,147-152.
12. Helmuth, R.(1990). Nonisotopic detection of PCR products In : PCR protocols. Eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., Academic Press, Toronto p 119-128.
13. Dell Portillo, P., Murillo, L.A. and Patarroyo, M.E. (1991). Amplification of a species specific DNA fragment of *Mycobacterium tuberculosis* and its possible use in diagnosis. *J.Clin. Microbiol.* 29, 2163-2168.
14. Eisenach, K.D., Siford, M.D., Care, M.D., Bates, J.H. and Crawford, J.T. (1991). Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction *Am.Rev.Respir.Dis.* 144, 1160-1163.
15. Folgueira, L., Delgado, R., Palenque, E. and Noriega, A.R., (1993). Detection of *Mycobacterium tuberculosis* DNA in clinical samples by using a simple lysis method and polymerase chain reaction. *J.Clin.Microbiol.* 31, 1019-1021.
16. Codfrey-Faussett, P.E., Wilkins, G.L., Khoo, S. and Stoker, N.G. (1991). Tuberculosis pericarditis confirmed by DNA amplification. *Lancet* 337, 176-177.
17. Reddy, L.V., Kumar, A. and Kurup, V.P. (1993). Specific amplification of *Aspergillus fumigatus* DNA by polymerase chain reaction. *Mol.Cell. Probes* 7, 121-126.
18. Spreadbury, C., Holden, D., Aufauvre-Brown, A., Bainbridge, B. and Cohen, J. (1993). Detection of *Aspergillus fumigatus* by polymerase chain reaction *J.Clin.Microbiol.* 31, 615-621.
19. Laure, F., Courgnaud, V., Rouzioux, C., Blanche, S., Verber, F., Burgard, M., Jacomet, C., Griscelli, C. and Brechot, C. (1988). Detection of HIV-1 DNA in infants and children by means of the polymerase chain reaction. *Lancet* 2, 538-540.
20. Manos, M.M., Ting, Y., Wright, D.K., Lewis, A., Broker, T.R. and Wolinsky, S.M. (1989). The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells* 7, 209-214.
21. Manjunath, N., Shankar, P., Rajan, L., Bhargava, A., Saluja, S. and Shriniwas, X. (1991). Evaluation of a polymerase chain reaction for the diagnosis of tuberculosis. *Tubercle* 72, 21-27.