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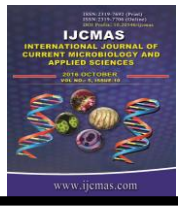

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Detection of *Aspergillus* Species by Polymerase Chain Reaction

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A B S T R A C T

Aspergillus species have recently caused increasing numbers of acute invasive infections in immunocompromised patients. This prospective and experimental study was carried out at Department of Microbiology and Central Research Laboratory, MGM Medical College and Hospital, Navi Mumbai. Detection of *Aspergillus* species was done using Polymerase Chain Reaction. This detection level (sensitivity 25%) is higher than Culture (19.5%), Fluorescence (17%) and Microscopy (10%) and difference in sensitivity is statically significant P value (0.001) <0.05, Significant. Species detection in nested PCR was *A. niger* (50%), *A.fumigatus* (35%) and A.*flavus* (15%). This shows more species specificity of PCR. Molecular methods are more sensitive and specific methods. It gives results for detection and speciation in short time which helps early diagnosis and treatment of patient.

Introduction

Aspergillus species have recently caused increasing numbers of life-threatening acute invasive infections in immunocompromised patients (Kontoyiannis *et al*., 2002). The steadily increasing incidence of invasive aspergillosis over the last few decades is ascribable to the increasing number of patients undergoing chemotherapy, bone marrow or solid organ transplantation and intensive corticosteroid therapy (Yamazaki *et al*., 1999).

Conventional diagnosis of fungal infection relies on the identification of pathogens by means of morphological characters specific to the genus and species. This is sometimes unsuccessful, however, because of the atypical features of some isolates. Molecular biological identification systems for pathogenic aspergilli have been suggested as a solution to this problem: for example, a PCR based diagnostic method for detecting the genus *Aspergillus* using 18S rDNA (Makimura *et al*., 1994; Yamakami *et al*., 1996; Gaskell *et al*., 1997) has been designed. Systems have also been described for specific detection of *Aspergillus fumigatus* with primers based on regions of the 28S rDNA or of the internal transcribed spacer (ITS) 1 and 2 regions of ribosomal DNA (rDNA) (Radford, 1998; Henry *et al*., 2000). These PCR systems described to date

are useful only in identifying the genus *Aspergillus* as a whole or the single species *A. fumigatus*. The ITS region contains variable elements that allow for sequence-based identification of *Aspergillus* species (Iwen *et al*., 2002); therefore, the region offers a possible template for design of species-specific primers for identification of the major pathogenic species.

Because the number of species of pathogenic fungi known to infect immunocompromised patients is growing, (Sugita *et al*., 2004) it is essential that quick and reliable methods of identification be found for the most common pathogenic species of aspergilli. This means that not just *A. fumigatus*, but also *Aspergillus flavus* and *A. niger*, should be rapidly identified by a successful system.

Materials and Methods

This prospective and analytical study was conducted at Microbiology laboratory and Central Research laboratory, MGM Medical College and Hospital, Navi Mumbai, Maharashtra, India. Total 200 sputum samples were collected from Aspergillosis suspected patients. KOH mount and examined by light microscopy and all samples were also subjected to Calcofluor white stained examined by Fluorescence microscope test and PCR.

Sample collection

3-5 ml of sputum was collected from patient attending a tertiary care hospital in a sterile container taking all sterile precaution and properly labeled the container with patient's name, date and time.

Primer sequences

The oligonucleotide primers used in this study are as described in the table 1. The primers were obtained from Sigma, USA.

PCR specification

PCR amplifications were performed in accordance to a procedure as followed by Sugita *et al*., (2004) According to the procedure master mix "BioMix Red" (Bioline, India), 5µl DNA, 20 pmol of primers were added and mixed to obtain 50µl final volume of the PCR mix.

Results and Discussion

PCR-based detection or identification systems for *Aspergillus* species were based on using 18S or 28S rDNA as target DNA. However, the sequences in these regions are conserved across a wide range of fungi; it is therefore difficult to design truly species-specific primers. As reported previously, the more variable ITS regions have proven more useful for identification of fungal species (Henry *et al*., 2000).

The present study using specific PCR amplification to allow identification not just of *A. niger* but also *A. fumigatus* and *A. flavus*, the second and third most frequently significant *Aspergillus* species in opportunistic infection, using specific PCR amplification. The importance of these species should not be underestimated. The number of infections they cause is increasing (Hoshino *et al*., 1999; Nenoff *et al*., 2002).

