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ORIGINAL ARTICLE

Five-year surveillance study of clinical and environmental Triazole-Resistant *Aspergillus fumigatus* isolates in Iran

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Abstract

Background: Invasive aspergillosis is one of the most common fungal infections and azole resistance in *Aspergillus fumigatus* (ARA*f*) is a growing medical concern in high-risk patients. To our knowledge, there is no comprehensive epidemiological surveillance study on the prevalence and incidence of ARA*f* isolates available in Iran.

Objectives: The study aimed to report a five-year survey of triazole phenotypes and genotype patterns concerning the resistance in clinical and environmental *A. fumigatus* in Iran.

Methods: During the study time frame (2016–2021), a total of 1208 clinical and environmental *Aspergillus* species were collected. Isolates were examined and characterised by in vitro antifungal susceptibility testing (CLSI M38 broth microdilution) and *cyp*51A sequencing.

Results: In total, 485 *Aspergillus* section *Fumigati* strains were recovered (clinical, $n = 23$; 4.74% and environment, $n = 462$; 95.26%). Of which *A. fumigatus* isolates were the most prevalent species ($n = 483$; 99.59%). Amphotericin B and the echinocandins demonstrated good in vitro activity against the majority of isolates in comparison to triazole. Overall, 16.15% ($n = 78$) of isolates were phenotypically resistant to at least one of the azoles. However, 9.73% of *A. fumigatus* isolates for voriconazole were classified as resistant, 89.03% were susceptible, and 1.24% were intermediate. While, for itraconazole and posaconazole, using the epidemiological cut-off value 16.15% and 6.83% of isolates were non-wild types, respectively. Remarkably, in 21.79% ($n = 17$)

phenotypically resistant isolates, no mutations were detected within the cyp51A gene.

Conclusion: Although the incidence of ARA*f* varies from country to country, in Iran the rate has ranged from 3.3% to 18%, significantly increasing from 2013 to 2021. Strikingly, a quarter of the phenotypically resistant isolates harboured no mutations in the *cyp*51A gene. It seems that other mechanisms of resistance are importantly increasing. To fill a gap in our understanding of the mechanism for azole resistance in the non-*cyp51A* strains, we highly recommend further and more extensive monitoring of the soil with or without exposure to fungicides in agricultural and hospital areas.

KEYWORDS

Aspergillus fumigatus, azole resistance, *cyp*51A gene, Iran, surveillance study

1 | INTRODUCTION

Globally *Aspergillus fumigatus*, a ubiquitous and opportunistic human fungal pathogen, is an important cause of invasive aspergillosis (IA) among immunocompromised individuals, especially in severely neutropenic patients, haematopoietic stem cells/solid organ transplant recipients and those with lung allografts.[1,2] Remarkably, breakthrough infections due to *Aspergillus* species in patients receiving antifungal prophylaxis or treatment are significant concerns.[3] Recently, IA in patients suffering from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and severe influenza infections have also been reported and are associated with high mortality despite the application of antifungal therapy.[4–6] Likewise, chronic pulmonary aspergillosis is also a growing public health concern.[7,8] Although triazole agents (e.g. voriconazole, itraconazole, posaconazole and isavuconazole) are the first-line options for the treatment of IA, reports of the emergence of triazole-resistant *A. fumigatus* (ARA*f*) isolates have raised major concerns in the management of this infection due to the increased number of treatment failures.[2,9,10] The first clinical cases of ARA*f* were reported from the U.S,[11] but the extent and burden of azole resistance vary from country to country (<5%–30%) with the highest rates being reported in parts of Europe.[10] The widespread use of azole fungicides in agricultural fields to optimise food production could favour ARA*f* selection with cross-resistance to medical azoles.[12,13] There is a potential link between residues of azole fungicides with similar chemical structures to triazole medicines in soil and the emergence of triazole-resistance.[14,15] In addition, the dispersal activity of ARA*f* spores leads to geographical transfer is an argument in support of the emergence of these cross-resistant isolates in different countries.[14,16–20] One major mechanism of azole resistance in *A. fumigatus* is mutations in the target enzyme sterol demethylase (*cyp51A*) gene. The enzyme 14- sterol demethylase is the main target for triazoles encoded by the *cyp51A* gene. Triazole antifungals interact with the active site of the *cyp*51A enzyme (sterol demethylase), preventing the conversion of lanosterol to ergosterol. This results in the accumulation of hazardous sterol intermediates and the depletion of ergosterol, resulting in the inhibition of fungal cell growth and death.[21] TR_{34}/L98H and TR_{46}/Y121F/T289A are the most frequently identified resistance mechanism found in environmental *A. fumigatus* strains and azole-naïve patients and may be resulted from the agricultural use of azole fungicides,[13,22] while several other hot spot single nucleotide polymorphisms (e.g. G54, G138, G434, M220, H147, Y121, G448 and P216) in the *cyp*51A gene have been identified in ARA*f* from the patients under long-term antifungal therapy.[23] Nevertheless, studies have reported that over 40% of ARA*f* may be related to non-*cyp*51A mutations that lead to azole resistance among *A. fumigatus* isolates.[24] One of the issues is the mutations in the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase-encoding gene (*hmg*1) associated with triazole-resistance *A. fumigatus* isolates.[25] However, overexpression of the *cyp51A* gene and efflux pump genes are also involved in resistance to triazole agents.[21]

Surveillance studies on the antifungal susceptibility of *Aspergillus* isolates are limited in Iran.[26] However, environmental ARA*f* with predominant TR_{34}/L98H mutations in the *cyp51A* gene followed by TR46/Y121F/T289A mutation has been importantly reported earlier.[18,27–30] Surveillance studies are beneficial to inform decisions concerning health services and research funding and antifungal stewardship policies and guidelines.[31] Therefore, understanding how resistance develops requires special considerations for the prophylaxis and treatment of patients with aspergillosis. In the current study, we aimed to report a five-year survey of triazole phenotypes and genotype patterns concerning the resistance in clinical and environmental *A. fumigatus* in Iran.

