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Frequency of azole resistance in clinical and environmental strains of *Aspergillus fumigatus* in Turkey: a multicentre study

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Objectives: *Aspergillus fumigatus* causes several diseases in humans and azole resistance in *A. fumigatus* strains is an important issue. The aim of this multicentre epidemiological study was to investigate the prevalence of azole resistance in clinical and environmental *A. fumigatus* isolates in Turkey.

Methods: Twenty-one centres participated in this study from 1 May 2018 to 1 October 2019. One participant from each centre was asked to collect environmental and clinical *A. fumigatus* isolates. Azole resistance was screened for using EUCAST agar screening methodology (EUCAST E.DEF 10.1) and was confirmed by the EUCAST E.DEF 9.3 reference microdilution method. Isolates with a phenotypic resistance pattern were sequenced for the *cyp51A* gene and microsatellite genotyping was used to determine the genetic relationships between the resistant strains.

Results: In total, resistance was found in 1.3% of the strains that were isolated from environmental samples and 3.3% of the strains that were isolated from clinical samples. Mutations in the *cyp51A* gene were detected in 9 (47.4%) of the 19 azole-resistant isolates, all of which were found to be TR34/L98H mutations. Microsatellite genotyping clearly differentiated the strains with the TR34/L98H mutation in the *cyp51A* gene from the strains with no mutation in this gene.

Conclusions: The rate of observed azole resistance of *A. fumigatus* isolates was low in this study, but the fact that more than half of the examined strains had the wild-type *cyp51A* gene supports the idea that other mechanisms of resistance are gradually increasing.

Introduction

Aspergillus fumigatus strains cause a wide spectrum of diseases and invasive aspergillosis is the most severe manifestation.[1] Triazoles are the active agents against this mould that can be used for evidence-based treatment as well as the prevention of *Aspergillus* infections.[2] However, the occurrence of triazole-resistant *A. fumigatus* isolates has been reported globally in

both environmental and clinical settings.[3] The most commonly identified reason for azole resistance in both clinical and environmental isolates is the occurrence of point mutations in the lanosterol-14α-demethylase gene (*cyp51A*) in combination with tandem repeats in the promoter region of this gene.[4]

The prevalence of azole resistance in *A. fumigatus* isolates in Turkey is not well known and the aim of this multicentre prospective study was to investigate the prevalence of azole resistance in clinical and environmental *A. fumigatus* isolates from different regions in Turkey.

Methods

Twenty-one centres from various regions in Turkey participated in this study from 1 May 2018 to 1 October 2019. A participant from each centre was asked to collect environmental and clinical *A. fumigatus* species complex (SC) isolates and send them to one of the two coordinating centres (Hacettepe University Medical School or Bursa Uludağ University Medical Centre). A thermotolerance test for phenotypic identification of *A. fumigatus* sensu stricto was performed at the coordinating centres.[2]

Ethics approval

The study protocol was approved by the Bursa Uludağ University Clinical Research and Ethics Committee (Date: 24 April 2018; Decision No.: 2018-8/7).

Environmental isolates

Each centre's participant collected environmental samples from the flower beds surrounding that individual hospital and from the agricultural areas. One to three samples were taken per flower bed/field/garden. The samples consisted mainly of soil. Two to five grams of each sample was placed in a 50 mL conical vial and 8 mL of 0.2 M NaCl solution with 1% Tween 20 was poured in. The sample was thoroughly dissolved and homogenized through vortexing and the resulting suspension was kept at 45–50°C for 2–3 h. Afterwards, 100 μL of the supernatant was inoculated on Sabouraud dextrose agar medium with chloramphenicol and gentamicin (Oxoid, Istanbul, Turkey).[5] The plates were incubated at 37°C and were pre-evaluated in terms of the growth of possible *A. fumigatus* SC isolates.

Clinical isolates

All *A. fumigatus* SC strains that were isolated from clinical samples were collected during the study period. A questionnaire was completed for every collected isolate that included questions about the specimen types (tissue biopsies and respiratory samples) from which the *A. fumigatus* SC strains were isolated. Since susceptible and resistant strains can be isolated from the very same sample, at least five colonies were asked for to be sent separately from samples with growth of multiple colonies.

Screening procedure for the detection of azole resistance

Azole resistance was screened for by subculturing each isolate on 4-well plates, each containing RPMI 1640/2% glucose agar supplemented with 4 mg/L itraconazole, 2 mg/L voriconazole, 0.5 mg/L posaconazole or no antifungal. Plates were prepared in-house according to EUCAST E.DEF 10.1 recommendations.[6] Any growth on one or more azole-containing agar wells was noted and confirmed using the reference antifungal microdilution method.

Antifungal susceptibility tests

Antifungal susceptibility tests were performed for *A. fumigatus* strains using the EUCAST E.DEF 9.3 reference microdilution method[7] and interpreted using the revised EUCAST clinical breakpoints (Version 2.0, valid from 24 September 2020).

