Molecular characterization of Aspergillus infections in an Iranian educational hospital using RAPD-PCR method

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ARTICLEINFO	ABSTRACT
	Objective(s): The nosocomial infections by Aspergillus species are associated with constructions and increased dust loads in hospital indoors. Our main object was to find the environmental sources of Aspergillus species causing hospital acquired infections.
	Materials and Methods: The clinical and environmental samplings were performed during 18 months from spring 2010 to summer 2011 in Imam educational hospital, Urmia, Iran. A morphological diagnosis was performed including microscopic characterization of isolated aspergillus from cultured specimens and polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) for the identification in the level of species. Random amplified polymorphic DNA – PCR RAPD-PCR using random primers for rDNA gene was performed to compare Aspergillus isolates of clinical cases with the relevant environmental sources.
	Results: Use of RAPD method resulted various differential patterns, so that some Aspergillus isolates from the clinical and hospital indoor were completely matched (matched pairs) and some other Aspergillus isolates were not matched. In the case of matched pairs, *Aspergillus niger* and *A. flavus* isolated from broncoalveolar lavage and sinus discharge were relevant to those of air conditioner and walls surfaces, respectively. ***Conclusion:*** The hospital sources for the Aspergillus clinical isolates included air condition and walls. RAPD-PCR analysis can play a trivial role to find the hospital sources of Aspergillus clinical isolates.

Introduction

The incidence of life threatening invasive aspergillosis has been developed following the increased immune compromised cases, bone or organ transplant receivers and patients with leukemia, lymphoma and other malignances (1). Incidence rate of invasive Aspergillus infections is about 17 to 26%, 5 to 24% and 5 to 15% in lung transplant, acute leukemia and allogenic bone marrow transplant patients, respectively. *Aspergillus fumigatus* is the most common species isolated from invasive Aspergillus infections, although *Aspergillus flavus, Aspergillus terreus, Aspergillus niger* and other species also frequently cause invasiveaspergillosis (1). Hospital acquired aspergillosis is known to be an airborne infection due to constructions and renovations which make the higher rates of airborne conidia. Detailed studies have found Aspergillus spores in many parts of hospital, including shower heads, dusty air conditioners, hospital plants, clothing and other sources (1). Small outbreaks of nosocomial aspergillosis have been reported, sometimes in association with environmental sources of conidia. Elucidation of the complex epidemiology in such cases requires detailed molecular typing studies. However, not all of the molecular procedures are applicable to fungal typing (2). Recently, a study of *A. terreus* infections in immunosuppressed patients showed that environmental and some patient isolates had identical band patterns by RAPD analysis (3).

A recent study on the immune suppressed patients at the nephrology and transplant ward of Imam University Hospital, Urmia, Iran showed 36 (66.6%) and 17 (31.4%) frequencies of Candida and Aspergillus species (respectively) isolated from clinical specimens. Moreover, 110 fungal isolates of Candida, Saccharomyces, Aspergillus, Penicillium, Alternaria and Cladosporidium species were obtained from hospital indoor and outdoor. Among all, 35(31.5%) Candida and 48(43.2%) Aspergillus species were detected. Regarding high frequently of

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Aspergillus spp. isolated from clinical and environmental sites in the nephrology ward, the authors tried to perform a molecular epidemiologic study to find accurate and exact environmental sources for Aspergillus infections and colonization in the large, general hospitals. Other studies have used molecular methods for the identification and epidemiological aims on pathogenic bacteria and viruses including Mycobacteria (4, 5), Escherichia coli (6) and Rotavirus (7). Present study uses the molecular method of random amplification of polymorphic DNA (RAPD) to study the environmental sources of Aspergillus species which recently isolated from all cases with hospital acquired infections (HAIs) or colonization.