2 | MATERIALS AND METHODS

2.1 | Environmental and clinical sample collection

A total of 1153 *Aspergillus* species from soil samples (depth of 0–5 cm) at the different agricultural areas including citrus garden (orange, sour orange, mandarin, pomegranate, peach), watermelon, melon, sour cherry, corn, okra, vegetables (squash, tomato, eggplant,

cucumber, leek, parsley, pepper, bell pepper, sweet pepper, sesame, beans, green beans, pinto beans, bergamot, lettuce, radish, mint, spring onion, fenugreek, zucchini, broad bean, basil, beetroot, spinach and garden cress), rice farm and strawberry fields were collected during 5 years (2016–2021) and examined in terms of the growth of ARA*f* isolates. Briefly, 150-gram soil samples were suspended in 5 ml sterile normal saline solution with Tween 20 (Sigma-Aldrich) at a final concentration of 0.05%, vortexed thoroughly and allowed to settle down. Subsequently, 100 µl of the suspension was inoculated on three malt extract agar (MEA, Difco) plates each supplemented with one of the following triazoles: itraconazole at a final concentration of 4 mg/L, voriconazole at 1 mg/L and posaconazole at 0.5 mg/L. Additionally, a plate without an antifungal agent was inoculated as growth control. All plates were incubated at 45°C for up to 72 h to maximise the selective yield for azole-resistant *Aspergillus* species.[32] The mimicking *Aspergillus* species complexes that were able to grow at least on one azole-containing agar were sub-cultured and were identified by morphological characteristics (e.g. macroscopic and microscopic features) followed by molecular identification as previously described.[18] In addition, during this period 55 clinical *Aspergillus* isolates received for species identification or antifungal susceptibility testing by the Invasive Fungi Research Center (IFRC) at Sari, Iran was included as were those cultured from the lower respiratory tracts, upper respiratory tracts, sinus, nails, ears and biopsy samples. Isolates were cultured onto the Sabouraud dextrose agar plate (SDA) on the day of receipt prior to further testing.[33]

2.2 | Identification of *Aspergillus fumigatus* species complex

Aspergillus isolates were identified by conventional (e.g. macroscopic, microscopic and temperature studies) and molecular analysis as previously described.[33] Briefly, all isolates were sub-cultured onto MEA plates at 27°C. A sterile blade was used to scrape the portions of the mycelia from individual isolates and genomic DNA was extracted using an UltraClean Microbial DNA isolation kit (Mo bio) according to the manufacturer's instructions. DNA extracts were stored at −20°C prior to use. Sequencing of the partial b-tubulin gene was performed using the primer pairs TUB2a (5- GACCCAGCAGATGTT-3) and TUB2b (5-GTTGTTGGGAATCCACTC-3). BLASTn searches of these sequences were then performed in GenBank, and results were considered significant with an E-value of >98% identity and with at least 90% query coverage.[34]

2.3 | In vitro Antifungal Susceptibility Testing

Susceptibility testing for amphotericin B (Bristol-Myers-Squib), itraconazole (Janssen, Beerse), voriconazole (Pfizer), posaconazole (Merck Sharp & Dohme BV), anidulafungin (Pfizer) and caspofungin (Merck Sharp & Dohme BV) was performed by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) M38-A2 standard.[35,36] Briefly, stock solutions of each antifungal drug were prepared by dissolving the powders in dimethyl sulfoxide (DMSO), followed by dilutions in RPMI medium (Gibco) with the final concentrations ranges of 0.03–16 mg/L for amphotericin B and azoles and 0.008–8 mg/L for echinocandins. Inoculum suspensions were prepared from non-germinated colonies (3-day cultures) in a sterile normal saline solution containing Tween 40 (0.05%) by slightly scraping the surface of mature colonies. The supernatants were adjusted spectrophotometrically at a wavelength of 530 nm to 80–82% transmittance to twice the final inoculum ($0.5–3.1\times10^6$ CFU/ml) and inocula were diluted 1:50 in RPMI 1640 medium, and the final inoculum in assay wells was between $0.5–3.1\times10^4$ CFU/ml. MICs were read visually as the lowest concentration, which provided complete inhibition of growth after 48 h of incubation at 35°C for amphotericin B and azoles. For echinocandins, minimum effective concentrations (MECs) were determined microscopically as the lowest concentration of drug promoting the growth of small, round, compact hyphae relative to the appearance of the filamentous forms seen in the growth control. *Candida krusei* (ATCC 6258) and *Hamigera insecticola* (ATCC 3630, *formerly Paecilomyces variotii*) were included as quality controls and was included in each day of testing.

