

ASPERGILLUS MONITORING PROJECT IN A LARGE EDUCATIONAL HOSPITAL USING MOLECULAR ASSAY

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Abstract

Background: It is important to find reliable and accessible methods for the diagnosis and identification of fungal species causing hospital acquired infections. Our main objective was using a rapid and accessible molecular method for the monitoring of Aspergillus infections and identification of causing agents in the level of species.

Material and Methods: The study subjects were primarily clinical specimens collected from suspected HAI patients with clinical symptoms after hospitalization. Also some environmental specimens were collected from air and instruments of health care facilities for the investigation of Aspergillus sources in a university hospital of UMSU, Urmia. All specimens were transported to Medical Mycology Center for the detection and identification of Aspergillus species using morphological methods. Also molecular method, PCR-RFLP using single restriction enzyme as a rapid and available method was performed to investigate environmental sources of Aspergillus infections.

Results: Total of 110 clinical fungal isolates included Candida and Aspergillus species and some other opportunistic fungi. Among the clinical Aspergillus findings, *Aspergillus flavus* (47%), *Aspergillus fumigatus* (29.4%) and *Aspergillus niger* (23.6%) were the most frequent species respectively and also *Aspergillus niger* (43.7%), *Aspergillus flavus* (41.8%), *Aspergillus fumigatus* (14.7%) were isolated as the most frequent species from environmental sources.

Conclusion: Because of accessibility, speed and high sensitivity of diagnosis, the PCR-RFLP was very useful for the identification of medically important *Aspergillus* species and epidemiological approaches.

Key words: Aspergillus, identification, molecular, hospital.

Introduction

Aspergillus species as opportunistic fungi are found in soil, water and decaying vegetation. They are also isolated from unfiltered air, ventilation systems, false ceiling dust, contaminated dust dislodged during hospital renovation and construction. Catheters and implants might be colonized by Aspergillus species (Warris and Verweij, 2005). There is plenty of evidence supporting the role of these fungi as agents of hospital acquired infections (HAIs) (Dancer et al., 2009). The primary route of acquiring Aspergillus infection is by inhalation of the fungal spores. In severely immunosuppressed patients, pneumonia results from local lung tissue invasion. In addition, the fungus may disseminate through the bloodstream to deep organs. Patients with severe, prolonged, granulocytopenia, especially bone-marrow transplant recipients, are at the highest risk. *A. fumigatus* and *A. flavus* are the most frequently isolated species in patients with proven aspergillosis (Verweij et al., 2002). The presence of invasive disease may be inferred by the clinical symptoms and signs, and images obtained by CT scanning etc.

It is important to find reliable and accurate methods for the identification of fungal agents of HAIs and epidemiological approaches as well. Aspergillus infections are difficult to diagnose at early stages and conventional microbiological, serologic or imaging techniques are often insufficient to ensure the early diagnosis and identifications. Tissue invasion, as demonstrated by examination of biopsy material, provides unequivocal evidence of invasive disease, and blood cultures are usually negative (Healy et al., 2004).

Recently, some immunological and molecular tests were used for the identification of Aspergillus species causing HAIs (Verweij et al., 2002; Healy et al., 2004). Different molecular methods such as restriction fragment length polymorphism (RFLP) based on amplification of ribosomal RNA have been employed to identify Aspergilli in the level of species (Moody and Tyler, 1990). Because of facility in use, speed, availability and high sensitivity of diagnosis, PCR-RFLP using single restriction enzyme was studied in this study for the identification of Aspergillus species isolated from the HAI patients and the hospital indoor environments (Mirhendi and Diba, 2007).

