A 9-Year Experience of Aspergillus Infections from Isfahan, Iran

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Purpose: Aspergillosis is an important fungal disease affecting millions of individuals worldwide. The genus of Aspergillus consists of various complexes, causing a wide spectrum of diseases from superficial infections in immunocompetent hosts to life-threatening disseminated infections among immunocompromised patients. This study aimed to identify Aspergillus species by phenotypic (total isolates) and molecular tests (35 isolates), obtained from patients in Isfahan (the third-largest city of Iran) between 2010 and 2018, and determine the susceptibility of 35 clinical isolates to itraconazole (ITR), amphotericin-B (AMB), and voriconazole (VOR).

Patients and Methods: Based on clinical signs, a total of 2385 suspected cases were included in this retrospective study from January 2010 to December 2018. Direct microscopic examination with potassium hydroxide, sabouraud dextrose agar with chloramphenicol, and czapekdox agar media was applied to identify etiologic agents. Thirty-five Aspergillus species collected from January 2016 to December 2018 were identified by PCR-sequencing of ITS1-5.8SrDNA-ITS2 region, and their susceptibility to ITR, AMB, and VOR was determined using E-test.

Results: Based on direct microscopy and positive culture, 132 out of 2385 suspected cases had Aspergillus infection (5.5%). Fifty-four patients were male, and 78 patients were female. Patients in the age groups of 41–50 and 21–30 years had the highest and lowest frequencies, respectively. Aspergillus flavus/oryzae (n=54), A. fumigatus (n=24), A. niger (n=15), and A. terreus (n=12) were the most prevalent Aspergillus species, respectively. Among 35 Aspergillus species, the MIC ranges of AMB, ITR, and VOR for A. flavus/oryzae, A. niger, and A. terreus were (0.5–4 μg/mL; 0.5–16 μg/mL; 0.25–8 μg/mL), (1 μg/mL, 1 μg/mL, 1 μg/mL; 1 μg/mL), and (4–4 μg/mL, 0.5–1 μg/mL, 0.5–1 μg/mL), respectively.

Conclusion: Aspergillus infections have a wide spectrum of clinical manifestations and often occur in immunocompromised patients. Accurate identification at the species level is essential since the emergence of cryptic species is connected to different patterns of AFST that affect patient treatment outcomes. Azole-resistant Aspergillus spp. is a global concern, and the detection of the route of resistance is pivotal to prevent and control infection.

Keywords: Aspergillus, causative agents, E-test, itraconazole, amphotericin-B, voriconazole

Introduction

Aspergillosis is a wide-spectrum fungal disease affecting millions of people worldwide. It ranges from mycotoxicosis, onychomycosis, and allergic syndromes in immunocompetent hosts to life-threatening invasive or systemic infections among immunosuppressed patients, such as those undergoing a solid organ transplant, hematopoietic stem cell transplant (HSCT), chemotherapy, besides those taking corticosteroids.1,2 Approximately 1.6 million people die due to Invasive fungal diseases (IFDs), such as systemic aspergillosis, annually.3 The European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and
Research Consortium (EORTC/MSGERC) consensus definitions of “proven,” “probable,” and “possible” IFDs have been updated in 2019. These classifications have been of great value to researchers, who manage diagnostic tests, perform clinical trials of antifungal agents, and undertake epidemiologic investigations. Aspergillus species are ubiquitous saprophytic molds with four subgenera, which are subdivided into several sections. They can be found in hospital surroundings, such as ventilation equipment, surfaces, water, food, trees, and decaying vegetation. Among the hyaline filamentous fungi, Aspergillus is the most important gender, generating enormous airborne conidia that when inhaled, it could cause nosocomial outbreaks.

The genus Aspergillus is a large and important genus mainly connected to medical relevance as fungal pathogens (A. niger, A. terreus, A. fumigatus), biotechnologic and industrial applications (A. oryzae, A. niger), and food spoilage and mycotoxin production (A. parasiticus, A. flavus). The most routinely occurring human pathogenic Aspergillus species is Aspergillus fumigatus (67–73%), followed by A. flavus (10–16%), A. niger (5–9%), A. terreus (3–4%), and the others.