Materials and Methods
Study subjects
Clinical and environmental samples were obtained during 18 months from spring 2010 to summer 2011 at the large, training hospital, Imam Khomeini. The clinical specimens including sputum, bronco alveolar lavage (BAL), urine, sinus discharge, skin and nail scrapping were obtained from the cases with proved nosocomial infections hospitalized in a large educational hospital in Urmia, Iran. All clinical specimens were directly collected from the central laboratory of hospital, so that there was no ethical limitation for obtaining and testing the cases' specimens.

The environmental specimens were collected from cases' rooms and hospital indoors included air, air conditioners, walls, curtains, beds, trolleys, carpets and medical instruments. Air samples were collected from various places close to patient's bed by the culture media plates left uncovered. Other specimens were taken by using sterile swabs touched on the surfaces and wall splits.

Culture and Identification
All specimens were transported to Medical Mycology Center, Urmia Medical Sciences University (UMSU). A morphological diagnosis was performed using culture media, Czapecdox agar (CZA) and Sabouraud glucose agar (SGA) for the primary identification of isolated molds (8). The diagnosis was then confirmed by molecular identification using restriction fragment length polymorphism (RFLP) followed by random amplified polymorphic DNA (RAPD) for the epidemiological linkage among clinical and environmental sources.

DNA extraction
Aspergillus mycelial mass was harvested from the 12 to 24 hr fungal liquid cultures, filtered and purified. The genomic DNA was extracted by glass beads and phenol-chloroform method,(a solution of 1mM EDTA, 1% SDS, 100mM NaCl, 10 mM Tris-HCl and 2% Triton X-100, in distilled water, pH 8.0 was used as LysisBuffer). The extracted DNA was checked by using 1.5% agarose gel electrophoresis (3).

PCR for identification
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 mMTris- HCl at pH 8.0], 50 m MKCl, 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 Lab, 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') (9).The reactions were performed in a thermo cycler (XP Cycler, BIOER, China). Thermal program included an initial DNA denaturation at 95℃ for 5 min that followed by 30 cycles, consisting of the stages; denaturation at 95℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min following the last cycle. The DNA fragments were length separated by electrophoresis through 1.5% agarose gels in Tris Borate EDTA (TBE) buffer and 0.50 mg of ethidium bromide per ml. Results were documented by using a UV tarns illuminator (SynGene SYDR4/ 680X, UK) (10).

Digestion of PCR products using RFLP method
Digestion of amplified ITS fragments by using a novel restriction enzyme, *Mwo*I at 37°C enabled us to differentiate most of Aspergillus isolates in level of species. The method was performed for both clinical and environmental isolates. For the restriction enzyme digestion, 13 μl of each PCR product was directly digested by 5 U (0.5 μl) of the restriction enzyme *Mwo*I, 1.5 μl of the enzyme buffer, and then incubated at 37°C for 180 min (10). Digested PCR products were subjected to a 2% agarose gel electrophoresis and visualized in trans illuminator (Gel Doc system). Aspergillus isolates were identified comparing the electrophoretic DNA patterns with standard measures (Table 1).

Random amplification of polymorphic DNA (RAPD)
For the RAPD-PCR, six single primers which successfully tested before were used as random primers on Aspergillus genomic DNA. The primers: P1 (Rp4): 5´- CAGATGCTTC -3´, P2 (Rp1): 5´- TAGGGATCGGA -3´, P3 (SOY): 5´- AGGTCACTGA -3´, P4 (RP2): 5´- AAGGATCAGA -3´, P5 (R108): 5´- GATTTGCCCT -3´, P6 (UBC90): 5´- GGGGGTTAGG-3´were randomly selected from many available primers (Purchased from Molecular Biology Lab, TUMS). The primers were run into a PCR master mix containing 3 mM MgCl₂, 200 pmol of each primer, and 5 ng of DNA, in final volume of 100 μl. The thermal protocol used in thermo cycle system included 5 min at 95℃ ,45 cycles of 94℃ for 45 sec,

Table 1. Frequency of Aspergillus and other opportunistic fungi isolated from hospital indoor and outdoor