2.4 | cyp1A gene Sequencing and Microsatellite Typing

According to the CLSI clinical breakpoints for voriconazole, *A. fumigatus* isolates were classified as resistant (MIC ≥2 mg/L), susceptible (MIC≤0.5 mg/L) and intermediate (MIC1 mg/L).[35–37] In contrast due to the lack of clinical breakpoint for itraconazole and posaconazole, isolates were designated non-wild type to itraconazole (1 mg/L) and posaconazole (≤0.5 mg/L) by published epidemiological cut-off values (ECVs). Genomic DNA was extracted and to explore the underlying mutations of isolated non-WT *A. fumigatus*, the *cyp51A* and promoter regions were amplified and sequenced as previously described.[33,37] Sequences were compared to the *cyp*51A reference sequence from *A. fumigatus* strain ATCC 36607 (GenBank accession no. AF222068) using the MEGA software version 11. Microsatellite typing has been previously performed to investigate the genetic relatedness of *A. fumigatus* strains due to its reproducibility and high resolution (data not shown). STR typing was conducted with a panel of nine short tandem repeats (STRs) to compare the genetic relationship between Iranian ARA*f* isolates and plenty of non-wild-type (ARA*f*) strains obtained from different countries using Bio-Numerics software v7.6.1 (Applied Maths, Saint-Martens-Latem) as previously described.[38]

2.5 | Statistical analysis

The MIC/MEC values that inhibited 50% and 90% of isolates (MIC_{50} and MIC_{90}, respectively), MIC/MEC ranges and geometric mean (GM) MIC/MEC values were calculated. The differences in GM MIC

values were assessed for significance by a paired t-test. *p*-values of <.05 were considered significant.

3 | RESULTS

During 5 years (2016–2021), a total of 1208 clinical and environmental *Aspergillus* isolates were collected. Of which 485 *Aspergillus* section *Fumigati* strains were recovered (clinical, *n* = 23; 4.74% and environment, *n* = 462; 95.26%). The majority of clinical isolates recovered from the lower and upper respiratory tracts followed by other sites (e.g. cutaneous, orbital, ear and central nervous system) in which the isolates were cultured. *A. fumigatus* sensu stricto isolates were the most prevalent species (*n* = 483), accounting for over 99.59% of the isolates, although *Aspergillus thermomutatus* (0.21%) and *Aspergillus udagawae* (0.21%) isolates were the next most common species in this section recovered from clinical samples (Figure 1A). Table 1 summarises the MIC results for each drug against all *A. fumigatus* sensu stricto isolates (*n* = 483). The MICs of tested triazole ranged from ≤0.03 to >16 mg/L for itraconazole and voriconazole and ≤0.008–8 mg/L for posaconazole, compared to 0.016–4 mg/L for amphotericin B, ≤0.008–0.5 μg/ml for anidulafungin and ≤0.008–1 mg/L for caspofungin. The MIC values revealed that amphotericin B had potent in vitro activity (GM MIC of 0.5195 mg/L) against the majority of isolates in comparison to triazole. Of which the lowest GM MICs were observed with posaconazole (0.1321 mg/L), voriconazole (0.3343 mg/L) and itraconazole (0.4399 mg/L). Although clinical *A. fumigatus* isolates were more azole susceptible than environmental isolates, a statistically significant difference was detected (*p* ≥ .05). In addition, echinocandins demonstrated good in vitro activity against *A. fumigatus* tested isolates and they are superior to those of amphotericin B and other triazoles. In terms of GM MIC, anidulafungin demonstrated the lowest GM MEC value (0.0369 mg/L) followed by caspofungin (0.0812 mg/L). Remarkably, 78 out of 483 *A. fumigatus* isolates had a high MIC value of at least one azole by in vitro antifungal susceptibility testing. Based on the CLSI clinical breakpoints for voriconazole, 9.73% of *A. fumigatus* isolates were classified as resistant, 89.03% were susceptible and 1.24% were intermediate during this study period. While, for itraconazole and posaconazole, using the epidemiological cut-off value 83.85% and 93.17% of isolates were wild types, respectively, and for non-wild types, the percentages were 16.15% and 6.83%, respectively. Interestingly, the rate of observed azole resistance of clinical *A. fumigatus* isolates was relatively low in this study, in contrast, the percentage rate of environmental *A. fumigatus* isolates that were the resistant or non-wild type has gradually increased and was relatively unstable during the last 5 years (2016–2021) (Figure 1B). The major causes of triazole-resistance in *A. fumigatus* are the mutation in the *cyp51A* gene and its promoter. The results of *cyp*51A gene sequencing of *A. fumigatus* isolates that were phenotypically resistant to at least one of the azoles are shown in Table 2. Interestingly, 21.79% of isolates were without nonsynonymous *cyp*51A substitutions, but 78.20% of isolates had different nonsynonymous *cyp*51A substitutions. Of which, TR_{34}/L98H was the most frequently observed resistance mechanism and was present in 54 (69.23%) of the ARA*f* isolates, whereas TR_{46}/Y121F/T289A was present in 5 (6.41%) isolates and M172V, E427K was detected in 2 (2.56%) isolates. Remarkably, numerous point *cyp*51A gene polymorphisms were found in susceptible and WT *A. fumigatus* isolates that harboured the previously described amino acid substitutions. The other unknown point mutations and multi-azole resistance mutations were not detected in resistant isolates. Moreover, 21.79% of isolates that were phenotypically resistant to at least one azole was found to have wild-type CYP51A sequences, which is similar to our previous experience and those of others. Table 2 summarised the MICs and mutations of non-wild-type Environmental *A. fumigatus* isolates for 5 years period.

