Aspergillus diversity in the environments of nosocomial infection cases at a university hospital

Kambiz Diba1,2, Farzaneh Jangi2, Khadijeh Makhdoomi2, Naser Moshiri2, Fatemeh Mansouri2,4
1. Department of Medical Mycology and Parasitology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
2. Cellular and Molecular Research Center, Urmia University of Medical Sciences, Urmia, Iran
3. Imam Khomeini Hospital, Urmia University of Medical Sciences, Urmia, Iran
4. Department of Genetics and Immunology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran

Corresponding Author:
Kambiz Diba
Department of Medical Mycology and Parasitology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
E-mail: kambiz37diba@gmail.com

Abstract
Aspergillus species (sp.) that causes opportunistic infections have been increasingly found in human mainly immunosuppressive patients around the world every year. The main objective was to use a rapid and cheap molecular method for monitoring Aspergillus infections and epidemiological approaches. In order to identify Aspergilli species (spp.), a number of molecular methods including restriction fragment length polymorphism (RFLP) have been employed in accordance with ribosomal RNA amplification. The focus of this study — a group of hospitalized patients with clinical and subclinical signs of infection. All of the collected clinical specimens were transported to the medical mycology lab and examined for Aspergillus identification. The environmental specimens were collected from air and surfaces inspected for the Aspergillus within the hospital sources. At first, growth characteristics and microscopic features on mycological media for the identification of Aspergillus sp. were performed. For the confirmation of Aspergillus isolates which similarly found in clinical and environmental sources, molecular method polymerase chain reaction/restriction fragment length polymorphism was carried out. From the mentioned specimens, 102 fungal isolates included Candida spp., Aspergillus spp. and other fungi. Aspergillus flavus (47%), Aspergillus fumigatus (29.4%) and Aspergillus niger (23.5%) all were found as the most common clinical isolates. In addition, Aspergillus isolates from environmental were Aspergillus fumigatus (43.7%), Aspergillus flavus (41.7%), Aspergillus fumigatus (14.6%). Therefore, polymerase chain reaction-restriction fragment length polymorphism with a single restriction enzyme can be very useful in the identification of Aspergillus spp., because of its facility in use, speed, robust, and high sensitivity of diagnosis.

Keywords: Aspergillus, identification, molecular, hospital

Introduction
Aspergillus sp. falling under opportunistic fungi are often distributed and observed in soil, water, and decomposing vegetation. These species can also be isolated from unfiltered air, ventilation devices, dropped ceiling dirt, and polluted dust released from hospital renovation and construction operations. Aspergillus sp. may also colonize in catheters and implants [1]. As research findings suggested that these fungi underlay hospital-acquired infections (HAI) [2–5].

Aspergillus sp. are very profuse and most often found in soil, water, air, seed and, food. Some of these species can contribute to the development of a number of diseases including allergic bronco pulmonary disease, invasive infection, nasal sinusitis, mycotic keratitis, and otomycosis [11]. Aspergillus infection are developed through a path formed due to the inhalation of the fungal spores. And also patients with immunodeficient and immunosuppressed systems, pneumonia can be induced by local lung tissue invasion. In addition, the fungus may also spread into the bloodstream and affect various inner organs. Patients suffering from acute, prolonged granulocytopenia, especially those who undergo a bone-marrow transplant are most likely to be at the risk of developing filamentous fungi diseases, especially Aspergillus sp. Besides, patients diagnosed with aspergillosis are most likely to be infected by A. fumigatus and A. flavus which are regarded as the most commonly isolated species [3].

At least 30 species including A. flavus, A. terreus, A. niger, A. nidulans, A. ustus and A. versicolor underlie human diseases, while A. fumigatus is known as the most common cause of invasive aspergillosis. Therefore, choosing a reliable technique for early diagnosis of Aspergillus sp. leading to HAI is essential and can contribute to monitor diseases — the effective use of epidemiological approaches. The early diagnosis of Aspergillus infections is a challenging task. During the past decades, with the
increase in the number of antifungal agents, more drug resistant *Aspergillus* sp. have been developed [6–8]. The use of conventional microbiological, serologic, and imaging techniques do not lead to early diagnosis and effective treatment of *Aspergillus* infections. Clinical symptoms and signs and CT scan images can be employed to detect invasive diseases. Tissue invasion can be diagnosed by examining biopsy materials which enhances the chance of detecting invasive disease where the blood cultures are normally negative [9]. Besides, a set of clinical and pathological tests normally used for identification are time-consuming or less effective for routine surveys.

