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Molecular epidemiology and antifungal susceptibilities of Aspergillus species isolated from patients with invasive aspergillosis

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SUMMARY

OBJECTIVE: The aim of this study was to evaluate the demographic data, molecular epidemiology, and in vitro antifungal susceptibility results of patients with *Aspergillus* isolated from various clinical specimens.

METHODS: A total of 44 *Aspergillus* strains were studied. The definition of invasive aspergillosis in patients was made according to European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria. Strains were phenotypically and molecularly identified. Demographic characteristics of patients and genotypes of strains were evaluated. Phylogenetic analysis was done by the The Unweighted Pair-Group Method with Arithmetic Mean (UPGMA). Antifungal susceptibility of strains was determined according to The Clinical and Laboratory Standards Institute (CLSI)-M61-Ed2 and The European Committee on Antimicrobial Susceptibility Testing (EUCAST).

RESULTS: A total of 11 patients were classified as proven and 33 as probable invasive aspergillosis. There was a statistically significant difference in age groups, subdisease, neutropenic, and receiving chemotherapy between groups. A total of 23 strains were identified as *Aspergillus fumigatus*, 12 as *Aspergillus niger*, 6 as *Aspergillus flavus*, and 3 as *Aspergillus terreus*. Phylogenetic analysis revealed five different genotypes. No statistical difference was found in the comparisons between patients groups and genotype groups. There was a statistically significant difference between genotype groups and voriconazole, posaconazole, and itraconazole Minimum Inhibition Concentration (MIC).

CONCLUSION: Accurate identification of strains and antifungal susceptibility studies should be performed due to azole and amphotericin B resistance. Genotyping studies are important in infection control due to identifying sources of infection and transmission routes.

KEYWORDS: Aspergillus. Microbial sensitivity tests. Molecular epidemiology. Sequence analysis.

INTRODUCTION

Aspergillus spores are commonly found in our environments and generally enter the body through respiration. These spores easily lead to invasive infection in immunocompromised individuals, and infections are associated with high mortality[1].

The diagnosis of *Aspergillus* infections is usually delayed due to the lack of reliable and easy-to-apply diagnostic tests, and effective treatment cannot be started in a timely manner. In order to prevent delays in the diagnosis of invasive fungal infections in patients with immunosuppressed, EORTC/MSG diagnostic criteria were established[2].

The identification of *Aspergillus* strains is performed by conventional, molecular methods, and serological tests[1]. Genotyping methods allow the epidemiological relationship between clinical and patient environmental isolates[3-6].

Antifungal resistance is increasing in clinical strains due to the intensive use of azole group pesticides as pesticides[1]. The mold should be produced from the clinical sample to detect antifungal resistance; a susceptibility test should be performed by reference methods (CLSI, EUCAST)[7-9].

The aim of this study was to retrospectively evaluate the demographic data, molecular epidemiology, and in vitro antifungal susceptibility results of patients with *Aspergillus* mold growth isolated from various clinical specimens over a period of 2 years.

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METHODS

Strains were isolated in the Erciyes University Medical Faculty Hospital, Mycology Laboratory, in Kayseri-Turkey in the past 2 years. All 44 strains were late from clinical samples of patients with suspected invasive aspergillosis (IA) infection. The galactomannan antigen (GMA) was detected in 22 patients.

The definition of IA was made according to the EORTC/MSG criteria. The data of the patients were compared with the data of 19 control patients who were negative for direct microscopy, culture growth, histopathology, and GMA.

Phenotypic identification of the strains was performed according to microscopic and macroscopic features. Molecular identifications of strains were determined by sequence analysis of the ITS1-4, D1D2-NL1-4 region. DNA sequence analysis was performed using the ABI 3130XL analyzer device (Applied Biosystems, USA). The ITS1-4 and D1/D2 nucleotide sequences of *Aspergillus* were analyzed using the BLASTN program provided on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis: ITS and D1/D2 nucleotide sequences of *Aspergillus* strains were obtained from GenBank. The sequences were aligned using the Clustal X software. Nucleotide sequences were then used for phylogenetic analysis by the UPGMA method with 1000 bootstrap replication to ensure robustness using the MEGA software (MEGA Inc., Englewood, USA).

