

Azole resistance and *cyp51A* gene mutations in clinical and environmental isolates of *Aspergillus fumigatus* complex from Pakistan

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ABSTRACT

Background and Purpose: Azole resistance in *Aspergillus* spp. is of significant clinical concern. We aimed to determine azole resistance in *A. fumigatus* complex from clinical and environmental sources in Pakistan.

Materials and Methods: 144 clinical and 25 environmental *A. fumigatus* complex isolates from across Pakistan were used for determination of minimum inhibitory concentrations (MICs) for posaconazole, voriconazole and itraconazole by broth microdilution using CLSI standards. For 39 isolates, mutations in the *cyp51A* gene were determined by sequencing.

Results: Amongst the clinical isolates, 13 (9%) had non-wild-type MICs for posaconazole, 1 isolate had non-wild-type MIC (2 µg/ml) for voriconazole and none of the isolates had non-wild-type MICs for itraconazole. Of the environmental isolates, 3 (12%) had non-wild-type MICs to posaconazole, while none of the isolates had non-wild-type MICs for voriconazole or itraconazole. Amongst the 39 isolates sequenced, no commonly known mutations in the *cyp51A* gene were identified. Several single nucleotide polymorphisms (SNPs) were found in isolates that showed no correlation with the MICs data.

Conclusion: We found *A. fumigatus* complex isolates with non-WT MIC profiles and an upward shift in MICs over time in our study. However, mutations in the *cyp51A* gene were not identified. These signals, while not definitive for resistance, indicate evolving selective pressure and justify continued, systematic surveillance that integrates longitudinal phenotypic testing with targeted genomics.

Keywords: Azole resistance, *cyp51A* gene, *Aspergillus fumigatus* complex, Pakistan



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Introduction

Aspergillus fumigatus is a filamentous mold that is widespread in the environment. It is responsible for causing a disease spectrum ranging from an aspergilloma to invasive aspergillosis, most particularly in immunocompromised patients. Azoles inhibit the synthesis of ergosterol in the fungal cell walls by binding to CYP51 proteins and are used to treat *Aspergillus* infections [1]. As with other microorganisms, *Aspergillus* also poses a concern of antimicrobial resistance. One of the first azole-resistant *A. fumigatus* strains was reported in clinical samples from 1980s in USA with high itraconazole minimum inhibitory concentration (MIC) [2]. The acquisition of azole-resistant strains may occur by two routes. They can be acquired directly from the environment or emerge as a result of prolonged azole therapy leading to selection of resistant strains. Literature shows that the environmental route of acquisition is responsible for >90% of azole-resistant cases [3].

Azole resistance in *Aspergillus* spp. is of significant clinical concern. Invasive *Aspergillus* infections are responsible for 40-50% of mortality in patients of acute leukemia or hematopoietic stem cell transplants; however, azole-resistant *Aspergillus* leads to 21% higher mortality compared to infections with azole-

susceptible strains [4-6]. In several resource limited countries, including Pakistan, it is not routine practice to determine susceptibilities of *Aspergillus* spp. and treatment is based on empirical therapy recommended by guidelines. These guidelines consider the rates of environmental resistance to recommend empirical options [7]. While azole resistance in environmental and clinical specimens has been considerably explored in several countries, including the neighboring countries Iran, India and China, in Pakistan, there is a paucity of literature on azole-resistance [8-10].

Our study aims to bridge this gap and provide evidence for effective clinical applicability of guidelines for empirical treatment in Pakistan. We determined azole resistance in *Aspergillus fumigatus* complex isolated from environmental and clinical isolates and evaluated *cyp51A* gene mutations [1].

Materials and Methods

Clinical isolates

144 *Aspergillus fumigatus* complex isolated from clinical specimens received at Aga Khan University Hospital clinical microbiology laboratory from December 2019 to June 2023 were included in the study. All clinically relevant isolates from respiratory tract,

eye, CSF and other sterile body sites, from both hospitalized and non-hospitalized patients were included. Duplicate isolates from the same patient were not included. Isolates were identified phenotypically by colony morphology and microscopic features as visualized on lactophenol cotton blue smears.