The chance of detecting HAI-inducing *Aspergillus* sp. can also be increased by employing a set of immunological and molecular tests [3, 9]. Molecular techniques which include the restriction fragment length polymorphism (RFLP) developed based on the amplification of ribosomal RNA have been used to recognize *Aspergillus* spp. and make effective treatment decisions [10]. PCR-RFLP method was employed in this study with a single restriction enzyme to identify *Aspergillus* sp. isolated from HAI patients and the hospital interior environments. The advantages of this technique are its ease of use, cost-effectiveness, high speed, and its diversity power [6, 11, 12]. PCR-RFLP which is based on single restriction enzyme *Mwo* has been proposed previously due to its reliability and accuracy than conventional techniques [13]. The data in this literature is insufficient for different countries with various circumstances. Therefore, this study addresses the identification of *Aspergillus* sp. in patients referring to a university hospital in Iran.

**Materials and Methods**

**Clinical and environmental specimens**

Most of the clinical specimens collected in this study are from patients suffering from acute infection symptoms hospitalized at a large university hospital. The invasive, acute disease was diagnosed using the clinical symptoms and CT scan images. Colonization of the patients was done by isolating the organism in the culture of specimens such as bronchiolar lavage, sinus discharge, sputum, synovial fluid, and urine. Our target group in this study included all patients with clinical symptoms of fungal infections 48–72 h after hospitalizing in the ward. All specimens were taken to the Medical Mycology Center, UMS University. Standard morphologic techniques were used to diagnose and identify *Aspergillus* elements as well as the growth characteristics and microscopic features of sabouraud glucose agar 4%. Besides, PCR-RFLP is a molecular method which is employed to identify isolated *Aspergillus* [13]. Additional specimens were collected for each positive case from places such as hospital interior spaces and equipment including air, surfaces of the walls and coverings, beds, sheets, trolleys, air conditioning, medical equipment and devices, finger touch samples of cases, staffs, and visitors. Air samples were taken by using sabouraud glucose agar 4% (SGA) plates left uncapped and exposed to air flow. Furthermore, the other samples were collected with sterile swabs and then were inoculated on a transport medium such as SGA 4% [12]. All samples including transport cultures were transferred to the Medical Mycology Center, Urmia Medical Sciences University (UMSU).

**DNA extraction**

*Aspergillus* mycelia mass was prepared by filtering fungal liquid cultures for 12–24 h. Genomic DNA was taken from isolates by using glass beads in a lysis buffer (1 mM EDTA, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, 2% Triton X-100, pH=8.0). Then it was processed by the conventional phenol-chloroform method and was also checked by using agarose gel electrophoresis [14].

**PCR amplification**

Universal primers were used in this study to amplify *Aspergillus* ITS regions forward Primer: 5'- TCC GTA GGT GAA CCT GCG G - 3' and Reverse Primer: 5'- TCC TCC GCT TAT TGAT TAT GC-3'. In addition, PCR assay was performed using 5 µl of the DNA template in a PCR buffer with the reaction volume of 50 µl (20 mM Tris- HCl, pH=8.0), 50 mM KCl, 0.1 mM each of forward and reverse primers for the ITS regions of ribosomal DNA, and 1.5 U of *Taq* DNA polymerase. All reactions occurred in a thermal cycler (XL model, Bioer, China). PCR program began by denaturing DNA at 95 °C for 5 min followed by 30 cycles. Each cycle includes three steps: denaturation, annealing, and extension steps at 95, 55, and 62 °C lasting 30 s, 1 min, and 5 min, respectively and a final extension at 72 °C for 5 min after the last cycle. Besides, a negative control, i.e., double deionized water (DDW, Merck, Germany), and positive control, DNA template extracted from standard *Candida* strain: *C. albicans* (ATCC 10261) (Boiling Phenol– Chloroform method), were employed. Electrophoresis was used to length separate DNA fragments via 1.5% agarose gels in Tris-Borate EDTA buffer and 0.50 mg ethidium bromide per ml. The results were documented using a tams illuminator gel doc System (Figure 1).