Environmental specimens	Case	HCWs	Visitor	Carpet	Walls	Bed & blanket	Sink	Trolleys	Medical devices	Air	Air conditioner	Out door
Contaminants												
Candida	1	5	1	2	4	4	3	1	0	0	0	2
Aspergillus	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

HCWs: health care workers

Table 2. List of Aspergillus spp. Commonly isolated from cases and hospital indoor

Aspergillus clinical isolates	Species	Clinical	Environmental
KDH3	*A. niger*	BAL	carpet
KDH16	*A. flavus*	nail scrapping	walls, carpet
KDH24	*A. niger*	sputum	carpet, walls
KDH27	*A. flavus*	sputum	air conditioner
KDH28	*A. fumigatus*	BAL	trolleys
KDH30	*A. flavus*	air	air
KDH31	*A. fumigatus*	BAL	carpet
KDH32	*A. niger*	BAL	air conditioner
KDH33	*A. niger*	BAL	carpet
KDH34	*A. flavus*	BAL	walls
KDH36	*A. fumigatus*	BAL	carpets
KDH38	*A. flavus*	BAL	beds
KDH45	*A. flavus*	sinus discharge	walls
KDH37	*A. niger*	sputum	trolleys

35°C for 1 min, 72°C for 1.5 min and a final 72°C for 5 min (11). Agarose gel electrophoresis of the PCR products followed by an ethidium bromide staining showed various patterns making DNA bands which analyzed by using Image Master software (Gene Snap Tool, SynGene, version: 4.01.0, UK). Reproducibility of DNA patterns was demonstrated by the analysis of two to three Aspergillus subcultures. The RAPD-PCR patterns were compared between the clinical and environmental Aspergillus isolates for each case and the similarity of RAPD patterns was analyzed.

Results

Totally 454 samples including 198 clinical and 256 environmental specimens were collected. The experimental studies on the clinical specimens resulted total of 93(47%) fungal and bacterial isolates. Among all, 54 (58%) were fungal isolates which obtained from cases with symptomatic infection started after hospitalization. Fungi included Candida spp. 36 (66.6%), and Aspergillus spp. 17 (31.4%). Aspergillus isolates included *A. flavus* (47%), *A. fumigatus* (29.4%) and *A. niger* (23.6%) as the most frequent species. Our findings of Aspergillus morphologic identifications were confirmed by PCR-RFLP method. Also, 256 specimens were collected from the relevant environmental sites including beds, carpets, walls, trolleys, sinks, medical devices, air

condition systems and air samples. Morphologic examinations resulted to 110 fungal isolates including Candida, 35(31.5%) and Aspergillus, 48(43.2%) species and other fungi (saprophytic molds), 28(20.3%) such as Alternaria, Saccharomyces, Mucorals, Penicillium, Cladosporidium and Pheohyphomycetes. Aspergillus isolates included *A. niger* (43.7%), *A. flavus* (41.8%), and *A. fumigatus* (14.7%) (Table 2). It means that five *A. fumigatus* isolates, eight *A. flavus* and four *A. niger* were obtained from the clinical specimens of cases with invasive or non-invasiveaspergillosis mostly in the intensive care units, nephrology and surgery wards. Also 7, 20 and 21 isolates of *A. fumigatus*, *A. flavus*, and *A. niger* (respectively) were detected in samples and other environmental specimens.

From 65 clinical and environmental isolates of Aspergillus species, 28 isolates were common between cases and their environments in the level of species. For making an exact correlation among the clinical and environmental isolates, the findings of RAPD-PCR were analyzed. The random primers, P1-P6 made different electrophoretic DNA patterns for most cases. For example, application of P1 resulted in different DNA patterns for the pairs (clinical and environmental isolates) 16, 36 and 37 from 65 cases of Aspergillosis. Just in one case (pair 31) same DNA patterns were observed (Figure 1c). Use of primer P2 resulted in identical patterns for three pairs: 16, 31 and 37 as shown in Figure 1 d. At the same way, the other primers created common patterns not more than one. Comparison of clinical and environmental pairs with RAPD-PCR using six random primers revealed similar electrophoretic DNA patterns for clinical and environmental Aspergillus isolates (Figures 1 a, b) just in two of 28 pairs including 32 and 45. *A. niger* and *A. flavus* were included in the pairs 32 and 45 respectively. The Aspergillus isolate of bronco alveolar lavage of case (32) was completely similar to that of relevant air conditioner used in the private room. Also, DNA pattern of *A. flavus* isolated from sinus discharge of case (45) was similar to the isolate of wall swabs. Other Aspergillus pairs showed no similarity (Table 3).