Environmental isolates

For environmental isolates, strains recovered in a previous study from soil specimens across Pakistan, covering agricultural and urban environments, were used [11]. The study identified 249 *Aspergillus* isolates (*A. niger*: 99 [39.7%], *A. flavus*: 84 [33.7%], *A. fumigatus*: 31 [12.4%], other *Aspergillus* species: 35 [14.1%]) from 171 soil specimens between November 2021 and June 2023, recovered using previously described methods with minor modifications [12]. Briefly, two grams of each sample was suspended in 8 ml of normal saline, vortexed rigorously, allowed to settle and then re-vortexed. A sterile swab was dipped in the suspension and streaked on Sabouraud's Dextrose Agar (SDA) in 4 quadrants and incubated at 37°C. *Aspergillus* isolates were identified based on the colony morphology and microscopic features. Twenty-five of these *Aspergillus fumigatus* complex isolates were used in the current study for MIC determination.

MIC determination

For MIC determination by broth microdilution (BMD), CLSI protocols were followed according to the CLSI M38-A2 document that yielded in serial 2-fold dilutions of the three antifungals in Roswell Park Memorial Institute (RPMI) 1640 media in 96 well microtiter plates [13]. The addition of 100 µL of a 0.5 McFarland solution of the *A. fumigatus* complex isolates yielded a final concentration of the antifungals ranging from 0.03 to 16 µg/ml. The results were interpreted according to epidemiological cutoff values (ECVs) recommended by CLSI in the document M59 (voriconazole ≤1 µg/ml as wild-type (WT), ≥2 µg/ml as non-wild-type (non-WT); itraconazole ≤1 µg/ml as wild-type, ≥2 µg/ml as non-wild-type) [14]. Since no recent breakpoints or ECVs are provided by CLSI for posaconazole, EUCAST epidemiological cut-off value (ECOFF) was used (≤0.25 µg/ml as WT, ≥0.5 µg/ml as non-WT) [15]. Even though clinical breakpoints are provided by CLSI for voriconazole against *A. fumigatus*, they apply only to *A. fumigatus sensu stricto*, hence could not be applied to our study [16]. *A. flavus* ATCC 204304, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, and a clinical strain of *Candida auris* with known resistance to posaconazole, voriconazole and itraconazole, were used as controls with every batch of test isolates and were found to be within range. MICs of strains exhibiting non-WT values were retested. Pearson correlation test was used to determine the trend of MIC values with time (*r* value 0-0.29: negligible correlation, 0.30-0.49: weak correlation, 0.50-0.69: moderate correlation, 0.70-0.89: high correlation, 0.90-1: very high correlation).

cyp51A mutation analysis

A subset of isolates (30 clinical and 9 environmental) was screened for *cyp51A* mutations. Apart from non-wild-type isolates, wild-type isolates were also sequenced to determine if any genetic patterns identified had correlation with phenotypic resistance. These isolates were randomly selected. Fungal DNA was extracted using a commercially available DNA isolation kit (Qiagen). The *cyp51A* gene, from nucleotide -462 of the promoter region through nucleotide 1696 in the 3' untranslated region was amplified and sequenced by using 4 sets of primers. Amplicons were sequenced by Sanger sequencing. Sequence quality was assessed by visual inspection of ABI chromatograms, and low-quality regions at the beginnings and ends of reads were excluded from analysis. Only nucleotide positions showing clear, well-resolved single peaks were considered. Sequences were assembled and compared to the reference sequences available in GenBank (accession number AF338659) using sequence analysis software BioEdit (<https://bioedit.software.informer.com/7.2/>). The promoter region was specifically analyzed for tandem repeat insertions, and all detected nucleotide changes were annotated for predicted amino acid substitutions. Analysis focused on mutations previously associated with azole resistance.

Results

Clinical isolates

A total of 144 clinical *A. fumigatus* complex isolates were included in the study. MICs for all 144 isolates ranged from 0.03 µg/ml to 0.5 µg/ml, from 0.03 µg/ml to 2 µg/ml and 0.03 µg/ml to 1 µg/ml for posaconazole, voriconazole and itraconazole respectively. Thirteen (9%) had non-WT MICs for posaconazole, one isolate (0.7%) had non-WT MICs for voriconazole, and none of the isolates had non-WT MICs for itraconazole (Table 1, Supplementary table 1). Temporal trends in azole MICs are illustrated in Figure 1 and showed a negligible positive correlation of MICs trend with time for all three antifungals (correlation coefficient=0.05, 0.06 and 0.10 for posaconazole, voriconazole and itraconazole respectively). No mutations that are commonly known to be associated with azole resistance were detected in 30 clinical *A. fumigatus* complex isolates sequenced for *cyp51A* gene mutations. Certain single nucleotide polymorphisms (SNPs) were found in both wild-type and non-wild-type isolates (Table 2). Among the clinical cases that had non-WT MICs for voriconazole, 4/18 had prior azole exposure, 10/18 did not and for 4/18 this information was not available.

