Detection of *Aspergillus* Species by Polymerase Chain Reaction

Raksha Singh¹, Gurjeet Singh²* and A.D. Urhekкар¹

¹Department of Microbiology, MGM Medical College and Hospital, MGM Institute of Health Sciences, Kamothe, Navi Mumbai-410209, Maharashtra, India

²Department of Microbiology, NC Medical College and Hospital, Israna, Panipat-132107, Haryana, India

*Corresponding author

**Abstract**

*Aspergillus* species have recently caused increasing numbers of life-threatening 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.

**Keywords**

*Aspergillus*, Polymerase Chain Reaction, Sensitivity, Patient

**Article Info**

Accepted: 14 September 2016

Available Online: 10 October 2016

**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.*, 1999) 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.

| Name of organism | Primer set | Primers | Oligonucleotides | Gene accession No. |
|------------------|------------|---------|------------------|-------------------|
| *Aspergillus* species | ASAP | ASAP1  | 5’-CAGCGAGTACATCACCTTGG-3’  | KP987074.1 |
|  |  | ASAP2  | 5’-CCATTGTTGAAAGTTTAACTGATT-3’ | KP657690.1 |
| *A. fumigatus* | Fmi | ASPU  | 5’-ACTACCGATTGAATGGCTCG-3’  | KR023997.1 |
|  |  | Af3r  | 5’-CATACTTTTCAAGACGCGTCA-3’  | LC133095.1 |
| *A. niger* | Nig | ASPU  | 5’-ACTACCGATTGAATGGCTCG-3’  | KF304798.1 |
|  |  | Ni1r  | 5’-ACGCTTTCAGACAGATGCTCG-3’  | LC133092.1 |
| *A. flavus* | Fla | ASPU  | 5’-ACTACCGATTGAATGGCTCG-3’  | KP784374.1 |
|  |  | Fl2r  | 5’-TTCACCTACAGACACAGAGT-3’  | LC133097.1 |
| Pan-fungal primers | 18S rDNA | B2F  | 5’-ACTTTCGATGTTAGGATAG-3’  | KT935264.1 |
|  |  | B4R  | 5’-TGATCGTCTTCGATCCCTA-3’  | KT935264.1 |

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

| Reactiona | 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-A5 → A. niger, A-6-A7 → A. fumigatus).
Fig.3 Showing Aspergillus species detection by various methods.

![Bar chart showing Aspergillus species detection by various methods](chart1.png)

Fig.4 Showing Aspergillus speciation by PCR methods.

![Bar chart showing Aspergillus speciation by PCR](chart2.png)

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.

References

Bagyalakshmi, R., Therese, K.L., Madhavan, H.N. 2007. Application of semi-nested polymerase chain reaction targeting internal transcribed spacer region for rapid detection of panfungal genome directly from ocular specimens. Indian J. Ophthalmol., 55: 261-5.

Christoph, M., Tang, David, W., Holden, Agnès Aufauvre-Brown, Jonathan Cohen. 1993. "The Detection of Aspergillus spp. by the Polymerase Chain Reaction and Its Evaluation in Bronchoalveolar Lavage Fluid", American Rev. Respiratory Dis., 148(5): 1313-1317. doi: 10.1164/ajrccm/148.5.1313

Deshpande, P., Hedge, A., Kapadia, F., Mehta, A., Rodrigues, C., Shetty, A., Soman, R. 2011. Standardization of fungal polymerase chain reaction for the early diagnosis of invasive fungal infection. Indian J. Med. Microbiol., 29(4): 406-410.

Diba, K., Mirhendi, H., Kordbacheh, P., Rezaie, S. 2014. Development of RFLP-PCR method for the identification of medically important Aspergillus species using single restriction enzyme MwoI. Brazilian J. Microbiol., 45(2): 503-507.

Gaskell, G.J., Carter, D.A., Britton, W.J., Tovey, E.R., Benyon, F.H., Lovborg, U. 1997. Analysis of the internal transcribed spacer regions of ribosomal DNA in common airborne allergenic fungi. Electrophoresis, 18: 1567-1569.

Hayette, M., Vaira, D., Susin, F., Boland, P., Christiaens, G., Melin, P., Mol, P.D. Detection of Aspergillus Species DNA by PCR in Bronchoalveolar Lavage Fluid. J. Clin. Microbiol., 39(6): 2338–2340.