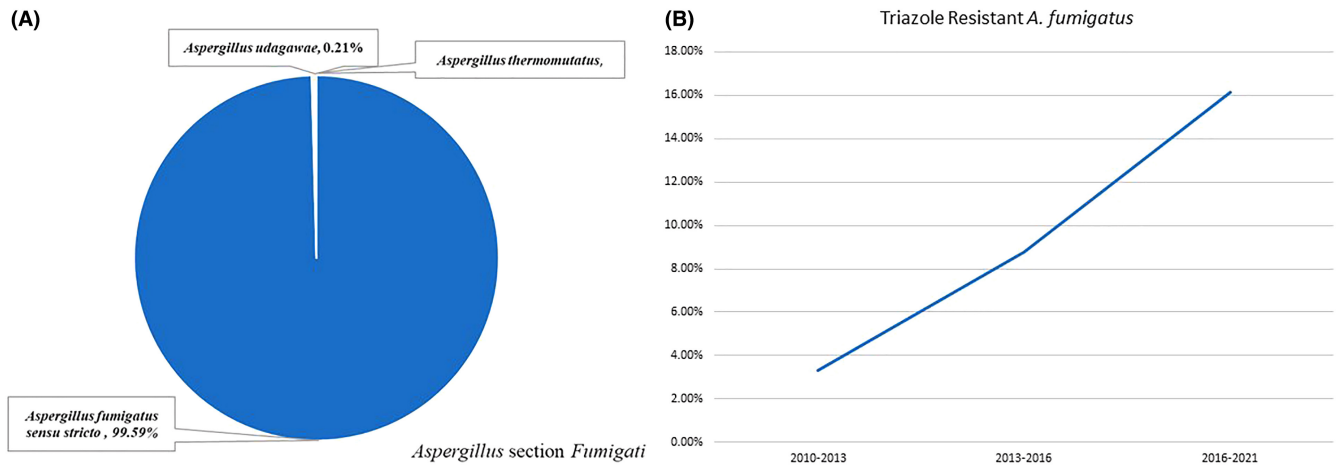

FIGURE 1 (A) Species distribution of 485 *Aspergillus* section *Fumigati* isolates identified to the species level by DNA sequence analysis (2016–2021); (B) Frequency of azole resistance *Aspergillus fumigatus* based on the CLSI clinical breakpoint and epidemiologic cut-off values from 2013 to 2021

TABLE 1 Minimum inhibitory concentration (MIC/MEC) values of amphotericin B, itraconazole, voriconazole posaconazole, anidulafungin and caspofungin against 483 *Aspergillus fumigatus*

Antifungal (no. isolates tested)	MIC/MEC range	MIC/MEC_{50}	MIC/MEC_{90}	Modal MIC/MEC	GM MIC/MEC
All *A. fumigatus*, sensu stricto ($n = 483$)					
Amphotericin B	0.016–4	0.5	1	0.5	0.5195
Itraconazole	≤0.03–>16	0.5	2	0.5	0.4399
Voriconazole	≤0.03–>16	0.25	1	0.25	0.3343
Posaconazole	≤0.008–8	0.25	0.5	0.25	0.1321
Anidulafungin	≤0.008–0.5	0.03	0.125	0.015	0.0369
Caspofungin	≤0.008–1	0.125	0.25	0.125	0.0812
Environmental *A. fumigatus* isolates ($n = 460$)					
Amphotericin B	0.016–4	0.5	1	0.5	0.5461
Itraconazole	≤0.03–>16	0.5	2	0.5	0.4639
Voriconazole	≤0.03–>16	0.25	2	0.25	0.3508
Posaconazole	≤0.008–8	0.25	0.5	0.25	0.14227
Anidulafungin	≤0.008–0.5	0.03	0.125	0.015	0.03853
Caspofungin	≤0.008–1	0.125	0.25	0.125	0.08582
Clinical *A. fumigatus* isolates ($n = 23$)					
Amphotericin B	0.016–1	0.25	0.5	0.25	0.1912
Itraconazole	≤0.03–1	0.125	1	0.125	0.1521
Voriconazole	≤0.03–0.25	0.125	0.25	0.125	0.1281
Posaconazole	≤0.008–0.125	0.03	0.25	0.03	0.0300
Anidulafungin	≤0.008–0.125	0.008	0.125	0.008	0.0163
Caspofungin	≤0.008–0.125	0.03	0.125	0.03	0.0274

Note: MIC/MEC_{50} and MIC/MEC_{90}–MIC concentrations at which 50% and 90% of the isolates were inhibited.

Abbreviations: GM MIC/MEC, geometric mean MIC.

TABLE 2 The MICs range and mutations of non-wild-type environmental *A. fumigatus* isolates from Iran, for 5 years period

MIC Range (mg/L)					
Nr of isolates (78)	Itraconazole	Voriconazole	Posaconazole	*cyp51A* mutations	Source of samples
54 (69.23%)	2–>16	2–>16	0.5–8	TR34/L98H	Environmental
5 (6.41%)	>16	>16	0.5–8	TR46/Y121F/T289A	Environmental
2 (2.56%)	8–>16	>16	0.5	M172V, E427K	Environmental
17 (21.79%)	2–>16	2–>16	0.5–8	No mutation	Environmental