Environmental isolates

MICs determined for 25 isolates ranged from 0.06 µg/ml to 0.5 µg/ml, from 0.06 µg/ml to 0.5 µg/ml and 0.25 µg/ml to 1 µg/ml for posaconazole, voriconazole and itraconazole respectively. Among these 25 isolates, three (12%) had non-WT MICs for posaconazole, while none of the isolates had non-WT MICs for voriconazole and itraconazole (Table 1, Supplementary table). Nine

isolates underwent sequencing for *cyp51A* mutations; no commonly known mutations were detected. One

isolate with WT MICs showed the presence of two SNPs (Table 2).

Table 1. Analysis of azole minimum inhibitory concentrations (MICs) for *Aspergillus fumigatus* complex

Clinical isolates	Range	Posaconazole	Voriconazole	Itraconazole
		0.03 – 0.5 µg/ml	0.03 – 2 µg/ml	0.03 – 1 µg/ml
	MIC ₅₀ (µg/ml)	0.25	0.5	0.5
	MIC ₉₀ (µg/ml)	0.25	1	0.5
	%NWT	9%	0.7%	0%
Environmental isolates	Range	0.06 - 0.5 µg/ml	0.06 – 0.5 µg/ml	0.25 – 1 µg/ml
		MIC ₅₀ (µg/ml)	0.25	0.5
		MIC ₉₀ (µg/ml)	0.5	0.5
		%NWT	12%	0%

NWT=Non-Wild-Type

Table 2. *cyp51A* mutation analysis in *Aspergillus fumigatus* complex isolates

Strain No.	Source	City of origin	MICs (µg/ml)			<i>cyp51A</i> mutations identified
			Posaconazole	Voriconazole	Itraconazole	
C_AFPK_01	LRT	Karachi	0.25	2	0.5	None
C_AFPK_02	LRT	Karachi	0.5	1	0.5	M172V
C_AFPK_03	LRT	Karachi	0.25	1	0.5	M172V
C_AFPK_04	LRT	Karachi	0.25	1	1	None
C_AFPK_05	LRT	Karachi	0.25	1	0.5	Q26H
C_AFPK_06	Nasal Tissue	Rahim Yar Khan	0.25	1	1	None
C_AFPK_07	LRT	Karachi	0.25	1	0.5	F495C
C_AFPK_08	LRT	Karachi	0.25	1	0.25	None
C_AFPK_09	LRT	Karachi	0.25	1	0.25	N248K
C_AFPK_10	LRT	Lahore	0.25	1	1	None
C_AFPK_11	LRT	Karachi	0.125	1	0.5	None
C_AFPK_12	LRT	Karachi	0.125	1	0.5	F349L
C_AFPK_13	LRT	Karachi	0.06	1	1	M172V
C_AFPK_14	LRT	Lahore	0.5	0.5	0.5	Y121H
C_AFPK_15	Pus	Karachi	0.5	0.5	1	None
C_AFPK_16	LRT	Karachi	0.5	0.5	1	None
C_AFPK_17	CSF	Karachi	0.5	0.5	0.5	None
C_AFPK_18	LRT	Karachi	0.5	0.5	0.25	Q26H
C_AFPK_19	LRT	Karachi	0.5	0.5	0.125	None
C_AFPK_20	LRT	Karachi	0.5	0.5	1	None
C_AFPK_21	Nasal Tissue	Karachi	0.25	0.5	0.12	None
C_AFPK_22	LRT	Lahore	0.5	0.25	0.25	N248K
C_AFPK_23	LRT	Karachi	0.5	0.25	0.06	Q26H
C_AFPK_24	LRT	Karachi	0.25	0.25	0.5	None
C_AFPK_25	LRT	Karachi	0.25	0.25	0.5	None
C_AFPK_26	LRT	Lahore	0.25	0.25	1	None
C_AFPK_27	LRT	Karachi	0.5	0.125	0.5	None
C_AFPK_28	LRT	Karachi	0.25	0.12	0.5	None
C_AFPK_29	Femoral clots	Karachi	0.25	0.12	0.5	None
C_AFPK_30	LRT	Karachi	0.12	0.06	0.25	None
E_AFPK_01	Soil	Karachi	0.25	0.5	1	M172V, F349L
E_AFPK_02	Soil	Akhtarabad	0.25	0.5	0.5	None
E_AFPK_03	Soil	Okara	0.25	0.5	0.5	None
E_AFPK_04	Soil	Karachi	0.25	0.25	0.5	None
E_AFPK_05	Soil	Phool Nagar	0.12	0.12	0.25	None
E_AFPK_06	Soil	Sujawal	0.06	0.12	0.25	None
E_AFPK_07	Soil	Nagarparkar	0.25	0.12	0.5	None
E_AFPK_08	Soil	Lahore	0.25	0.12	0.5	None
E_AFPK_09	Soil	Karachi	0.5	0.12	0.5	None