In clinical mycology laboratories, the identification of Aspergillus species relies principally on morphological criteria, including microscopic traits (eg, the shape of conidiogenous cells, conidiophore, conidial germination, and mycelial structures) and macroscopic features (eg, color, surface topography, and texture of colonies). Species identification based on morphological features is controversial, being now challenged versus molecular techniques. Identification of fungal species on the basis of phylogenetic relationships has unveiled “cryptic” species within morphologically indiscernible microorganisms such as in A. flavus. Molecular data progressively indicate that some known single “species” of fungi unambiguously consist of disparate species. Further, phylogenetics has uncovered cryptic species in the genus of Aspergillus containing A. flavus, A. bombycis, and A. niger. The significance of the cryptic species lies in differences in antifungal susceptibility. In recent years, molecular diagnosis in clinical laboratories has been applied for the precise identification of causal agents. The impressive antifungal treatment has become exclusively significant as the number of immunosuppressed patients has been increased in parallel with life-threatening invasive fungal infections (IFIs). It is indeed challenging to treat fungal infections due to the growing emergence of drug-resistant isolate and also immune system status of patients. Over the last decades, in vitro antifungal susceptibility testing for some antifungal drugs, such as echinocandins and azoles, have been developed to modify the treatment of aspergillosis. Due to the extensive use of antifungals and emerging azole-resistant Aspergillus species, the in vitro susceptibility testing of clinical isolates is essential both for the global surveillance of Aspergillus susceptibility to antifungal agents and appropriate treatment of patients. The Clinical and Laboratory Standards Institute (CLSI) has introduced a reference broth microdilution (BMD) method for antifungal susceptibility testing (AFST) of molds; however, this method is labor intensive. Commercialized E-test strips comprising defined gradients of antifungal agents are a more easy and favorable approach for AFST; however, the ability of E-test to detect azole-resistant strains has not yet proven.

This retrospective study aims to identify Aspergillus clinical isolates obtained from patients referred to mycology reference laboratory in Isfahan (the third-largest city of Iran) by phenotypic (all isolates) and molecular tests (35 isolates), between 2010 and 2018, and determine the susceptibility of 35 clinical isolates to itraconazole (ITR), amphotericin-B (AMB), and voriconazole (VOR).

**Patients and Methods**

**Patients**

A total of 2385 suspected cases (1386 males versus 999 females) were included in this retrospective study from January 2010 to December 2018. The study protocol was reviewed and approved by the Ethics Committee of Isfahan University of Medical Sciences (IR.MUL.MED.REC.1398.012). Demographic and clinical data, including age, sex, job, and clinical signs, were documented for each subject. These cases were housekeepers (n=691), farmers (n=471), freelancers (n=382), employees (n=308), students (n=193), unemployed (n=106), retirees (n=98), children (69), and unknown (n=67). Patients who had taken antifungal drugs for the past 7 days were excluded from the study.

**Phenotypic Tests**

Direct microscopic examination (DME) was performed for each sample using potassium hydroxide (KOH) 10%-20%, according to the type of clinical samples. Sabouraud dextrose agar (SDA) with chloramphenicol (0.04 g/L) and cycloheximide free (Difco, Detroit, MI, USA), together with czapekdox agar (QUELAB, Quebec, Canada) media, were applied for culture. The specimens obtained from superficial lesions and deep tissues were incubated at 30°C and 37°C, respectively, and then were checked for the fungal growth up to 21 days. Morphological
characters for identification of *Aspergillus* species are colony growth rate, sporulation degree, cleistotheca or sclerotia production, colony texture, hypha color pigmentation, and colony reverses. Discriminating features of conidiophore and conidial heads among various *Aspergillus* species are dimension, shape, texture, besides the color of stipes, the shape and size of vesicles, phialides (uniseriate or biseriate), the absence or presence of metulae between phialides and vesicle, conidia formation, and Hülle-cells (if present).