Nested PCR for *Aspergillus* species (*Niger*, fumigates, *flavus*) was performed by using species specific primer sets Nig, Fmi and Fla. The method followed by as per Sugita C *et al*., (2004). Out of 200 PCR test runs on sputum samples, with 50 samples showed presence of *Aspergillus* genome. This detection level (sensitivity 25%) is higher than Culture (19.5%), Fluorescence (17%) and Microscopy (10%) and difference in sensitivity is statically significant. Microscopy Vs. PCR , P value (0.001) <0.05, Significant.

Table.1 Primers for nested PCR of 18S rRNA gene in *Aspergillus* species.10

Name of organism	Primer set	Primers	Oligonuleotides	Gene accession No.
Aspergillus species	ASAP	ASAP1	5'-CAGCGAGTACATCACCTTGG-3'	KP987074.1
		ASAP2	5'-CCATTGTTGAAAGTTTTAACTGATT-3'	KP657690.1
A. fumigatus	Fmi	ASPU	5'-ACTACCGATTGAATGGCTCG-3'	KR023997.1
		Af3r	5'-CATACTTTCAGAACAGCGTTCA-3'	LC133095.1
A. niger	Nig	ASPU	5'-ACTACCGATTGAATGGCTCG-3'	KF304798.1
		Ni1r	5'-ACGCTTTCAGACAGTGTTCG-3'	LC133092.1
A. flavus	Fla	ASPU	5'-ACTACCGATTGAATGGCTCG-3'	KP784374.1
		Fl2r	5'-TTCACTAGATCAGACAGAGT-3'	LC133097.1
Pan-fungal primers	18S rDNA	B2F	5'-ACTTTCGATGGTAGGATAG-3	KT935264.1
		B4R	5'-TGATCGTCTTCGATCCCCTA-3'	KT935264.1

Table.2 Cycling conditions of first- and nested-step PCR reactions.10

Reaction[a]	**Cycling conditions**
First step (*Aspergillus* species)	Initial Denaturation at 94°C for 4 min, Denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 90 Sec (30 cycles). Thermal cycling was terminated by polymerization at 72°C for 10 min.
For the species specific primer sets Fmi, Nig, and Fla.	Initial Denaturation at 94°C for 4 min, Denaturation at 94°C for 1 min, annealing at 60°C for 15 Sec, and extension at 72°C for 15 Sec (25 cycles). Thermal cycling was terminated by polymerization at 72°C for 10 min.

Table.3 Showing different methods for detection of *Aspergillus* species.

Methods	Positive	Negative	Percentages
Microscopy (n=200)	20	180	10%
Fluorescence microscopy (n=200)	34	166	17%
Culture (n=200)	39	161	19.5%
PCR (n=80)	20	60	25%

Table.4 Showing *Aspergillus* speciation by PCR method.

Aspergillus species	Positive No.	Percentages
A. niger	10	50%
A. fumigatus	7	35%
A. flavus	3	15%
Total	20	100%

Fig.1 Showing confirmation of *Aspergillus* species.