LRT: Lower Respiratory Tract, MIC: Minimum Inhibitory Concentration. MIC values in bold indicate being in the non-wild-type range.

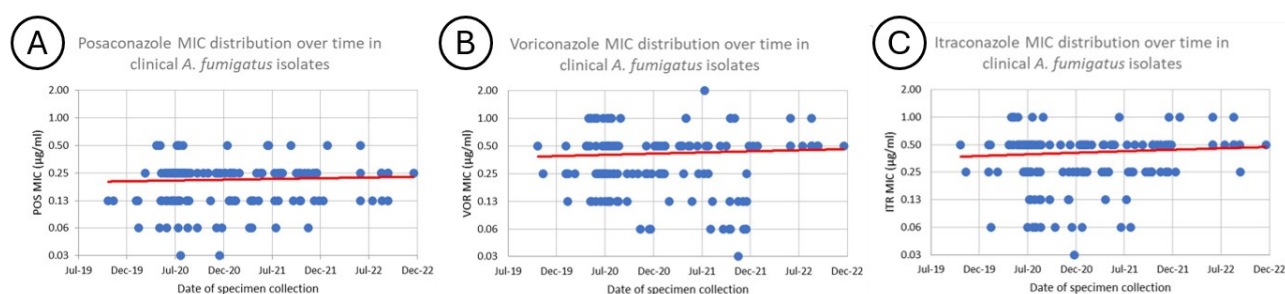


Figure 1. Trends in posaconazole (a), voriconazole (b) and itraconazole (c) minimum inhibitory concentrations (MICs) for *Aspergillus fumigatus* complex over time.

Discussion

Our study provides important findings related to azole susceptibilities of *A. fumigatus* complex in Pakistan. While certain studies from Pakistan have previously reported azole susceptibility results [17-19], the biggest limitations have been the interpretation of the reported MICs and the lack of evidence of molecular mechanisms of resistance. The challenge in interpretation lies in the lack of standardization of breakpoints. Both CLSI and EUCAST provide slightly different cutoffs, with certain gaps in interpretation. Adding to this, several studies, both at national and international scales, have provided their own MIC distribution data to evaluate epidemiological cutoffs and clinical breakpoints [20, 21]. Factors for this non-uniformity include lower prevalence of invasive fungal infections (that translates into less data for breakpoint determination), differences in antifungal susceptibility testing (AFST) methodology and PK/PD data inferred from animal models [22]. As a consequence of this variety of cutoffs, different studies report azole susceptibilities using different yardsticks, leading to heterogeneity, difficulties in inter-study comparisons and possible false impressions of resistance. To overcome this and get a valid interpretation of MIC distribution data, it is important to explore molecular mechanisms of resistance. To our knowledge, this is the first study from Pakistan providing this aspect in azole susceptibility data.