Materials and Methods

Clinical and environmental specimens

Our main subjects were clinical specimens including; sputum, bronchoalveolar lavage (BAL), sinus discharge, urine and synovial fluid collected from HAI suspected patients with clinical symptoms after hospitalization. The presence of invasive disease was inferred by the clinical symptoms and signs, and images obtained by CT scanning etc. and fungal colonization was suggested by frequently isolation of organisms from cultures while there are no clinical symptoms. All specimens were transported to Medical Mycology Center, UMSU, Urmia. The morphological diagnosis was performed using growth characteristics on Sabouraud glucose agar 4% and microscopic features for the identification of Aspergillus species (Raper and Fennell, 1965). Also some environmental specimens were collected from air and instruments of health care facilities including: surfaces of the walls, carpets, beds and blankets, trolleys, air condition, medical devices and also finger touch samples of

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cases, health care workers (HCWs) and visitors. Air samples were collected by using Sabouraud glucose agar (SGA4%) plates which left uncapped expose to blowing air and the other samples were taken with sterile swabs and inoculated on a transport medium like SGA4% (Diba et al., 2007). Also the molecular method, PCR-RFLP using single restriction enzyme as a rapid and available method was performed to investigate environmental sources of *Aspergillus* infections.

DNA extraction

It was necessary to prepare *Aspergillus* mycelia mass which is filtered and purified from the 12-24 hours fungal liquid cultures. Genomic DNA was extracted from *Aspergillus* mycelia mass by glass beads in a lysis buffer (1mM EDTA, 1% SDS, 100mM NaCl, 10mM Tris-HCl, 2% Triton X-100, PH: 8.0) followed by conventional phenol-chloroform method. The extracted DNA was checked by using agarose gel electrophoresis (Loeffler et al., 2002).

PCR amplification

The PCR assay was performed using 5 µl of the DNA template in a total reaction volume of 50 µl (consisting of PCR buffer (20 mM Tris- HCL at pH 8.0), 50 mM KCL, 0.1 mM each of forward (ITS: 5-TCC GTA GGT GAA CCT GCG G-3) and reverse (ITS-4 5-TCC TCC GCT TAT TGA TAT GC-3) primers for ITS regions of rDNA (purchased from Mirhendi Molecular Biology Center, TUMS), and 1.5 U of Taq DNA polymerase. We used universal primers for the amplification of *Aspergillus* ITS regions (Forward Primer: 5'- TCC GTA GGT GAA CCT GCG G - 3' Reverse Primer: 5'- TCC TCC GCT TAT TGAT TAT GC - 3'). All reactions were performed in a Thermocycler model XL (Bioer, China). Thermal program included an initial DNA denaturation at 95 °C for 5 min that followed by 30 cycles. Each cycle consisted of a denaturation step at 95 °C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72 °C for 5 min following the last cycle. For all PCRs, we had a negative control and a positive control as well. Double deionized water (DDW, Merck, Germany) was used as negative control and DNA template extracted (Boiling, Phenol-Chloroform method) from standard *Candida* strain: *C. albicans* (ATCC 10261), was employed as positive control. The DNA fragments were length separated by electrophoresis through 1.5% agarose gels in Tris Borate EDTA buffer and 0.50 mg ethidium bromide per ml. Results were documented by using a tams illuminator, Gel Doc System (Figure 1).

Digestion of PCR products using RFLP method

The method of restriction fragment length polymorphism was used to make differential pattern for the identification of *Aspergillus* spp. (Martínez-Culebras and Ramón, 2007). Digestion of amplified ITS fragments with our restriction enzyme, *Mwo*I at 37 °C enabled us to differentiate some medically relevant *Aspergillus* species. It was performed on all clinical and environmental *Aspergillus* isolates. For the restriction digestion, 13 µl of each PCR product was directly digested by 5 U (0.5 µl) of the restriction enzyme, 1.5 µl of the enzyme buffer, and incubated at 37 °C for 180 min (Almirante et al., 2005). Digested PCR products were subjected to electrophoresis in a 2% agarose gel and visualized with gel doc system. Molecular identification of *Aspergillus* species was performed comparing the electrophoresis bands with differential patterns (Figure 2).