Sequencing

Isolates with a phenotypic resistance pattern were sequenced for the *cyp51A* gene. DNA extraction was performed using a commercial kit (GeneMATRIX Plant & Fungi; EURx Ltd, Gdansk, Poland).[5,8] Primers for PCR and cycling conditions were as previously described.[9] The PCR products were purified using the Omega E.Z.N.A.® Cycle Pure (CP) Kit (Omega Bio-Tek, Norcross, GA, USA) and sequenced using a Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter Inc., Brea, CA, USA). The sequences obtained were compared with the sequence from an azole-susceptible strain (GenBank accession no. AF338659) and mismatches were identified using ClustalW analysis.[10]

Genotyping

Microsatellite genotyping was used to determine the genetic relatedness of the resistant strains.[5,11] Fragments from six loci (3A, 3B, 3C, 4A, 4B and 4C), each consisting of three trinucleotide and three tetranucleotide repeats, were amplified using fluorescently labelled primers.[11] PCR products were then subjected to fragment analysis using an automatic sequence analyser (Beckman Coulter Inc.). The assignment of repeat numbers to each marker was determined using the software of the automatic sequencing device (CEQ software). Data were analysed using Bionumerics v8.0 (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram was generated using the similarity coefficient followed by the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis; additionally, similarity matrices were generated from the tandem repeat numbers and were used as the input for generating the neighbour-joining tree.[12]

Results

In this study, both clinical and environmental isolates from nine centres, only environmental isolates from nine centres and only clinical isolates from three centres were analysed.

Environmental isolates

Eighteen centres from which environmental samples were collected are shown in Figure 1. See Table S1 (available as Supplementary data at *JAC* Online). A total of 2288 environmental samples were screened for azole resistance; 20% of them had *A. fumigatus* growth and 1.3% of these growths exhibited an azole resistance pattern. Most of the environmental samples (85.3%) were taken from agricultural soil; the resistance rate (0.8%) of *A. fumigatus* isolates grown in these samples was found to be significantly lower than the resistance rate (4.6%) of isolates collected from hospital environments (χ^2 test; $P=0.03$).

Clinical isolates

Clinical isolates were collected from 12 centres; ultimately, a total of 392 clinical *A. fumigatus* isolates were screened for azole resistance. Azole resistance was observed in 3.3% of the isolates. See Table S2.

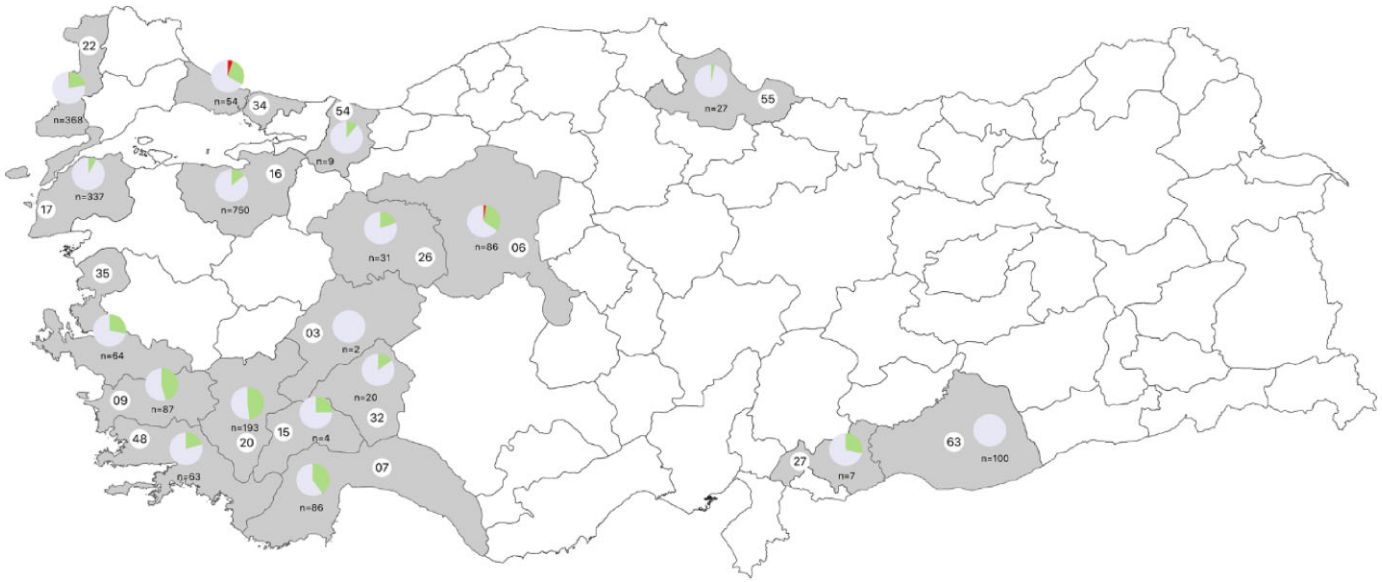

Figure 1. Distribution of participating centres. The grey circles show where the environmental samples were collected; the green section of each circle indicates the growth percentage of that sample, while the red indicates the resistance rate. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 1. MICs determined using the EUCAST microdilution method (E.DEF 9.3) and mutations in the *cyp51A* gene for the strains that were resistant by the agar screening method