Table 3. Frequency of RAPD based correlation between the clinical and environmental Aspergillus isolates

Species	Total C&E by RFLP	Common species in C&E	Same C&E isolates by RAPD	Different C&E by RAPD
A. fumigatus	12	4	0	5
A. flavus	28	6	1	7
A. niger	25	5	1	6
Total	65	28	2	18

C: clinical aspergillus isolates; E: environmental aspergillus isolates
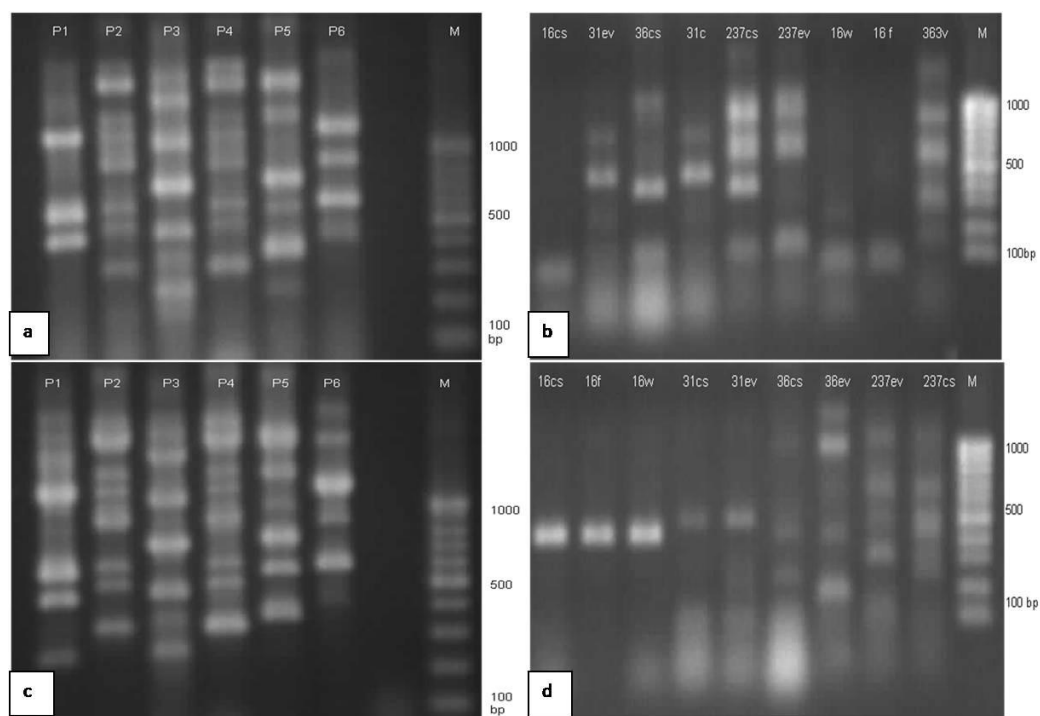
Figure 1. RAPD patterns of clinical and environmental for the strains 32 and 45 are similar using each tested primers (a, c). Application of P1 in RAPD resulted different patterns for the pairs of 16, 36 and 37 (b). P2 resulted same patterns for Aspergillus groups 16, 31 and 37 (d)