**Digestion of PCR products by RFLP method**

The restriction fragment length polymorphism technique was employed to produce differential patterns in order to identify *Aspergillus* spp.[14]. When amplified ITS fragments are digested with the proposed restriction enzyme, *Mwo*...
A. flavus (47%), A. fumigatus (29.4%) and A. niger (23.5%) were found to represent the most frequent clinically isolated Aspergillus sp., in descending order, as shown in Table 1. The black filamentous mold ‘Alternaria alternata’ was the only non-Candida, non-Aspergillus isolated fungus derived from a synovial sample of a patient suffering from septic arthritis. As it is shown in Figure 2, all identified samples were confirmed using PCR-RFLP technique.

A total number of 256 specimens were collected from finger touches and body surface samples of the patients, hospital employees, and visitors, hospital devices and equipment including beds, floors, walls, trolleys, sinks, medical devices and air samples (Table 2). The samples collected containing 102 fungal isolates including Candida spp., Aspergillus spp., and other fungi as saprophytic molds: Alternaria sp., Saccharomycetes sp., Mucorals, Penicillium sp., Cladosporidium sp., and Pheohyphomycetes.

Besides, A. flavus (47%), A. fumigatus (29.4%) and A. niger (23.5%) were found to represent the most frequent clinically isolated Aspergillus sp., in descending order, as shown in Table 1. The black filamentous mold ‘Alternaria alternata’ was the only non-Candida, non-Aspergillus isolated fungus derived from a synovial sample of a patient suffering from septic arthritis. As it is shown in Figure 2, all identified samples were confirmed using PCR-RFLP technique.

A total number of 256 specimens were collected from finger touches and body surface samples of the patients, hospital employees, and visitors, hospital devices and equipment including beds, floors, walls, trolleys, sinks, medical devices and air samples (Table 2). The samples collected containing 102 fungal isolates including Candida spp., Aspergillus spp., and other fungi as saprophytic molds: Alternaria sp., Saccharomycetes sp., Mucorals, Penicillium sp., Cladosporidium sp., and Pheohyphomycetes.

Among the total cases which were under study, 35 (31.5%) Candida spp., 48 (43.2%) Aspergillus spp. and 28 (20.3%) other fungi were isolated. In addition, the environmental Aspergillus isolates included A. niger (43.7%), A. flavus (41.7%), and A. fumigatus (14.6%) (Table 1).

### Results

The analysis of experimental findings for 198 clinical samples suggested that 93 (47%) of the cases were positive for the presence of fungal or bacterial infections. Besides, 54 (58%) positive cases had a fungal infection. This indicates that all of the isolated fungi can lead to nosocomial infections. The isolated fungi contained 36 Candida spp. (66.6%) and 17 Aspergillus spp. (31.4%).
Table 2: Isolation and frequency of opportunistic fungi in relation to hospital indoor contamination

| Environmental specimens | Case | Staff | Visitor | Carpet | Walls | Bed and blanket | Sink | Trolleys | Medical devices | Air conditioner | Air conditioner | Outdoor |
|-------------------------|------|-------|---------|--------|-------|-----------------|------|----------|-----------------|----------------|---------------|---------|
| **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       |
| Others                  | 0    | 0     | 0       | 3      | 5     | 4               | 1    | 4        | 6               | 4              | 1             | 0       |
| Total                   | 4    | 6     | 1       | 14     | 16    | 15              | 6    | 10       | 7               | 9              | 4             | 10      |

Discussion

There are many evidences to support the role of opportunistic fungi that act as main agents underlying in HAIs. For instance, in the time period from 1980 to 1990, *Aspergillus* spp. was identified as the main factor leading to life-threatening infections in immunocompromised patients [16]. *Aspergillus* elements are typically isolated from soil, water, and compost. The spores have also been found in unfiltered air, air conditioning systems, and dust released through hospital renovation and construction activities.