4 | DISCUSSION

Invasive aspergillosis is one of the most severe life-threatening fungal infections associated with high morbidity, mortality and prolonged hospital stay among immunocompromised patients.[2,39] Globally the exact estimated incidence of IA has not been clearly understood.[3,40] Although *Aspergillus* section *Flavi* isolates are the most common species isolated from superficial infections, *A. fumigatus* is typically the most common agent of IA.[28,41] Nevertheless, some studies have reported an increased prevalence of cryptic and azole-resistant *Aspergillus* species, which have been associated with the increased numbers of treatment failures.[33,42,43] Therefore, ESCMID-ECMM-ERS *Aspergillus* guidelines importantly recommend the detection and monitoring of antifungal resistance in *Aspergillus* species recovered from patients requiring antifungal treatment.[44] In addition, identification at the species level for clinically relevant isolates is strongly recommended, because misidentification and delays in the diagnosis, and inappropriate management of IA may have negative impacts on survival. In the current study, *A. fumigatus* (99.59%) was the predominant species, followed by cryptic species (0.21%), which are almost consistent with other findings.[33] Likewise, in previous retrospective studies in Portugal, the frequency of cryptic species within the *Fumigati* section was 5.3% (18/337), as follows *A. felis*, *A. lentulus*, *A. udagawae*, *A. hiratsukae* and *A. oerlinghauensis*.[45] Recently, in a multicentre prospective study, 369 cryptic *Aspergillus* isolates were identified, of which less than 15 cryptic species have been linked to IA, and <1% of cases were involved by the *Fumigati* section.[46] Moreover, in a study in Mexico, cryptic species of *Aspergillus* were 30% of the isolates, of which three cryptic species of section *Fumigati* were identified (e.g. *A. fumisynnematus*, *A. hiratsukae* and

A. lentulus).[47] Ironically, there have also been numerous case reports of IA due to azole-resistant *Aspergillus* species in critically ill patients with SARS-CoV-2 infection (COVID-19-associated pulmonary aspergillosis or CAPA).[48,49] Therefore, azole-resistant infections have remarkably increased and are an emerging and public health concern.[18,27–30,41] However, reports of clinical and environmental isolates harbouring different resistance mechanisms are currently limited, and studies involving large numbers of isolates are lacking.[24,25]

Studies showed that triazole-resistant *Aspergillus* strains compared to susceptible ones have elevated mortality rates (>20%), despite the application of appropriate antifungal therapy.[2,50,51] Recently, Cao et al., showed that the prevalence rates of *A. fumigatus* were 45.8%, 64.7% and 62.5% in environmental samples collected in 2014, 2016 and 2018, respectively, which is consistent with their previous reports showing a range of 35% to 77.8%.[52–54] Interestingly, azole-resistant *A. fumigatus* in agricultural areas with a previous history of azole fungicide use was detected in 6.9% of samples in Mexico, 8.3% in Paraguay, 9.8% in Peru and 2.2% in Nigeria.[55] This was consistent with our previous reports showing a range of 3.3% to 8.8% in environmental samples collected in 2013 and 2016, respectively.[18,28] In addition, these data concur with previous soil studies in Germany, Denmark and the United Kingdom that reported an overall low incidence of ARA*f* in agricultural samples before and after exposures to azole fungicide.[56] Recently Ahangarkani et al., investigated three ARA*f* isolates (voriconazole MICs ≥16 mg/L) harboured the TR_{46}/Y121F/T289A mutation in the *cyp*51A gene among compost soil samples in Iran.[27,29] Our results revealed that the prevalence of ARA*f* in soils around agricultural and hospital areas dramatically increased during the 5 years (Figure 1B). Similar to what others have found, mutations within the *cyp*51A gene associated with azole resistance were found in 16.15% (n = 78) of *A. fumigatus* sensu stricto isolates that were phenotypically resistant, and sequenced isolates showed wide variability in point mutations. Interestingly, the rate of azole-resistant in *A. fumigatus* isolates in Iran ranged from 3.3% to 18%, significantly increasing from 2013 to 2021. During these 5 years, the percentage of isolates that were resistant to voriconazole was 9.73%, but different rates were observed for itraconazole (16.15%) and posaconazole (6.83%) for non-wild-type isolates. Similar to our previous experiences,[18,28,29] the majority of isolates 54 (69.23%) harboured TR_{34}/L98H in the *cyp*51A gene, whereas TR_{46}/Y121F/T289A was only in 5 (6.41%) isolates. However, two isolates (2.56%) had M172V and E427K polymorphisms.