Antifungal susceptibility testing was performed according to the CLSI-M61-Ed2 and EUCAST-v10.0[7,8]. AmphotericinB (AMB), voriconazole (VOR), and itraconazole (ITR) (Sigma, USA) were tested by the broth microdilution test. Caspofungin (CAS), anidulafungin (ANI), and posaconazole (POS) (Etest, bioMerieux, France) were tested by the gradient strip test. There were reported VOR breakpoints for only *Aspergillus fumigatus* strain in CLSI-M61-Ed2[7]. ECOFF values and susceptibilities breakpoints for AMB, ITR, POS, and VOR of *A. fumigatus*, *Aspergillus niger*, *Aspergillus flavus,* and *Aspergillus terreus* strains were evaluated according to EUCAST[8,9].

Ethics statement

Ethical approval was not considered necessary as the isolates were stock samples taken during routine laboratory activities.

Statistical analysis

Statistical analyses were performed using IBM-SPSS-Statistics V22.0. Chi-square test was used for the comparison between the characteristics of the patients and their diagnosed groups, and t-test and one-way ANOVA tests were used for the comparison between antifungal MIC means and genotypes. $p<0.05$ was considered significant.

RESULTS

A total of 44 patients (12 females, 32 males) were included in the study. Most of the patients were hospitalized for chest diseases (29.5%) and hematology-oncology services (18.1%). In total, 20 (45.45%) of the samples were found to be bronchoalveolar lavage, 10 tissue, 5 wounds, 5 sterile body fluids, and 4 sputa. Most patients have an underlying factor such as malignancy (n.24, 54.5%).

According to EORTC/MSG criteria, 11 patients were classified as proven and 33 as probable IA. The clinical samples of 11 patients with proven IA were three sterile body fluids and eight tissues, and hyphae were seen in the direct microbiological and histopathological examination of all of them. The mean age of patients with IA was higher than that of the control group. The rate of association with hematological cancer in patients with proven IA was found to be higher than in other groups. There was a statistically significant difference in subdisease, neutropenic, and receiving chemotherapy between groups. There were no significant differences in having a catheter and prophylactic antifungal drugs between groups. VOR was used most frequently as a prophylactic antifungal agent. GMA test was above 0.5 ng/mL in 12 patients (27.2%). Demographic data of patients with IA and the control group are shown in Table 1.

A total of 44 strains were identified by conventional methods, and 23 were identified as *A. fumigatus*, 12 *A. niger*, 6 *A. flavus*, and 3 *A. terreus.* However, sequence analysis could be performed on 35 of 44 strains, and these strains were identified using the same by conventional methods (23 *A. fumigatus*, 4 *A. niger*, 5 *A. flavus*, 3 *A. terreus*). There was no statistical difference between IA patient groups and isolated strains (Table 1).

The 14 *A. fumigatus*, 8 *A. niger*, and 2 *A. flavus* were isolated from 24 respiratory tract samples. Five *A. fumigatus*, four *A. flavus*, and one *A. niger* strain were isolated from tissue samples. Three *A. fumigatus*, one *A. niger*, and one *A. terreus* strain were isolated from wound samples. Two *A. niger*, two *A. terreus*, and one *A. fumigatus* strain were isolated from a sterile liquid.

Result of phylogenetic tree analysis using UPGMA method

Main genotypes were designed A, B, C, D, and E. The most common genotype was genotype A, which accounted for 23 (65.7%) of the 35 isolates. Subgenotypes were determined in main clone A (A1–A2). Genotype A contained *A. fumigatus* strains. Genotype B contained three *A. terreus* strains. Genotype C contained four *A. flavus* strains. Genotype D contained one *A. flavus* strain. Genotype E contained four *A. niger*

Table 1. Evaluation of demographic characteristics, isolated strains, and genotypes of patients diagnosed with proven and probable IA, and control groups.