A couple of molecular techniques were employed recently to discover opportunistic fungi such as *Candida* and *Aspergillus* sp. [17, 18]. As a case in point, the PCR-RFLP technique was employed by Moody and Tyler [10] to analyze interspecies variations of *Aspergillus* group flavi such as *A. flavus*, *A. parasiticus*, and *A. nomius*. Dendis et al. [19] also used the same technique to detect some pathogenic fungi in febrile neutropenic patients. RFLP was also used with a single restriction enzyme by Mirhendi and team [20] to identify and differentiate pathogenic fungi including *Candida* spp. The PCR-RFLP method with a single restriction enzyme, *Mwo*, was also employed to discover *Aspergillus* sp. The whole molecular identification process including the isolation of the pathogen fungi from a short incubated culture, using DNA extraction, PCR, and RFLP profiles for 6–10 samples takes about several hours (12–18 h). However, the process lasts at least for 1–5 days with traditional methods. Therefore, this technique is faster than classic or morphologic identification approaches.

PCR-RFLP employing *Mwo* was used in this study as a rapid and reliable technique to identify *Aspergillus* spp. released from clinical specimens. Accordingly, it was found that over 50% of all samples in the environmental isolates were positive in terms of the presence of at least one opportunistic fungus. The floor coverings, walls, and beds were the most frequent samples that were contaminated by the above fungi. This finding can be attributed to the fact that the patient cases had contacts with the above surfaces frequently and for a long time.

The process used to identify the isolated hospital fungi is of high importance as it contributes to select the most effective antifungal therapy. Furthermore, the use of unreliable techniques leading to misdiagnosis and overtreatment may speed up the spread of hospital infections.

Collecting hospital fungi isolates from cases and employees’ finger touch samples is important (Table 2). *Aspergillus* spp. representing the second most important group of fungi underlying nosocomial infections was sampled from the air and indoor/outdoor places at the hospital. The most contaminated hospital indoor samples were collected from carpets, walls, and also air specimens in the hospital rooms [2]. *Aspergillus* spp. is inhaled mainly from the spores existing in aerosols suspended in the air. However, the present study did not address the isolation of *Aspergillus* spore from air samples. Respirable fungal spores can be released from building or construction projects and spread into the environment [1]. Because of their small size, *Aspergillus* spores can suspend in the air for a long time. Therefore, the use of antiseptic agents (fungicides) on a daily basis as a routine laboratory procedure is recommended.

Our results of *Aspergillus* isolation are in agreement with the above studies implying that *A. fumigatus* and *A. flavus* are the most commonly isolated *Aspergillus* spp. in the patients with diagnosed aspergillosis. Molecular assays that allow genetic characterization of *Aspergillus* phenotype diversity are likely to become incorporated into major mycologist guidelines that are widely applicable in clinical practice. In addition, early identification is important for discovering new antifungal drugs to control hospital and environmental infections [21].

Acknowledgements

The authors would like to thank the deputy student services for their kind help to collect the samples. This study as a scientific project (contract number: 1395-01-32-2781) was financed by the Research and Technology Deputy of Urmia University of Medical Sciences.
Conflict of Interest

The authors confirm that there are no conflicts of interest.