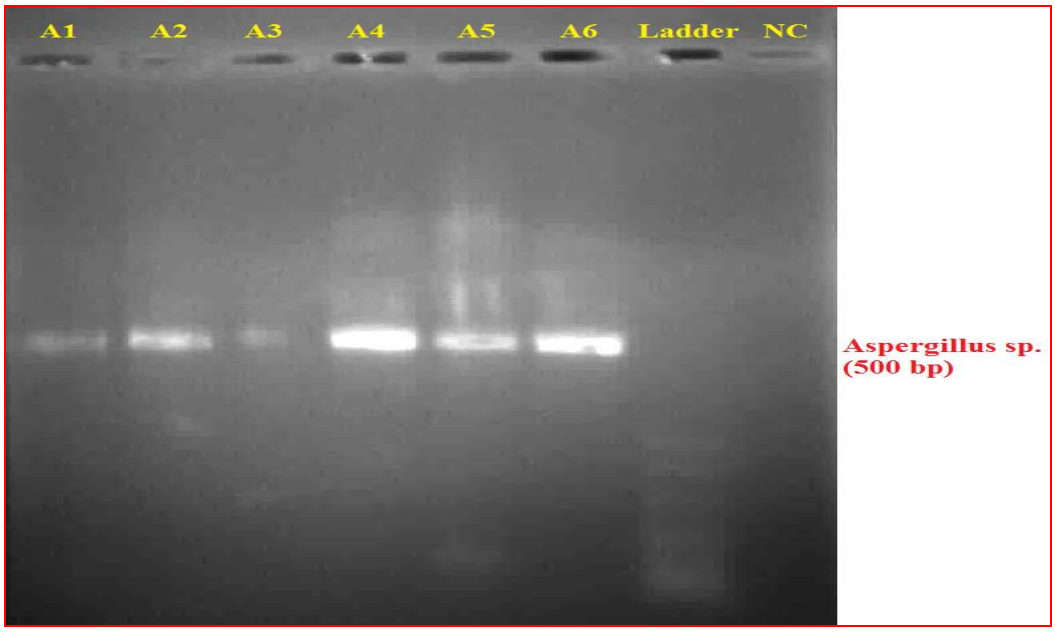


Fig.2 Showing species characterization of *Aspergillus* species (A1→A. flavus, A2-A 5→*A. niger*, A-6-A7 → *A. fumigatus*).

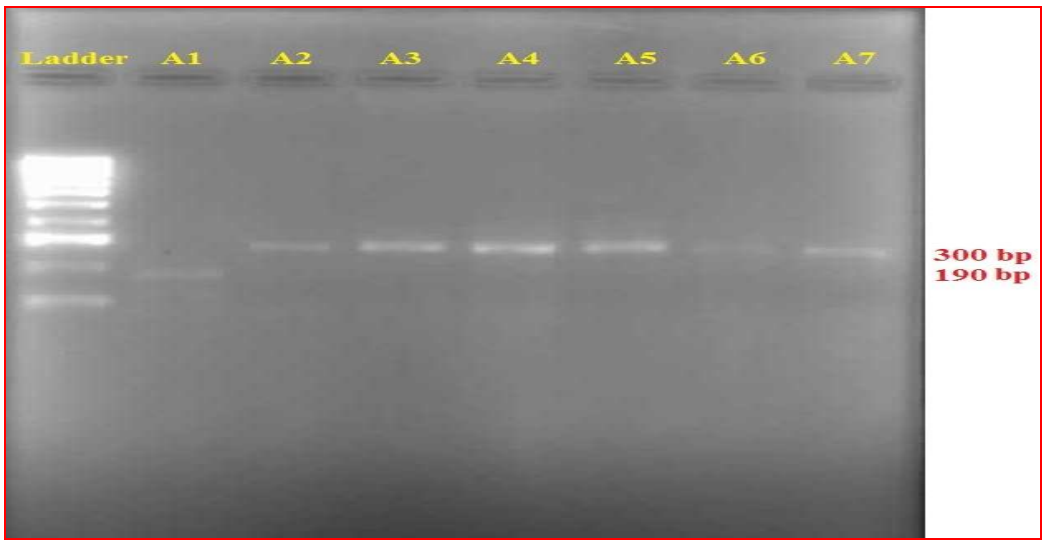


Fig.3 Showing *Aspergillus* species detection by various methods.