Discussion

There is plenty of evidence supporting the role of opportunistic fungi as important agents of hospital acquired infections. During 1980 to 1990, Aspergillus spp. emerged as causing agents of life-threatening infections in immune compromised patients (12). Nosocomial aspergillosis still poses a significant clinical problem (1). Although PCR based methods for detection of fungi have been described recently (13, 8, 2), there is a problem of finding the real source of opportunistic fungi such as Aspergillus spp. causing hospital acquired infections. In the present study, the PCR based technique, RAPD-PCR was employed to find a correlation between the environmental and cases isolated Aspergillus. Before using RAPD method for epidemiologic surveys, it has to be evaluated the extent of the differences in DNA patterns that can be generated. The previous data verify that RAPD analysis is useful for fingerprinting *A. fumigatus* (9, 15), *A. flavus* (3, 17), *A. niger* (18) and *A. terreus* (17, 19).

Use of RAPD in the present study was based on the previous studies on discriminatory power of this method using the random primers among medically important Aspergillus species (17). Comparing the findings of RAPD on the environmental isolates with those which obtained from cases shows that some Aspergillus isolates match completely in DNA patterns. In fact, we considered a 100% similarity of RAPD patterns between two relevant Aspergillus isolates. Moreover, among all Aspergillus isolates of

this study, only two cases were correlated with environmental sources. In these cases, *A. niger* and *A. flavus* were included as the Aspergillus species causing nosocomial apergillosis which obtained from hospital indoor. A. niger was isolated from both specimens of bronco alveolar lavage and air conditioner in the patient room and *A. flavus* from sinus discharge and walls.

Likewise our findings showed that of more than 27% of Aspergillus species have no correlations with the clinical and environmental Aspergillus isolates. From all tested environmental samples, the carpet, walls and beds were the most contaminated surfaces by the above fungi. Isolation of aspergillus spore from air samples was not considerable in our study, although 5 aspergillus cases were obtained from the air of wards. The main route of Aspergillus nosocomial infection is inhalation of airborne spores by immune compromised patients and pneumonia is the result of lung tissue invasion in the patients (1). Because of their size, aspergillus spores remain suspended in air for a long time. Moreover, it is supposed that Aspergillus contamination of other sources was due to air contamination of hospital indoors. Building works or constructions are capable of liberating large numbers of airborne fungal spores into the environment (20).

Our results of Aspergillus species identification in hospital specimens is confirmed by other studies, so that in a similar study, *A. fumigatus* and *A. flavus* were the most frequently isolated Aspergillus spp. in

the patients with proven aspergillosis (8). Some studies reported that Aspergillus airborne spores have also been isolated from unfiltered air, ventilation system, and dusts displayed during hospital renovations and constructions (21). Although our findings of RAPD analysis showed a molecular correlation between some clinical Aspergillus isolates and their environmental sources as mentioned before, no such sources were detected upon detailed screening of the other wards; no same strains were detected in carpets, trolleys and air samples, and no aspergilli were grown from the samples taken from medical sets.

Conclusion

As a conclusion, RAPD-PCR can be applied as a simple, rapid, and useful method, but it plays a trivial role in finding the hospital sources of Aspergillus clinical isolates. It should be noted, however, that selecting the random primers is an important point to find the highest level of molecular similarity.