References

1. Warris A, Verweij PE. Clinical implications of environmental sources for Aspergillus. Med Mycol. 2005;43 Suppl 1:S59–65.
2. Dancer SJ. The role of environmental cleaning in the control of hospital-acquired infection. J Hosp Infect. 2009;73(4):378–85.
3. Verweij PE, Te Dorsthorst DT, Rijs AJ, De Vries-Hopers HG, Meis JF. Nationwide survey of in vitro activities of itraconazole and voriconazole against clinical Aspergillus fumigatus isolates cultured between 1945 and 1998. J Clin Microbiol. 2002;40(7):2648–50.
4. Mirhendi H, Diba K, Kordbacheh P, Jalalizand N, Makimura K. Identification of pathogenic Aspergillus species by a PCR-restriction enzyme method. J Med Microbiol. 2007;56(Pt 11):1568–70.
5. Diba K, Kordbacheh P, Mirhendi H, Rezaie S, Mahmoudi M. Identification of Aspergillus species using morphological characteristics. Pak J Med Sci. 2007;23(6):867–72.
6. Brandolt TM, Klaflke GB, Goncalves CV, Bitencourt LR, Martinez AMBd, Mendes JF, et al. Prevalence of Candida spp. in cervical-vaginal samples and the in vitro susceptibility of isolates. Brazilian J Microbiol. 2017;48(1):145–50.
7. Salari S, Khorasavi AR, Mousavi SAA, Nikbakht-Brojeni GH. Mechanisms of resistance to fluconazole in Candida albicans clinical isolates from Iranian HIV-infected patients with oropharyngeal candidiasis. Journal de Mycologie Médicale. 2016;26(1):35–41.
8. Naji S, Diba K, Yosefzadeh R, Mansouri F. Interspecies differences of candida species causing recurrent vulvovaginal candidiasis in response to fluconazole treatment. Tehran University Medical Journal TUMS Publications. 2017;75(4):280–7.
9. Healy M, Reece K, Walton D, Huong J, Shah K, Kontoyiannis DP. Identification to the species level and differentiation between strains of Aspergillus clinical isolates by automated repetitive-sequence-based PCR. J Clin Microbiol. 2004;42(9):4016–24.
10. Moody SF, Tyler BM. Use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the Aspergillus flavus group: A. flavus, A. parasiticus, and A. nomius. Appl Environ Microbiol. 1990;56(8):2453–61.
11. Li RY, Li DM, Yu J, Liu W, Ji ZH, Wang DL. Application of molecular biology techniques in the identification of pathogenic fungi and the diagnosis of fungal infection. Beijing Da Xue Xue Bao. 2004;36(5):536–9.
12. EJ Anaissie MM, MA Pfaller. Clinical mycology: Churchill Livingston; 2003.
13. Diba K, Mirhendi H, Kordbacheh P, Rezaie S. Development of RFLP-PCR method for the identification of medically important Aspergillus species using single restriction enzyme Mwo. Brazilian J Microbiol. 2014;45(2):503–7.
14. Loeffler J, Kloepfer K, Hebart H, Najvar L, Graybill JR, Kirkpatrick WR, et al. Polymerase chain reaction detection of aspergillus DNA in experimental models of invasive aspergillosis. J Infect Dis. 2002;185(8):1203–6.
15. Martinez-Culebras PV, Ramon D. An ITS-RFLP method to identify black Aspergillus isolates responsible for OTA contamination in grapes and wine. Int J Food Microbiol. 2007;113(2):147–53.
16. Almirante B, Rodriguez D, Park BJ, Cuenca-Estrella M, Planes AM, Almeida M, et al. Epidemiology and predictors of mortality in cases of Candida bloodstream infection: results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. J Clin Microbiol. 2005;43(4):1829–35.
17. Mohammadi R, Mirhendi H, Rezaei-Matehkolaei A, Ghahri M, Shidfar MR, Jalalizand N, et al. Molecular identification and distribution profile of Candida species isolated from Iranian patients. Med Mycol. 2013;51(6):657–63.
18. Asadzadeh M, Ahmad S, Al-Sweih N, Khan ZU. Rapid molecular differentiation and genotypic heterogeneity among Candida parapsilosis and Candida orthopsilosis strains isolated from clinical specimens in Kuwait. J Med Microbiol. 2009;58(Pt 6):745–52.
19. Dendis M, Horvath R, Michalek J, Ruzicka F, Grijalva M, Bartos M, et al. PCR-RFLP detection and species identification of fungal pathogens in patients with febrile neutropenia. Clin Microbiol Infect. 2003;9(12):1191–202.
20. Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi H. A one-enzyme PCR-RFLP assay for identification of six medically important Candida species. Nihon Ishinkin Gakkai Zasshi. 2006;47(3):225–9.
21. Walsh TJ, Francesconi A, Kasisi M, Chanock SJ. PCR and single-strand conformational polymorphism for recognition of medically important opportunistic fungi. J Clin Microbiol. 1995;33(12):3216–20.