	Proven IA (n=11)	Probable IA (n=33)	Control group (n=19)	χ^2	p-value
	n (%)	n (%)	n (%)		
Gender*					
Men	8 (17.8)	24 (53.3)	13 (28.9)	0.121	0.941
Women	3 (16.7)	9 (50.0)	6 (33.3)		
Age (years)*					
<18	0 (0.0)	2 (66.7)	1 (33.1)	13.033	**0.043**
18–45	2 (10.5)	6 (31.6)	11 (57.9)		
46–64	5 (18.5)	16 (59.3)	(22.2)		
>65	4 (28.6)	9 (64.3)	1 (7.1)		
Neutropenia*					
Yes	7 (26.9)	16 (61.5)	3 (11.5)	8.069	**0.018**
No	4 (10.8)	17(45.9)	16 (43.2))		
Intravenous catheter*					
Yes	9 (25.7)	17 (48.6)	9 (25.7)	3.807	0.149
No	2 (18.2)	16 (57.1)	10 (35.7)		
Chemotherapy*					
Yes	10 (25.0)	16 (40.0)	14 (35.0)	7.625	**0.022**
No	1 (4.3)	17 (73.9)	5 (21.7)		
Prophylactic antifungal*					
Yes	7 (31.8)	10 (45.5)	5 (22.7)	4.920	0.085
No	4 (9.8)	23 (56.1)	14 (34.1)		
Mortality*					
Yes	4 (26.7)	5 (33.3)	6 (40.0)	2.952	0.229
No	7 (14.6)	28 (58.3)	13 (27.1)		
Subdisease (n=63)*					
Hematological cancers	7 (41.2)	3 (17.6)	7 (41.2)	31.978	**0.000**
Other cancers	4 (22.2)	10 (55.6)	4 (22.2)		
COPD	0 (0.0)	6 (85.7)	1 (14.3)		
DM	0 (0.0)	7 (100.0)	0 (0.0)		
RA	0 (0.0)	7 (100.0)	0 (0.0)		
Others	0 (0.0)	3 (30.0)	7 (70.0)		
Strains**					
A. fumigatus	4 (36.4)	19 (57.5)	0	6.821	0.078
A. niger	2 (18.1)	10 (30.3)	0		
A. flavus	4 (36.4)	2 (6.1)	0		
A. terreus	1 (9.1)	2 (6.1)	0		
Genotypes**					
A1	4 (36.4)	16 (48.6)	0	8.214	0.223
A2	0	3 (9.1)	0		
B	1 (9.1)	2 (6.1)	0		
C	3 (27.4)	1 (3.0)	0		
D	0	1 (9.1)	0		
E1	1 (9.1)	1 (3.0)	0		
E2	0	2 (6.1)	0		

*Line percentage. **Column percentage. Bold indicate statistically significant p-values. Hematological cancers: acute myeloid leukemia, multiple myeloma, and chronic lymphocytic leukemia. Other cancers: lung, bone, nasal, skin, kidney, breast, and stomach cancers. COPD: chronic obstructive pulmonary disease; DM: diabetes mellitus; RA: rheumatological disease.

strains and consisted of two subgenotypes (E1–E2) (Figure 1). The clinical origin and isolation date of strains were evaluated, and there was no clonal relationship found among strains to determine an outbreak. No statistical difference was found in the comparisons between the patient groups and the genotypes groups. Of 35 strains, 9 belonged to patients diagnosed with proven IA. Of the nine strains, four were of the A1 genotype, and three strains in the C, B, and E1 genotypes had one strain each (Table 1).

Antifungal susceptibility testing

There was a statistically significant difference between genotypes groups and VOR, POS, and ITR MIC GO.

MIC range, MIC_{50}, MIC_{90} values of strains, and wildtype (WT) strain number and susceptibility according to EUCAST-10.0v as shown in Table 2. The only one *A. fumigatus* strain was found to be resistant to AMB (1.5 µg/mL) and non-wildtype. According to CLSI, the VOR MIC value of all *A. fumigatus* strains was found low from 0.5 µg/mL.