**Molecular Species Identification**

From January 2016 to December 2018, all clinical isolates (no, 35) were identified using the polymerase chain reaction (PCR)-sequencing technique.

**PCR**

Genomic DNA from the clinical sample was extracted using phenol/chloroform technique and the amplified ITS1-5.8SrDNA-ITS2 region was sent for sequence analysis. Briefly, PCR mixture, including 2.5 μL of 10× reaction buffer, 1.5 mM MgCl₂, 0.4 mM dNTPs, 1.25 U of Taq polymerase, 30 pmol of ITS1 primer (5ʹ-TCC GTA GGT GAA CCT GCG G-3ʹ), 30 pmol of ITS4 primer (5ʹ-TCC TAT TGA TAT GC-3ʹ), and 3 μL of extracted DNA, was applied in a final volume of 25 μL. The PCR cycling conditions were an initial denaturation phase at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 1 min, and a final extension phase at 72°C for 7 min. Six microliters of PCR products were loaded on 1.5% agarose gel, stained with 0.5 μg/mL ethidium bromide, visualized by gel documentation system (UVITEC, UK), and then photographed.

**Sequencing**

Amplicons were purified with GenCatch TM PCR Cleanup Kit (Epoch Life Science, USA), and cycle sequencing reactions were performed in the forward direction (Bioneer, South Korea). Ultimately, the products were analyzed with Chromas 2.6.6 (https://technelysium.com.au/wp/chromas/) and evaluated using NCBI BLAST searches against fungal sequences existing in DNA databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Antifungal Susceptibility Testing (AFST)**

The antifungal susceptibility of *Aspergillus* species obtained from January 2016 to December 2018 (35 isolates) was determined by using the agar diffusion E-test (bioMérieux, France) with RPMI medium (Sigma Chemical Co., St. Louis, MO, USA), according to the supplier’s recommendations. Conidial suspensions were calibrated to an optical density yielded 10⁶ CFU/mL. The suspension was applied to spread fungi onto the surface of RPMI 1640 agar supplemented with 2% glucose and morpholinepropanesulfonic acid (MOPS) via direct inoculation with a sterile cotton swab. The minimum inhibitory concentration (MIC) was assessed at 100% inhibition for all antifungal agents, including ITR, AMB, and VOR, after incubation at 35°C for 48 hours. The lowest antifungal concentration was considered, at which the border of the elliptical inhibition separated the scale of the strip.

**Data Analysis**

The association between the clinical samples type and *Aspergillus* species was analyzed by chi-square (χ²) and Fisher’s exact tests in SPSS version 23. Further, MIC ranges, MIC50, MIC90, and geometric mean (GM) of antifungal agents were calculated for clinical isolates in the section of AFST.

**Results**

One hundred and thirty-two out of 2385 suspected cases had *Aspergillus* infection (5.5%). Fifty-four patients were male, and 78 patients were female. The age range of the patients was 21–76 years, with the mean age of 51.4 years. Patients in the age groups of 41–50 and 21–30 years had the highest and lowest frequencies, respectively. *Aspergillus flavus/oryzae* (n=54), *A. fumigatus* (n=24), *A. niger* (n=21), *A. terreus* (n=12), *A. nidulans* (n=6), and *A. clavatus* (n=3) were the most prevalent *Aspergillus* species (Table 1). Twelve isolates remained unidentified and reported as *Aspergillus* spp. All sequences related to molecular identified species were deposited in GenBank, and accession numbers were obtained for them (MT582514 and MT584269-MT584302). The most *Aspergillus* species were obtained from bronchoalveolar lavage (BAL) (38.6%), toanel (21.2%), sinus (15.9%), ear (9.1%), fingertip (9.1%), eye (5.3%), and skin (0.7%). *Figure 1* shows the frequency of different clinical types of *Aspergillus* infections from 2010 to 2018. According to EORTC/MSGERC criteria, there was no proven invasive aspergillosis; however, based on host factors, clinical features, and mycological evidence, 35 and 38 patients were recorded as probable and possible IFDs, respectively.
Statistical Analysis

Fisher’s exact test showed that the association between the clinical samples type and *Aspergillus* species was not statistically significant (p = 0.86). The MIC\textsubscript{50} and MIC\textsubscript{90} values were calculated as the minimum concentrations of agents being able to inhibit 50-90% of the clinical isolates, respectively. The high and low off-scale MICs were included in the analysis by conversion to the next higher and lower antifungal agent concentrations.