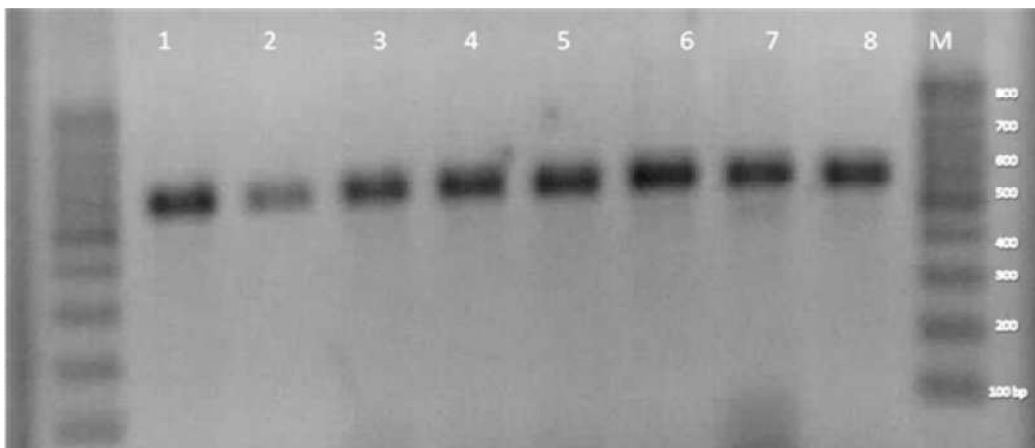


Figure 1: Agarose gel electrophoresis of amplified ITS fragments of *Aspergillus* species. , Lane I: *A.niger*, Lanes 2-5: *A. flavus* , Lanes: 6,7: *A. fumigatus* and Lane 8: A frequently tested *A. fumigatus* as positive control and M: 100 bp DNA ladder. As it shown, approximately all species make the same size PCR bands on agarose gel.

Results

The results of experimental studies on 198 clinical specimens showed totally 93 (47%) fungal or bacterial infections. Among all positive cases, 54 (58%) had a fungal infection, supposing that all of the isolated fungi were causing agents of nosocomial infections with clinical symptoms after hospitalization. The isolated fungi included, 36 *Candida* spp. (66.6%) and 17 *Aspergillus* spp. (31.4%). Among the clinical

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isolated *Aspergillus* species; *A. flavus* (47%), *A. fumigatus* (29.4%) and *A. niger* (23.6%) were the most frequent species respectively (Table 1). The black filamentous mold ‘*Alternaria alternata*’ was the only non *Candida*, non *Aspergillus* isolated fungus in this study which was isolated from synovial sample of a patient with septic arthritis. All identifications findings were confirmed by PCR-RFLP method (Figure 2). Among all environmental specimens, 256 specimens were collected from finger touch and body surface samples of patients, personnel and visitors, bed, floor, walls, trolleys, sink, medical devices and air samples (Table 2) which made up 110 fungal isolates including *Candida*, *Aspergillus* spp. and other fungi as saprophytic molds: *Alternaria*, *Saccharomyces*, *Mucorals*, *Penicillium*, *Cladosporidium* and *Pheohyphomycetes*. Among all isolates, *Candida* spp. 35(31.5%), *Aspergillus* spp. 48(43.2%) and other fungi 28(20.3%) were included. Environmental *Aspergillus* isolates were: *A. niger* (43.7%), *A. flavus* (41.8%) and *A. fumigatus* (14.7%) (Table 1).

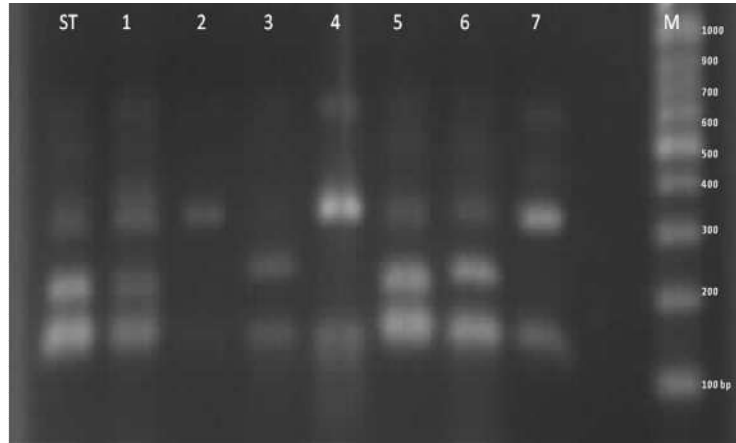


Figure 2: Agarose gel electrophoresis of ITS PCR products from standard *Aspergillus* species after digestion with the restriction enzyme *Mwo*I. Lanes ST: standard *Aspergillus* species as positive control, lanes 1, 3, 5, 6: *A. flavus*, lanes 2, 4, 7: *A. fumigatus*, Lane M: 100 bp DNA ladder.