Acknowledgment

References

1. Curtis L, Cali S, Conroy L, Baker K, Ou CH, Hershow R, *et al*. Aspergillus surveillance project at a large tertiary-care hospital.J Hosp Infect 2005; 59:188-196.
2. Leenders A, Van Belkum A, Janssen S, de Marie S, Kluytmans J, Wielenga J, et al. Molecular Epidemiology of Apparent Outbreak of Invasive Aspergillosis in Hematology ward. J ClinMicrobiol 1996; 34:345-351.
3. Rath PM, Petermeier K, Verweij PE, Ansorg R. Differentiation of Aspergillus ustus strains by Random Amplification of Polymorphic DNA. J Clin Microbiol 2002; 40:2231-2233.
4. Moghim S, Sarikhani E, Nasr Esfahani B, Faghri J. Identification of Nontuberculous Mycobacterium species isolated from water samples using phenotypic and molecular methods and determination of their antibiotic resistance patterns by E-test method, in Isfahan, Iran. Iran J Basic Med Sci 2012; 15:1076–1082.
5. Shojaei H, Hashemi A, Heidarieh P, Pourahmad F, Daei N. Molecular identification of rare clinical Myco-bacteria by application of 16S-23S spacer region sequencing. Iran J Basic Med Sci 2012; 15:661–668.
6. Nakhaei Moghaddam M, Forghanifard MM, Moshrefi S. Prevalence and molecular characterization of plasmid-mediated extended-spectrum β-Lactamase genes (bala$_{TEM}$, bla$_{CTX}$and blA$_{SHV}$) among urinary *Escherichia coli* clinical isolates in Mashhad, Iran. Iran J Basic Med Sci 2012; 15:833–839.
7. Kargar M, Zare M,Najafi A. Molecular epidemiology of rotavirus strains circulating among children with gastroenteritis in Iran. Iran J Pediatr2012; 22:63–69.
8. Diba K, Kordbacheh P, Mirhendi SH, Rezaie S, Mahmoudi M. Identification of Aspergillus species using morphological characteristics. Pak J Med Scien 2007; 23:867-872.
9. Loudon KW, Burnie JP, Coke AP, Matthews RC. Application of polymerase chain reaction to fingerprinting Aspergillusfumigatus by random amplification of polymorphic DNA. J ClinMicrobiol 1993; 31:1117-1121.
10. Mirhendi SH, Diba K, Kordbacheh P, Jalalizand N, Makimura K. Identification of pathogenic Aspergillus species by a PCR-restriction enzyme method. J Med Microbiol 2007; 56:1568-1570.
11. Mirhendi SH, Moazeni M, Nikaeen M, Makimura K. Typing of *Aspergillus fumigatus* and *Aspergillus niger* Strains by random amplification of polymorphic DNA analysis using a six primer set. Shiraz E Med J2009; 10:34-39.
12. Anaissie EJ. Clinical Mycology. London: Churchill Livingston; 2003.
13. Baddley JW, Pappas PG, Smith AC, Moser SA. Epidemiology of *Aspergillus terreus* at a University Hospital. J Clin Microbiol 2003; 41:5525-5529.
14. Martinez-Culebras PV, Ramon D. An ITS-RFLP method to identify black Aspergillus isolates responsible for contamination in grapes and wine. Int J Food Microbiol 2007; 113:147-153.
15. Bart-Delabesse E, Sarfati J, Debeaupuis JP, Van Leeuwen W, van Belkum A, Bretagne S, *et al*. Comparison of restriction fragment length polymorphism, microsatellite length polymorphism, and random amplification of polymorphic DNA analyses for fingerprinting *Aspergillus fumigatus* isolates. J Clin Microbiol 2001; 39:2683-2686.
16. AnjaiaV, Thakur RP, Rao VP. Molecular diversity in Trichoderma isolates with potential for bio control of *Aspergillus flavus* infection in groundnut. Intl Arachis Newsl 2001; 21:31-33.
17. Midorikawa GE, Pinheiro MR, Vidigal BS, Arruda MC, Costa FF, Pappas GJ JR, *et al*. Characterization of *Aspergillus flavus* strains from Brazilian nuts and cashew by RAPD and ribosomal DNA Analysis. Appl Microbiol 2008; 47:12-18.
18. Abed K. Differentiation of *Aspergillus niger* by random amplification of polymorphic DNA. J Ind Microbiol Biotechnol 2008; 35:1027-1032.
19. Symoens F, Bouchara JP, Heinemann S, Nolard N. Molecular typing of *Aspergillu sterreus* isolates by random amplification of polymorphic DNA. J Hosp Infect. 2000; 44:273-280.
20. Warris A, Verweij PE. Clinical implications of environmental sources for Aspergillus. Med Mycol 2005; 43:S59–S65.
21. Dancer SJ.The role of environmental cleaning in the control of hospital-acquired infection. J Hosp Infect 2009; 73:378-385.