AFST

The MIC ranges of antifungal agents were as follows: *A. flavus* (AMB, 0.5–4 μg/mL), (ITR, 0.5–16 μg/mL), (VOR, 0.25–8 μg/mL); *A. niger* (AMB, 1 μg/mL), (ITR, 1 μg/mL), (VOR, 1 μg/mL); *A. terreus* (AMB, 4–4 μg/mL), (ITR, 0.5–1 μg/mL), (VOR, 0.5–1 μg/mL). Figure 2 shows an amphotericin B E-test strip on RPMI medium, demonstrating an MIC of 1 μg/mL in a susceptible *A. flavus/oryzae*. MIC\textsubscript{50} (concentrations inhibiting ≥50% of growth), MIC\textsubscript{90} (concentrations inhibiting ≥90% of growth), and GM values for the 35 clinical isolates are presented in Table 2.

### Discussion

*Aspergillus* species, as the most important opportunistic fungi, extensively are found in nature. They can cause a wide spectrum of diseases in individuals with compromised immune systems.\textsuperscript{23} Changes in epidemiological aspects have occurred due to the increase in the number of immunosuppressed patients, advances in recognition, speciation of clinical and environmental isolates, and extensive use of broad-spectrum antifungal drugs.\textsuperscript{7,24} The genus *Aspergillus* comprises various complexes, such as *A. flavus* complex, *A. fumigatus* complex, *A. nidulans* complex, *A. terreus* complex, and *A. ustus* complex. After *A. fumigatus* complex, *A. flavus* is considered as the second most prevalent *Aspergillus* species in medical laboratories and hospitals of Europe,\textsuperscript{25} the United States,\textsuperscript{26} Brazil\textsuperscript{27} and arid regions of the Middle East.\textsuperscript{28} *A. niger* and *A. terreus* are ranked second in Korea, and Austria, respectively.\textsuperscript{29} Contrary to these findings, *A. flavus* is the most common *Aspergillus* species in the present investigation. Often, people are infected with this fungal disease by daily inhalation of hundreds of spores, as well as colonization of a fungus in the respiratory tract if the immune system is suppressed. In line with this concept, the most *Aspergillus* species were obtained from BAL samples in the present study. The fungal cell wall component galactomannans (GM) are polysaccharides, including a mannose backbone with galactose as side groups released during the hyphal growth.\textsuperscript{30} It is a biologic marker and used as a noninvasive method for detecting aspergillosis in blood or BAL. One of the
while we isolated only 7 *Aspergillus* spp. (5.3%) from keratomycosis. Otomyisis (swimmer’s ear) is an acute or chronic superficial fungal infection of the external auditory canal, occasionally involving middle parts of the ears. Gharaeghi et al, reported 5.7% to 81% of otomyisis (with a mean value of 51.3%) in different areas of Iran. The incidence of otitis externa in our study was 9.1%. Determination of antifungal susceptibility patterns for clinical strains is crucial step to develop an effective strategy for infection management. The first choice for treatment of human Aspergilloisis is azole drugs, such as VOR, ITR, and isavuconazole (ICZ). In the present study, 35 *Aspergillus* spp. containing *A. flavus/oryzae, A. terreus,* and *A. niger* were tested using E-test against three antifungal drugs, including ITR, VOR, and AMB. Some investigations have compared the CLSI BMD methods with E-test for susceptibility testing of molds; however, they have been confined to selected antifungal agents or a limited subcategory of fungal species. Lamoth et al showed good agreement between the two methods, depending on the fungus and antifungal tested. Nevertheless, some *Aspergillus* species, such as *A. terreus,* demonstrated higher MICs in the E-test method, possibly reflecting the innate resistance of these species to amphotericin B. Triazole MIC values for *Aspergillus* species are usually lower with the E-test compared to the CLSI BMD method; thus, the use of the E-test strip for antifungal susceptibility testing of posaconazole among *Aspergillus* species are not recommended. The E-test reveals to be comparable to the CLSI BMD method for in vitro antifungal testing of voriconazole among *Aspergillus* species, and it can detect triazole-resistant isolates. Another limitation of our research was the lack of AFST data for other species, such as *fumigatus, nidulans,* and *clavatus.* Resistance to azole drugs would develop in response to the continued exposure of fungus to azolic agents and transmission of gene mutations to the new conidia. In the absence of clinical breakpoints, the concept of epidemiological cut-off values (ECV) has been described by CLSI. The MIC separates wild and non-wild strains, considering the latter as those with possible mutational or acquired resistance based on their phenotypic MIC values. The ECV can predict possible resistance to an antifungal drug, for which there is not adequate information to establish breakpoints. According to CLSI M59 document, ECV of AMB, ITR, and VOR for *A. flavus/oryzae, A. terreus,* and *A. niger* is (4, 1, 2 µg/mL), (4, 2, 2 µg/mL), and (2, 4, 2 µg/mL), respectively. Although Alborz et al, showed that 36.1% of *Aspergillus* species were resistant to AMB, in the present study, it was seen in 12.5% of clinical isolates. Johnson et al, reported that clinical
In the present study, an 78% rate of ITC resistance to AMB was reached among A. terreus isolates. In agreement, 28% of isolates were resistant to AMB, ITR, and VOR.