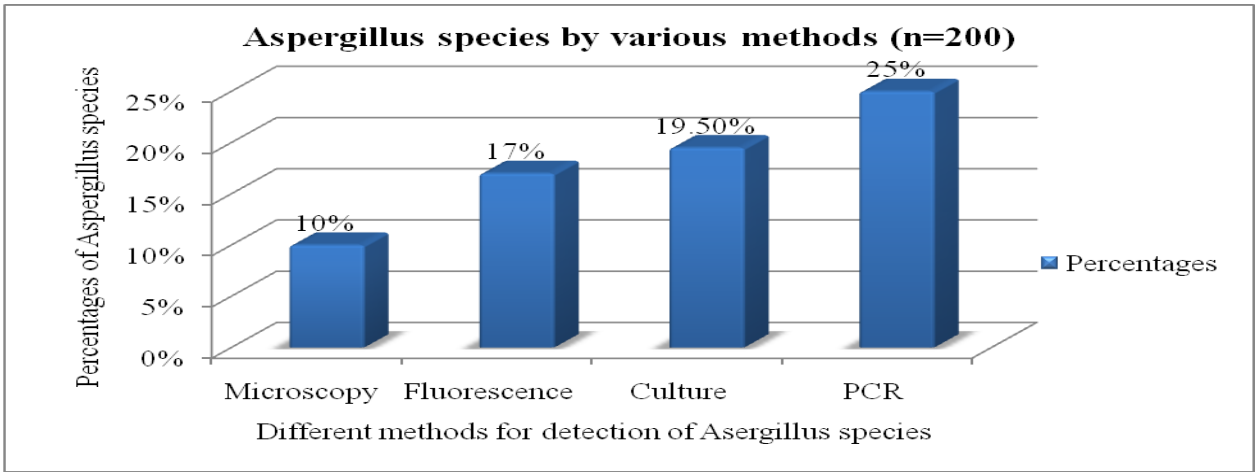
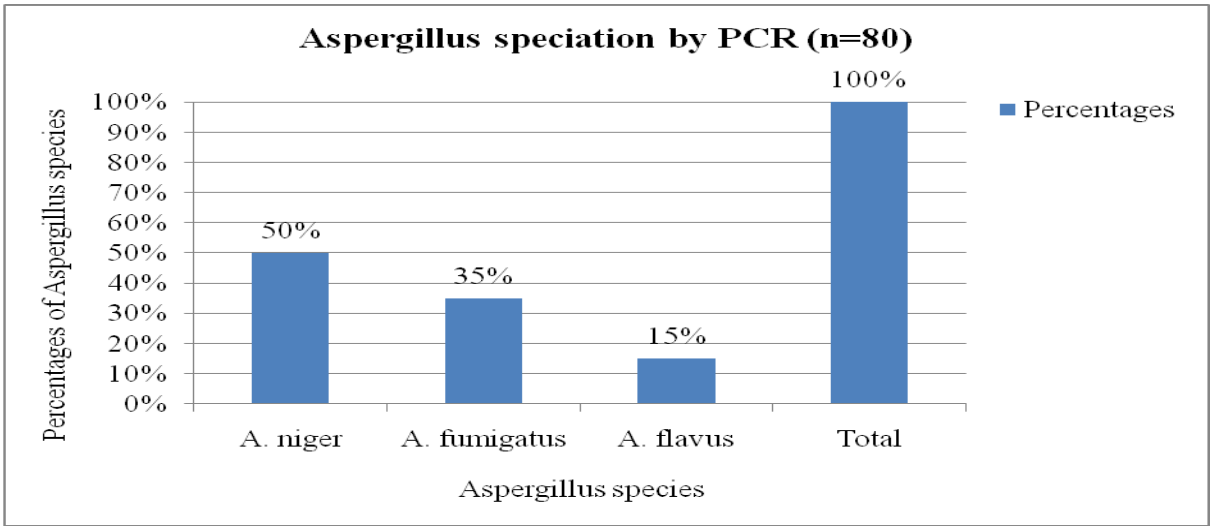


Fig.4 Showing *Aspergillus* speciation by PCR methods.



Species detection in nested PCR was *A. niger* (50%), A.fumigatus (35%) and A.*flavus* (15%). This shows more species specificity of PCR.

Most of the PCR studies for aspergillosis were done in other countries-Christop *et al*., [1993 from United Kingdom], Hayette *et al*., (2001 from Belgium), Loeffler *et al*., (2002 Germany), Sugita *et al*., (2004 from Japan),White *et al*., (2006 United Kingdom), Diba *et al*., (2014 from Iran).

Studies from India –

a) Bagyalakshmi *et al*., (2007 from Chennai) studied on 168 ocular specimen. Smear examination (20.23%), Culture (25%), PCR(53.57%).
b) Deshpande *et al*., (2011 from Maharashtra) studied on 71 cases . Out of which PCR positive (18.30%).

All the above mentioned studies showed high level of sensitivity and specificity of PCR for detection of *Aspergillus*.

In conclusion, nested PCR for *Aspergillus* species (*niger*, *fumigatus*, *flavus*) on sputum samples which showed presence of infection (pus cells) was performed by using species specific primer sets Nig, Fmi and Fla.

Comparison of nested PCR with other methods showed high sensitivity and specificity (Microscopy 10%, Fluorescence microscopy 17%, Culture 19.5% and PCR 25%).

Molecular methods are more sensitive and specific methods. It gives results for detection and speciation in short time which helps early diagnosis and treatment of patient.

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