Although there was numerous SNPs in the *cyp*51A enzyme, many of them have previously described as engendering an azole-resistant phenotype. Surprisingly, 21.79% of isolates were phenotypically resistant to at least one azole without synonymous *cyp*51A substitutions, which is similar to our previous experience and those of others.[33,57] We found that the prevalence of environmental ARA*f* in Iran with different *cyp*51A mutations has dramatically increased and might be a potential hotspot for the emergence of *A. fumigatus* azole resistance. Although in the current study, the rate of clinical azole resistance was relatively low, it should be noted that more resistant strains might have been detected if a larger number of isolates had been studied. Recently, a multicentre study by Ener et al., was conducted and revealed that ARA*f* isolates were found in 1.3% of environmental and 3.3% of clinical isolates. TR34/L98H mutations in the *cyp*51A gene were detected in 47.4% of ARA*f* isolates. However, in 52.6% of phenotypically resistant isolates, no mutations were detected within the *cyp*51A gene.[51] Moreover, the prevalence of ARA*f* in a Danish national surveillance study was 6.1% (66/1083) at patient level and TR_{34}/L98H was the most common alteration, but non-*cyp*51A-mediated resistance accounted for 19.7% (13/66).[58] Strikingly, the majority of studies have shown that over half of the phenotypically resistant isolates harboured no mutations in the *cyp*51A gene.[59] Although these mechanisms of resistance have been described from wide geographical areas among both clinical and environmental isolates, the frequency of these resistance alleles varies considerably from country to country (<5%–30%) and patient's underlying diseases.[50,60,61] Only limited data are available regarding the prevalence of ARA*f* isolates in IA subjects and their relationship to previous azole exposure. Because of this, there is increasing interest in understanding other mechanisms of azole resistance in *Aspergillus* species, and this may have implications for the diagnosis and treatment of IA. To fill a gap in our understanding of the mechanism for azole resistance in the non-*cyp51A* strains, we highly recommend further and more extensive monitoring of the soil exposure to fungicides in agricultural and hospital areas, to determine trends in the rate of ARA*f* and to investigate the other mechanisms of resistance such as overexpression of efflux pumps, gain-of-function mutations in transcription factors, mutations in regulatory and sterol biosynthesis elements, and mutations within the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase-encoding gene (HMG1), which encodes HMG-coenzyme A (CoA) reductase in *A. fumigatus*. In conclusion, the findings of this study suggest a high possibility for the transmission of ARA*f* isolates through environmental to the clinical setting, which could pose a great challenge for containing the problem of azole resistance. Therefore, the geographical variation in ARA*f* isolates suggests a need to include local drug resistance rates to devise public health policies and local guidelines for treatment and management. Furthermore, if the resistant isolates are prevalent, routine antifungal susceptibility testing should be performed for all clinically relevant *A. fumigatus* isolates to guide antifungal therapy against highly aggressive infections caused by these species and for epidemiological purposes. Although the current study represents a five-year surveillance study of clinical and environmental ARA*f* Isolates in Iran, there are some limitations that must be considered. No clinical outcome, no antifungal breakpoints data for itraconazole, isavuconazole and posaconazole were available, in addition, the majority of tested isolates were received from other hospitals or reference laboratories, we were unable to determine if there may be geographic areas or climates.

AUTHOR CONTRIBUTIONS

HB involved in conception of the research idea, study design, data collection and analysis and interpretation and drafting of the manuscript;

SHK, HB and SK involved in preparation of the first draft of the manuscript; SHK, MA, IH involved in study design and analysis, interpret the data and reviewed the manuscript; SHK, HZ, SGH, SK, KA, RV and FA involved in data collection, part of laboratory work, data analysis and reviewed the manuscript. All authors participated in the study implementation, contributed to editing and reviewed the manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Fuhren J, Voskuil WS, Boel CH, et al. High prevalence of azole resistance in *Aspergillus fumigatus* isolates from high-risk patients. *J Antimicrob Chemother*. 2015;70(10):2894-2898.