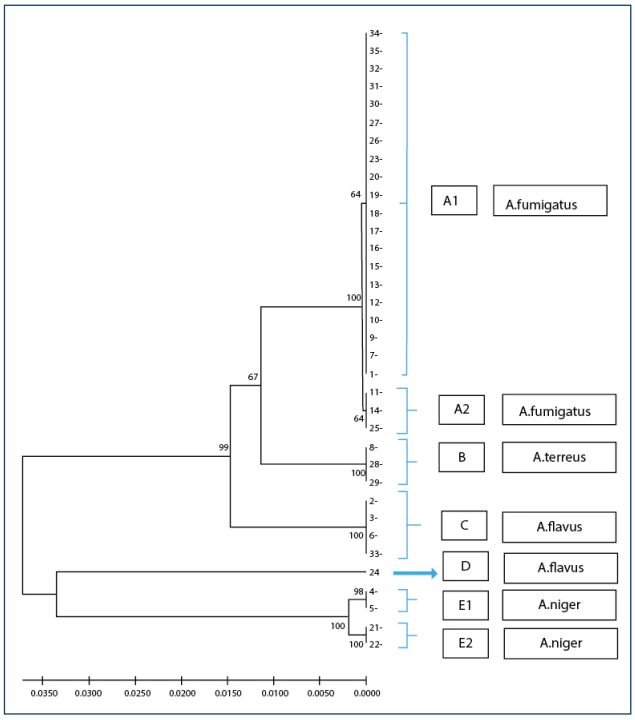

Figure 1. For phylogenetic analysis, the UPGMA of 35 *Aspergillus* strains with ITS1-4 and D1/D2 sequences available in GenBank. Strains tested were represented by isolation numbers. Strains with a similarity of 95% or high in the phylogenetic tree were considered main clones, and those in the main clone category with a similarity of 97% or above were considered subclone. Main clones were designed A, B, C, D, and E, respectively.

DISCUSSION

The incidence of IA has increased due to the increase in the number of immunocompromised patients. The incidence of IA reported in various European countries varies according to patient population and geography (0.4–23%). Patients with IA have high mortality of 30–85%. However, with early diagnosis and appropriate treatment, this rate drops below 50%[1]. The mortality rate in our patients was 20.4%.

Since the incubation period is not known precisely in IA, there is no consensus on the definition of hospital-acquired cases. In a France study, in which cases up to 10 days after hospitalization were considered hospital-acquired, it was reported that 23.8% of aspergillosis cases were hospital-acquired[10]. All the patients in our study were hospitalized for more than 10 days and had a subdisease or drug use that suppresses the immune system.

The species most commonly isolated in hospital-acquired aspergillosis are *A. fumigatus* and *A. flavus*. In a study by Atalay et al.[11] in our hospital, 12 of 24 *Aspergillus* strains were identified as *A. fumigatus*, 8 as *A. flavus*, 3 as *A. niger,* and 1 as *A. terreus,* and *A. fumigatus* was reported to be the dominant species in respiratory tract samples compared with other *Aspergillus* strains. The results obtained are similar to other results. The GMA test can also contribute significantly to the diagnosis of IA through serial follow-up[1]. In our study, there were no statistically significant differences in GMA test results between the patient groups.

Genotyping of *Aspergillus* strains allows investigation of nosocomial aspergillosis to identify outbreak-related strains, distinguish epidemic from endemic or sporadic strains, and determine the origins of infection. Refojo et al.[6] investigated whether there is a genetic relationship between the *A. flavus* strains isolated from the air of the hemato-oncology service and two patients. This study's results show that the *A. flavus* isolates recovered from the patients were not genetically related to those retrieved from the patient rooms. de Valk et al.[5] used two molecular methods for genotyping in their study and showed that the strains isolated from each patient were of different genotypes, while the strains isolated from the same patient were all of the same genotypes. Also, they also showed that there were multiple genotypes among the isolates obtained from respiratory tract samples. Similarly, Vanhee et al.[4] performed a genotypes analysis of 41 *A. fumigatus* isolates from nine patients with proven IA hospitalized in two different centers, and was shown in the identification of 11 distinct genotypes. The researchers reported the lack of a clear epidemiological link between patients, even between those hospitalized in the same center. The results of our study

Table 2. MIC range, MIC_{50}, MIC_{90} values of strains, and WT strain number and susceptibility according to EUCAST 10.0v.

	MIC (μg/mL)			Antifungal susceptibility, n (%)		
	Ranges	MIC_{50}	MIC_{90}	WT	S	R
A. fumigatus (n: 23)						
AMB	0.016–1.5	0.125	0.50	22	22	1
VOR	0.064–0.25	0.125	0.125	23	23	–
POS	0.002–0.25	0.032	0.125	23	23	–
ANI	0.002–0.032	0.002	0.032	ND	ND	ND
ITR	0.125–1	1	1	23	23	–
CAS	0.016–0.25	0.032	0.125	ND	ND	ND
A. niger (n: 12)						
AMB	0.125–0.5	0.125	0.25	12	12	–
VOR	0.036–0.25	0.125	0.25	12	ND	ND
POS	0.002–0.25	0.064	0.125	12	ND	ND
ANI	0.002–0.02	0.002	0.008	ND	ND	ND
ITR	0.125–1	1	1	12	ND	ND
CAS	0.002–0.25	0.008	0.016	ND	ND	ND
A. flavus (n: 6)						
AMB	0.25–0.5	0.5	0.5	6	ND	ND
VOR	0.064–0.5	0.125	0.125	6	ND	ND
POS	0.032–0.25	0.125	0.125	6	ND	ND
ANI	0.002–0.004	0.002	0.004	ND	ND	ND
ITR	0.25–1	0.25	0.5	6	6	–
CAS	0.008–0.064	0.016	0.016	ND	ND	ND
A. terreus (n:3)*						
AMB	0.002–0.50			3	ND	ND
VOR	0.064–0.125			3	ND	ND
POS	0.016–0.02			3	3	–
ANI	0.002–0.02			ND	ND	ND
ITR	0.25–0.25			3	3	–
CAS	0.032–0.125			ND	ND	ND