Table 1: Identification of *Aspergillus* species isolated from clinical and environmental sources by RFLP method as well as their frequencies.

	Clinical specimens		Environmental specimens		Total	
	No.	%	No.	%	No.	%
<i>A. flavus</i>	8	47	20	41.7	28	43.1
<i>A. niger</i>	4	23.5	21	43.7	25	38.5
<i>A. fumigatus</i>	5	29.5	7	14.6	12	18.4
Total	17	100	48	100	65	100

Table 2: The frequency of opportunistic fungi isolated from air, instruments, indoor /outdoor surfaces (number of isolations) and finger touch of patients, HCWs and visitors. The last specimens are very important in the transmission of *Candida* spp.

Environmental specimens	Case	HCW	Visitor	Carpet	Walls	Bed & Blanket	Sink	Trolleys	Medical devices	Air	Air conditioner	Out door
Contaminants												
<i>Candida</i>	1	5	1	2	4	4	3	1	0	0	0	2
<i>Aspergillus</i>	3	1	0	9	7	7	2	5	1	5	3	8
Other	0	0	0	3	5	4	1	4	6	4	1	0
Total	13	6	1	14	16	15	6	10	7	9	4	10

* Health care worker

Discussion

There is plenty of evidence supporting the role of opportunistic fungi as important agents of HAIs. During the period 1980-90 *Aspergillus* species emerged as causing agents of life-threatening infections in immuno-compromised patients (Martínez-Culebras and Ramón, 2007). Recently, some molecular techniques were used for the detection and identification of opportunistic fungi including *Aspergillus* spp. Susan F. Moody and co-worker (Moody and Tyler, 1995) used the PCR-RFLP for the analysis of inter species variations of *Aspergillus* group flavi: *A. flavus*, *A. parasiticus*, *A. nomius*. Also Dendis his group (Dendis et al., 2003) performed this method for identifying some pathogenic fungi isolated from febrile neutropenic patients. Molecular method PCR-RFLP with single restriction enzyme was used by Mirhendi et al. (Mirhendi et

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al., 2006), for the identification and differentiation of pathogenic fungi such as *Candida* spp. The PCR-RFLP method using a single restriction enzyme, *MwoI* has also been used for the identification of *Aspergillus* spp. A whole process of molecular identification including, isolation of fungal agent from a short incubated culture, a profile of DNA extraction, PCR and RFLP, for 6-10 samples takes a long day (12-18 hours) approximately. It should absolutely be more rapid than classic or morphologic methods of identifications. In the present study the rapid and reliable method of PCR-RFLP employing *MwoI* enabled us to identify *Aspergillus* spp. isolated from clinical specimens. Our results of *Aspergillus* isolation showed that *A. fumigatus* and *A. flavus* were the most frequent *Aspergillus* species isolated from cases with proven aspergillosis. Likewise our findings of environmental isolates showed that more than 50% of all samples were positive for at least an opportunistic fungus. From all of the tested samples, carpets, walls and beds were the most contaminated surfaces by the above fungi. Considering the frequent and long time contacts between the above surfaces and cases, personnel and visitors, It could be a reasonable explanation for that result. It is important the most hospital yeast isolates were obtained from cases and personnel finger touch samples (Table 2). *Aspergillus* species (the second group of medically important fungi causing HAIs) are obtained from air and indoor/outdoor hospital environment. The most contaminated hospital indoor places in our study were the carpets, walls and also air samples of rooms (Dancer et al., 2009). The primary route of acquiring *Aspergillus* spp. is inhalation of the spores within aerosols suspended in air. Hospital renovations or constructions are capable of liberating large numbers of fungal spores into the health care units (Warris and Verweij, 2005). Although isolation of *Aspergillus* spores from air samples was not considered in this study.

Comparing various differential methods for the identification of *Aspergillus* species including conventional and molecular tests, because of its accessibility, speed and high sensitivity of diagnosis, the PCR-RFLP was very useful for the identification of medically important *Aspergillus* species and epidemiological approaches.

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