2. Patterson TF, Thompson GR 3rd, Denning DW, et al. Practice guidelines for the diagnosis and Management of aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2016;63(4):e1-e60.
3. Vallabhaneni S, Benedict K, Derado G, Mody RK. Trends in hospitalizations related to invasive aspergillosis and mucormycosis in the United States, 2000–2013. *Open Forum Infect Dis*. 2017;4(1):ofw268.
4. Koehler P, Bassetti M, Chakrabarti A, et al. Defining and managing COVID-19-associated pulmonary aspergillosis: the 2020 ECMM/ISHAM consensus criteria for research and clinical guidance. *Lancet Infect Dis*. 2021;21(6):e149-e162.
5. Salmanton-García J, Sprute R, Stemler J, et al. COVID-19-associated pulmonary aspergillosis, march-august 2020. *Emerg Infect Dis*. 2021;27(4):1077-1086.
6. Crum-Cianflone NF. Invasive aspergillosis associated with severe influenza infections. *Open Forum Infect Dis*. 2016;3(3):ofw171.
7. Prattes J, Flick H, Prüller F, et al. Novel tests for diagnosis of invasive aspergillosis in patients with underlying respiratory diseases. *Am J Respir Crit Care Med*. 2014;190(8):922-929.
8. Denning DW, Cadranel J, Beigelman-Aubry C, et al. Chronic pulmonary aspergillosis: rationale and clinical guidelines for diagnosis and management. *Eur Respir J*. 2016;47(1):45-68.
9. Wiederhold NP, Verweij PE. *Aspergillus fumigatus* and pan-azole resistance: who should be concerned? *Curr Opin Infect Dis*. 2020;33(4):290-297.
10. Lestrade PPA, Meis JF, Melchers WJG, Verweij PE. Triazole resistance in *Aspergillus fumigatus*: recent insights and challenges for patient management. *Clin Microbiol Infect*. 2019;25(7):799-806.
11. Denning DW, Venkateswarlu K, Oakley KL, et al. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother*. 1997;41(6):1364-1368.
12. Alvarez-Moreno C, Lavergne RA, Hagen F, Morio F, Meis JF, Le Pape P. Azole-resistant *Aspergillus fumigatus* harboring TR(34)/L98H, TR(46)/Y121F/T289A and TR(53) mutations related to flower fields in Colombia. *Sci Rep*. 2017;7:45631.
13. Alvarez-Moreno C, Lavergne RA, Hagen F, Morio F, Meis JF, Le Pape P. Fungicide-driven alterations in azole-resistant *Aspergillus fumigatus* are related to vegetable crops in Colombia, South America. *Mycologia*. 2019;111(2):217-224.
14. Hollomon D. Does agricultural use of azole fungicides contribute to resistance in the human pathogen *Aspergillus fumigatus*? *Pest Manag Sci*. 2017;73(10):1987-1993.
15. Berger S, El Chazli Y, Babu AF, Coste AT. Azole resistance in *Aspergillus fumigatus*: a consequence of antifungal use in agriculture? *Front Microbiol*. 2017;8:1024.
16. Prigitano A, Esposto MC, Grancini A, et al. Azole resistance in aspergillus isolates by different types of patients and correlation with environment – an Italian prospective multicentre study (ARiA study). *Mycoses*. 2021;64(5):528-536.
17. Nakano Y, Tashiro M, Urano R, et al. Characteristics of azole-resistant *Aspergillus fumigatus* attached to agricultural products imported to Japan. *J Infect Chemother*. 2020;26(10):1021-1025.
18. Badali H, Vaezi A, Haghani I, et al. Environmental study of azole-resistant *Aspergillus fumigatus* with TR34/L98H mutations in the cyp51A gene in Iran. *Mycoses*. 2013;56(6):659-663.
19. Chang H, Ashu E, Sharma C, Kathuria S, Chowdhary A, Xu J. Diversity and origins of Indian multi-triazole resistant strains of *Aspergillus fumigatus*. *Mycoses*. 2016;59(7):450-466.
20. Wu CJ, Wang HC, Lee JC, et al. Azole-resistant *Aspergillus fumigatus* isolates carrying TR_{34}/L98H mutations in Taiwan. *Mycoses*. 2015;58(9):544-549.
21. Mellado E, Garcia-Effron G, Buitrago MJ, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. Targeted gene disruption of the 14-alpha sterol demethylase (cyp51A) in *Aspergillus fumigatus* and its role in azole drug susceptibility. *Antimicrob Agents Chemother*. 2005;49(6):2536-2538.
22. Vaezi A, Fakhim H, Javidnia J, et al. Pesticide behavior in paddy fields and development of azole-resistant *Aspergillus fumigatus*: should we be concerned? *J Mycol Med*. 2018;28(1):59-64.
23. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJ. Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. *Antimicrob Agents Chemother*. 2010;54(6):2425-2430.
24. Hagiwara D, Arai T, Takahashi H, Kusuya Y, Watanabe A, Kamei K. Non-cyp51A azole-resistant *aspergillus fumigatus* isolates with mutation in HMG-CoA reductase. *Emerg Infect Dis*. 2018;24(10):1889-1897.
25. Rybak JM, Ge W, Wiederhold NP, et al. Mutations in hmg1, challenging the paradigm of clinical triazole resistance in *Aspergillus fumigatus*. *mBio*. 2019;10(2):e00437-e00419.
26. Taheri Rizi Z, Abastabar M, Fakhim H, et al. Antifungal activity of a novel triazole, efinaconazole and nine comparators against 354 molecularly identified aspergillus isolates. *Mycopathologia*. 2020;185(2):357-365.