This high rate of resistance to azoles may be attributed to cross-resistance with agricultural triazoles and long-term azole therapy among patients with chronic aspergillosis. In the present study, a 78% rate of ITC resistance was shown among VOR-resistant isolates of A. flavus/oryzae earlier. Our study was limited by the fact that we were unable to detect resistance mechanism analysis for A. flavus/oryzae with high voriconazole and itraconazole MICs.

Conclusion

Aspergillus infections have a broad spectrum of clinical manifestations and often occur in immunocompromised patients. Since people with immunodeficiency disorders are on the rise, opportunistic fungi have been noticed as one of the main concerns of morbidity and mortality in such patients. Accurate identification at the species level is essential as the emergence of cryptic species is connected to specific patterns of AFST that affect patient treatment outcomes. In the present study, 12.5%, 78%, and 28% of A. flavus/oryzae isolates were resistant to AMB, ITR, and VOR, respectively. Azole-resistant Aspergillus spp. is a global issue. Understanding the route of resistance is essential to perform executable prevention and control measures. When the prevalence of environmental resistant isolates to azoles is above 10%, empirical therapy should not include voriconazole monotherapy.

Abbreviations

IFDs, invasive fungal diseases; EORTC/MSGERC, The European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium; HSCT, haematopoietic stem cell transplant; DME, direct microscopic examination; KOH, potassium hydroxide; SDA, sabouraud dextrose agar; PCR, polymerase chain reaction; MOPS, morpholinepropanesulfonic acid; ITR, itraconazole; AMB, amphotericin
B; VOR, voriconazole; AFST, antifungal susceptibility testing; GM, galactomannan; ICZ, isavuconazole; CLSI, Clinical and Laboratory Standards Institute; DMIs, demethylase inhibitors; ABC, ATP-binding cassette.

**Data Availability**
All sequences were deposited in the GenBank database, and accession numbers were obtained for them, and inserted into the manuscript (MT582514, and MT584269 – MT584302).

**Ethics Approval and Informed Consent**
Participants gave their consent for publication of the study. This investigation was approved by the Ethics Committee of Isfahan University of Medical Sciences. The code number is IR.MUI.MED.REC.1398.012.

**Consent for Publication**
All participants (patients) gave their consent for publication of the present investigation.

**Informed Consent**
Informed consent from all patients and parents/Legally authorized representatives of minor patients was obtained at the pint of data collection.

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**Author Contributions**
All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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**Disclosure**
The authors declare that there is no conflict of interest.

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