*MIK_{50} and MIK_{90} not calculated due to low number of *A. terreus* strains. ND: not done; WT: wild type; S: susceptible; R: resistant; AMB: amphotericin B; VOR: voriconazole; POS: posaconazole; ANI: anidulafungin; ITR: itraconazole; CAS: caspofungin.

were found to be similar to the studies mentioned. When the clinical origin and isolation date of strains were evaluated, there was no clonal relationship found among the *Aspergillus* strains to determine an outbreak. There was no statistically difference in the comparisons between the patient groups and the genotype groups. When we compared the geometric mean of antifungals and the genotypes, there was a statistically significant difference between the azole group antifungals (ITR, VOR, and POS) and genotypes.

In high-risk patients, prophylactic antifungal drug options are recommended by international guidelines. With the use of prophylactic antifungals, there may be a decrease in IA cases and a positive effect on the prognosis[1]. In a study conducted on patients with chronic obstructive pulmonary disease (COPD) diagnosed with IA in our hospital in 2013, researchers reported that VOR could be given as the initial treatment[12]. In a study conducted in our hospital in 2014, the MIC ranges of 26 *Aspergillus* isolates were found 0.004–2 μg/mL for AMB,

0.004–1 µg/mL for CAS, and 0.016–0.64 µg/mL for VOR[13]. These results are similar to our findings. Considering the toxic effects of AMB, we thought that the choice of VOR in prophylactic treatment in our hospital was appropriate. We can see that it had very low MIC values of azole group drugs for all strains. Although there were no statistically significant differences in receiving prophylactic antifungal between patient groups, there was no death in the receiving prophylactic antifungal drug group.

In the ESCMID guideline, VOR or isavuconazole is recommended in the treatment of IA as the first choice, but if high azole MIC values are detected, it is necessary to turn to AMB or combined treatment options[1]. According to the ESCMID, antifungal susceptibility tests should be performed[1]. In the ARTEMIS global surveillance study, the azole resistance rate was found to be 5.8% (29/497) in *A. fumigatus* isolates, which are the causative agents of IA, and it was stated that all resistant isolates were isolated in China[14]. In our study, all *A. fumigatus* strains were found to be susceptible to VOR according to CLSI. According to EUCAST 10.0 v, 43 *Aspergillus* strains were found as WT. Only one of the 23 *A. fumigatus* strains had the highest MIC value for AMB (1.5 µg/mL). Characteristics of the patient with AMB-resistant *A. fumigatus* isolated in the proven IA patient group: in genotype A1, 59 years old, male, clinical specimen sterile fluid (pleura), subdisease chronic lymphocytic leukemia, with a catheter and receiving chemotherapy, and the patient died. Also, when we look at the MIC values for echinocandin group antifungal drugs (ANI, CAS) of all strains, we saw that the MIC values are very low (0.002–0.25 µg/mL) (Table 2).

CONCLUSION

Early diagnosis of IA according to the EORT/MSG diagnostic criteria is very important, and early appropriate antifungal drug intake reduces mortality. Accurate identification of strains and antifungal susceptibility studies is required, especially due to azole and AMB resistance. Genotyping studies provide a better understanding of infection sources and transmission routes, and aid infection control.

Reference-resistant or susceptibility breakpoints for antifungal drugs for some *Aspergillus* strains are unclear. Such studies in the future will contribute to the necessary breakpoints for antifungal resistance for *Aspergillus* strains.

AUTHORS' CONTRIBUTIONS

FMS: Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. **ANK:** Methodology, Supervision, Writing – original draft, Writing – review & editing. **PS:** Writing – original draft, Writing – review & editing. **MAA:** Writing – original draft, Writing – review & editing **AB:** Methodology, Writing – original draft, Writing – review & editing. **OC:** Methodology, Writing – original draft, Writing – review & editing. **BD:** Methodology, Writing – original draft, Writing – review & editing.

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