Henry, T., Iwen, P.C., Hinrichs, S.H. 2000. Identification of Aspergillus species using internal transcribed spacer regions 1 and 2. J. Clin. Microbiol., 38: 1510-1515.

Henry, T., Iwen, P.C., Hinrichs, S.H. 2000. Identification of Aspergillus species using internal transcribed spacer regions 1 and 2. J. Clin. Microbiol., 38: 1510-1515.

Hoshino, H., Tagaki, S., Kon, H. et al. 1999. Allergic bronchopulmonary aspergillosis due to Aspergillus niger without bronchial asthma. Respiration, 66: 369-372.

Iwen, P.C., Hinrichs, S.H., Rupp, M.E. 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med. Mycol., 40: 87-109.

Kontoyiannis, D.P., Bodey, G.P. 2002. Invasive aspergillosis in: an update. Eur. J. Clin. Microbiol. Infect. Dis., 21: 161-172.

Loeffler, J., Kloepfer, K., Hebart, H., Najvar, L., Graybill, J.R., Kirkpatrick, W.R., Patterson, T.F., Dietz, K., Bialek, R., Eisele, H. 2002. Polymerase Chain Reaction Detection of Aspergillus DNA in Experimental Models of Invasive Aspergillosis. J. Infect. Dis., 185: 1203-6.
Makimura, K., Murayama, S.Y., Yamaguchi, H. 1994. Specific detection of *Aspergillus* and *Penicillium* species from respiratory specimens by polymerase chain reaction (PCR). *Jpn. J. Med. Sci. Biol.*, 47: 141-156.

Nenoff, P., Kliem, C., Mittag, M., Horn, L.C., Niederwieser, D., Haustein, U.F. 2002. Secondary cutaneous aspergillosis due to *Aspergillus flavus* in an acute myeloid leukaemia patient following stem cell transplantation. *Eur. J. Dermatol.*, 12: 93-98.

Radford, S.A., Johnson, E.M., Leeming, J.P., et al. 1998. Molecular epidemiological study of *Aspergillus fumigatus* in a bone marrow transplantation unit by PCR amplification of ribosomal intergenic spacer sequences. *J. Clin. Microbiol.*, 36: 1294-1299.

Rao, K., Saha, V. 2000. Medical management of *Aspergillus flavus* endocarditis. *Pediatr. Hematol. Oncol.*, 17: 425-427.

Sugita, C., Makimura, K., Uchida, K., Yamaguchi, H., Nagai, A. 2004. PCR identification system for the genus *Aspergillus* and three major pathogenic species: *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. *Med. Mycol.*, 42: 433-437.

White, P.L., Linton, C.J., Perry, M.D., Johnson, E.M., Barnes, R.A. 2006. The Evolution and Evaluation of a Whole Blood Polymerase Chain Reaction Assay for the Detection of Invasive Aspergillosis in Hematology Patients in a Routine Clinical Setting. *Clin. Infect. Dis.*, 42: 479-86.

Yamakami, Y., Hashimoto, A., Tokimatsu, I., Nasu, M. 1996. PCR Detection of DNA specific for *Aspergillus* species in serum of patients with invasive aspergillosis. *J. Clin. Microbiol.*, 34: 2464-2468.

Yamazaki, T., Kume, H., Murase, S., Yamashita, E., Arisawa, M. 1999. Epidemiology of visceral mycoses: analysis of data in annual of the pathological autopsy cases in Japan. *J. Clin. Microbiol.*, 37: 1732-1738.

Zhao, J., Kong, F., Li, R., Wang, X., Wa, Z., Wang, D. 2001. Identification of *Aspergillus fumigatus* and related species by nested PCR targeting ribosomal DNA internal transcribed spacer regions. *J. Clin. Microbiol.*, 9: 2261-2266.

---

**How to cite this article:**

Raksha Singh, Gurjeet Singh and A.D. Urhekar. 2016. Detection of *Aspergillus* Species by Polymerase Chain Reaction. *Int.J.Curr.Microbiol.App.Sci.* 5(10): 254-260. doi: [http://dx.doi.org/10.20546/ijcmas.2016.510.027](http://dx.doi.org/10.20546/ijcmas.2016.510.027)