27. Ahangarkani F, Badali H, Abbasi K, et al. Clonal expansion of environmental triazole resistant *aspergillus fumigatus* in Iran. *J Fungi (Basel)*. 2020;6(4):199.
28. Nabili M, Shokohi T, Moazeni M, et al. High prevalence of clinical and environmental triazole-resistant *aspergillus fumigatus* in Iran: is it a challenging issue? *J Med Microbiol*. 2016;65(6):468-475.
29. Ahangarkani F, Puts Y, Nabili M, et al. First azole-resistant *aspergillus fumigatus* isolates with the environmental TR(46) /Y121F/T289A mutation in Iran. *Mycoses*. 2020;63(5):430-436.
30. Seyedmousavi S, Hashemi SJ, Zibafar E, et al. Azole-resistant *Aspergillus fumigatus*, Iran. *Emerg Infect Dis*. 2013;19(5):832-834.
31. Verweij PE, Lestrade PP, Melchers WJ, Meis JF. Azole resistance surveillance in *Aspergillus fumigatus*: beneficial or biased? *J Antimicrob Chemother*. 2016;71(8):2079-2082.
32. Serrano-Lobo J, Gómez A, Reigadas E, Muñoz P, Escribano P, Guinea J. Screening of azole resistance in *Aspergillus fumigatus* using the EUCAST e.def 10.2 azole-containing agar method: a single study suggests that filtration of conidial suspensions prior to inoculum preparation may not be needed. *Mycoses*. 2022;65:1045-1049.
33. Badali H, Cañete-Gibas C, McCarthy D, et al. Species distribution and antifungal susceptibilities of aspergillus section Fumigati isolates in clinical samples from the United States. *J Clin Microbiol*. 2022;60(5):e0028022.
34. Peterson SW. Phylogenetic analysis of aspergillus species using DNA sequences from four loci. *Mycologia*. 2008;100(2):205-226.
35. Clinical and Laboratory Standards Institute, ed. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi*. 3rd ed. Clinical and Laboratory Standards Institute; 2017.
36. Clinical and Laboratory Standards Institute. *Performance Standards for Antifungal Susceptibility Testing of Filamentous Fungi*. 2nd ed.. CLSI supplement M61 Clinical and Laboratory Standards Institute; 2020.
37. Espinel-Ingroff A, Turnidge J, Alastruey-Izquierdo A, et al. Posaconazole MIC distributions for *Aspergillus fumigatus* species complex by four Methods: impact of cyp51A mutations on estimation of epidemiological cutoff values. *Antimicrob Agents Chemother*. 2018;62(4):e01916-e01917.
38. de Valk HA, Meis JF, Curfs IM, Muehlethaler K, Mouton JW, Klaassen CH. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J Clin Microbiol*. 2005;43(8):4112-4120.
39. Cadena J, Thompson GR 3rd, Patterson TF. Aspergillosis: epidemiology, diagnosis, and treatment. *Infect Dis Clin North Am*. 2021;35(2):415-434.
40. Pappas PG, Alexander BD, Andes DR, et al. Invasive fungal infections among organ transplant recipients: results of the transplant-associated infection surveillance network (TRANSNET). *Clin Infect Dis*. 2010;50(8):1101-1111.
41. Badiee P, Boekhout T, Zarei Mahmoudabadi A, et al. Multicenter study of susceptibility of aspergillus species isolated from Iranian university hospitals to seven antifungal agents. *Microbiol Spectr*. 2022;10(3):e0253921.
42. Guinea J, Peláez T, Alcalá L, Bouza E. Outdoor environmental levels of *Aspergillus* spp. conidia over a wide geographical area. *Med Mycol*. 2006;44(4):349-356.
43. Vaezi A, Fakhim H, Arastehfar A, et al. In vitro antifungal activity of amphotericin B and 11 comparators against aspergillus terreus species complex. *Mycoses*. 2018;61(2):134-142.
44. Ullmann AJ, Aguado JM, Arikan-Akdagli S, et al. Diagnosis and management of aspergillus diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline. *Clin Microbiol Infect*. 2018;24(Suppl 1):e1-e38.
45. Sabino R, Gonçalves P, Martins Melo A, et al. Trends on aspergillus epidemiology-perspectives from a National Reference Laboratory Surveillance Program. *J Fungi (Basel)*. 2021;7(1):28.
46. Imbert S, Cassaing S, Bonnal C, et al. Invasive aspergillosis due to aspergillus cryptic species: a prospective multicenter study. *Mycoses*. 2021;64:1346-1353.
47. Treviño-Rangel RJ, Villanueva-Lozano H, Bonifaz A, et al. Species distribution and antifungal susceptibility patterns of aspergillus isolates from clinical specimens and soil samples in Mexico. *Med Mycol*. 2021;59:1006-1014.
48. Meijer EFJ, Dofferhoff ASM, Hoiting O, Buil JB, Meis JF. Azole-resistant COVID-19-associated pulmonary aspergillosis in an immunocompetent host: a case report. *J Fungi (Basel)*. 2020;6(2):79.
49. Nasri E, Shoaei P, Vakili B, et al. Fatal invasive pulmonary aspergillosis in COVID-19 patient with acute myeloid leukemia in Iran. *Mycopathologia*. 2020;185(6):1077-1084.
50. Brito Devoto T, Hermida-Alva K, Posse G, Finquelievich JL, García-Effrón G, Cuestas ML. High prevalence of triazole-resistant *Aspergillus fumigatus* sensu stricto in an Argentinean cohort of patients with cystic fibrosis. *Mycoses*. 2020;63(9):937-941.
51. Ener B, Ergin Ç, Gülmez D, et al. Frequency of azole resistance in clinical and environmental strains of *Aspergillus fumigatus* in Turkey: a multicentre study. *J Antimicrob Chemother*. 2022;77(7):1894-1898.
52. Cao D, Wu R, Dong S, et al. Five-year survey (2014 to 2018) of azole resistance in environmental *Aspergillus fumigatus* isolates from China. *Antimicrob Agents Chemother*. 2020;64(10):e00904-e00920.
53. Mortensen KL, Mellado E, Lass-Flörl C, Rodriguez-Tudela JL, Johansen HK, Arendrup MC. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob Agents Chemother*. 2010;54(11):4545-4549.
54. Chowdhary A, Sharma C, Kathuria S, Hagen F, Meis JF. Azole-resistant *Aspergillus fumigatus* with the environmental TR46/Y121F/T289A mutation in India. *J Antimicrob Chemother*. 2014;69(2):555-557.
55. Resendiz-Sharpe A, Dewaele K, Merckx R, et al. Triazole-resistance in environmental *Aspergillus fumigatus* in Latin American and African countries. *J Fungi (Basel)*. 2021;7(4):292.
56. van der Torre MH, Whitby C, Eades CP, et al. Absence of azole antifungal resistance in *Aspergillus fumigatus* isolated from root vegetables Harvested from UK arable and horticultural soils. *J Fungi (Basel)*. 2020;6(4):208.
57. Howard SJ, Cerar D, Anderson MJ, et al. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg Infect Dis*. 2009;15(7):1068-1076.
58. Risum M, Hare RK, Gertsen JB, et al. Azole resistance in *Aspergillus fumigatus*. The first 2-year's data from the Danish National Surveillance Study, 2018-2020. *Mycoses*. 2022;65(4):419-428.
59. Castanheira M, Collingsworth TD, Davis AP, Deshpande LM, Pfaller MA. Isavuconazole nonwildtype *Aspergillus fumigatus* isolates from a global surveillance study display alterations in multiple genes involved in the ergosterol biosynthesis pathway not previously associated with resistance to other azoles. *Mycoses*. 2021;64(10):1279-1290.
60. Resendiz Sharpe A, Lagrou K, Meis JF, Chowdhary A, Lockhart SR, Verweij PE. Triazole resistance surveillance in *Aspergillus fumigatus*. *Med Mycol*. 2018;56(suppl_1):83-92.
61. Kidd SE, Goeman E, Meis JF, Slavin MA, Verweij PE. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses*. 2015;58(6):350-355.

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