				MIC (mg/L) - EUCAST E.DEF 9.3			
Isolate	City code	Sample type	Age (years)	itraconazole	voriconazole	posaconazole	*cyp51A* mutation
ÇK1	34	hospital environment	-	4	4	2	none
CRK1	34	hospital environment	-	4	4	2	none
CRK2	34	hospital environment	-	4	4	2	none
011KS06SN-B1	06	agricultural soil	-	>8	4	0.5	none
011KS06SN-B2	06	agricultural soil	-	>8	4	0.5	none
267MT22MR/B	22	agricultural soil	-	4	4	2	none
60986	16	sputum	45	>8	>8	2	TR34/L98H
61568	16	sputum	63	>8	>8	2	TR34/L98H
62946	16	bronchoalveolar lavage fluid	81	>8	>8	2	TR34/L98H
63413	16	sputum	74	>8	>8	2	TR34/L98H
63653	16	tracheal aspirate	67	>8	>8	2	TR34/L98H
64955	16	bronchoalveolar lavage fluid	80	>8	>8	2	TR34/L98H
2455	06	pleural fluid		>8	>8	2	TR34/L98H
457	06	pus		>8	>8	2	TR34/L98H
MY	27	bronchoalveolar lavage fluid		2	2	0.5	TR34/L98H
RT1	34	sputum	75	4	4	0.5	none
RT2	34	sputum	75	4	4	0.5	none
11b	07	sputum	18	4	4	2	none
13b	07	sputum	54	>8	4	2	none

Antifungal susceptibility and cyp51A gene mutation

Table 1 summarizes the results of the *in vitro* antifungal susceptibility tests with respect to the *cyp51A* gene mutation. Mutations in the *cyp51A* gene were detected in 9 (47.4%) of the 19 azole-resistant isolates, all of which were found to be TR34/L98H mutations.

Genotyping

Analysis of the microsatellite markers of the 19 azole-resistant isolates indicated the presence of two major independent genetic groups; the strains with TR34/L98H mutations in the *cyp51A* gene and those without any resistance-related mutations were clearly separated from each other. See Figure S1.

Discussion

This study was a multicentre study on the prevalence of azole-resistant *A. fumigatus* strains in environmental and clinical samples from Turkey. Varying resistance rates had previously been reported in single-centre studies from Turkey, necessitating multicentre data to clarify the range of resistance rates in the country.[9,13,14]

The epidemiological data gathered to date show that the frequency of environmental resistance acquisition ranges from 0.5% to 5% in sampled isolates.[15] The resistance rate of the environmental samples in this study was found to be on the lower end of this spectrum (1.3%), but the most striking finding of this study was that the resistance rate of the strains isolated from hospital environments was significantly higher than that of the strains isolated from agricultural soil. A recent study from southern England showed that urban flower beds (13.8%) have far more azole-resistant isolates than rural agricultural soils (1.1%).[16]

Various studies have shown that the azole resistance of *A. fumigatus* strains isolated from clinical samples is between 4% and 16%.[17] By contrast, the resistance rate in this study was found to be 3.3% and the strains were found in the three centres in which *A. fumigatus* isolation was high. While the azole resistance rate determined in this study of Turkish *A. fumigatus* isolates was low, it should be noted that more resistant strains might have been detected if a larger number of isolates had been studied.

Another important finding in this study was the absence of a resistance-related mutation in the *cyp51A* gene in approximately half of the observed resistant isolates. A recent study from the Netherlands emphasized that resistant strains without mutations in the *cyp51A* gene are increasing.[18] Another study used CRISPR-Cas9 technology to show that point mutations in an uncharacterized gene other than the *cyp51A* gene can also cause azole resistance in *A. fumigatus* isolates.[19] All of these findings indicate that mutations in the *cyp51A* gene are not the only mechanisms of azole resistance in *A. fumigatus* isolates and other mechanisms need to be investigated further. Overexpression of the sterol-demethylase gene and efflux pumps, *hapE* mutation and cholesterol import are the other mechanisms that might be involved in resistance to azoles in *A. fumigatus* strains.[3]

In conclusion, in this multicentre study conducted in Turkey, the level of azole resistance in *A. fumigatus* was found to be low in both environmental and clinical isolates. As a striking finding, more than half of the resistant isolates harboured no resistance-related mutations in the *cyp51A* gene, suggesting the possible existence and increasing significance of other mechanisms of azole resistance.

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Transparency declarations

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Supplementary data

Tables S1 and S2 and Figure S1 are available as Supplementary data at *JAC* Online.

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