None of the commonly known resistance genes (TR/L98H, G54W, M220I and F219C) were discovered on sequencing. The MIC ranges for *A. fumigatus* complex in our study were generally low with the maximum MIC of 2 µg/ml (of voriconazole) in 1 isolate. Higher MICs (4, 8 and >16 µg/ml) have been reported from India, Iran and China also harboring mutations in *cyp51A* gene known to confer resistance to azoles [8, 10, 23]. Although we did not find an overall high number of strains with non-WT azole MICs in our study, the presence of voriconazole non-WT strains in clinical specimens is worrisome, as this likely suggests developing resistance acquired due to prolonged azole exposure. Moreover, our study also finds that the distribution of azole MICs is in an upward trend which is concerning. Judicious use of voriconazole is warranted to prevent emergence and spread of azole resistant *A. fumigatus* complex in Pakistan.

The SNPs that were identified in our study (Q26H, Y121H, M172V, N248K, F349L, F495C) did not show

any pattern of association with the MICs, as they were found in both WT and non-WT strains. In India, while one study identified tandem repeats (TR34/L98H and TR46/Y121F/T289A) as the predominant mutations, other novel mutations (G54R, Y431C, and P216L) were also described in resistant isolates in another recent study [24, 25]. The M172V mutation was also found in two azole-resistant *A. fumigatus* isolates in an Iranian study [26]. In both these studies, the isolates had higher MICs to azoles (4-16 µg/ml). While our findings do not conclusively link any of the identified SNPs with high MICs (including M172V), further research by constructing structural protein homology models using these SNPs and their subsequent phenotypic effect when inserted in wild-type strains is needed to convincingly identify these SNPs as a cause of decreased azole susceptibility, as several mutations are known to alter a site on the CYP51 proteins that does not affect the docking of the azole compound or the biological activity of the protein [27].

The correlation of the route of azole resistance is also important, as this identifies the cause of azole resistance in the region (due to fungicidal use in agriculture vs due to clinical exposure). The more common route of resistance documented in literature is due to fungicidal use in agriculture [28-30]. Pakistan is an agrarian country and environmental acquisition of resistance may be anticipated. A major limitation of our study is the sample size, as *A. fumigatus* complex accounted for only 12.4% of *Aspergillus* species isolated from 171 soil specimens. Moreover, we did not have information regarding prior azole exposure in most clinical cases. Therefore, we cannot conclusively determine the route of development of resistance due to study limitations, and there is a need for more thorough clinical research with an emphasis on judicious voriconazole use.

There are other limitations of our study. Due to lack of financial resources, we were not able to carry out sequencing of all strains with increased MICs. Furthermore, recent research has identified newer mechanisms of resistance, such as the presence of efflux pump genes [31, 32]. Our study focused on sequencing of the *cyp51A* gene only, thus it is a possibility that other mechanisms of resistance such as HMG-CoA reductase-encoding gene mutations, *cyp51B* gene mutations and *hapE* gene mutations may be overlooked [33]. This prompts the use of whole genome sequencing. Another limitation is that this was a single center study; though

the Aga Khan University Hospital clinical microbiology laboratory does receive clinical specimens from all parts of the country, comprising of a wide variety of clinical backgrounds. Additionally, due to lack of available resources, organism identification was solely based on morphological characteristics, making it difficult to definitively identify the cryptic species within the *Aspergillus fumigatus* complex.

The utility of antifungal susceptibility testing is limited for case-by-case evaluation, as early treatment in such cases is critical and testing takes several days to weeks to report. The importance, however, of surveillance of MICs and genotypic resistance mechanisms is to establish epidemiological rates of resistance to guide antifungal therapy. This makes it a pressing concern to continue surveillance of MICs in order to monitor for emerging resistance beyond a threshold that would deem voriconazole monotherapy reconsideration according to Verweij et al [7]. Also, we find that the distribution of MICs is in an upward trend, which is a worrisome finding. Judicious use of azoles is warranted in light of our study.

Conclusion

We found *A. fumigatus* complex isolates with non-WT azole MIC profiles and an upward shift in MICs over time in our study. These signals, while not definitive for resistance, indicate evolving selective pressure and justify continued, systematic surveillance that integrates longitudinal phenotypic testing with targeted genomics. Ongoing monitoring will be critical to detect emerging reduced susceptibility early and to inform stewardship, diagnostics, and treatment choices.

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Conflicts of interest

The authors declare no conflict of interest related to this publication. The authors alone are responsible for the content and the writing of the paper.

Authors' contributions

SARN: Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization. SZ: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing. JF: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Review & Editing, Visualization, Funding acquisition. NG: Methodology, Validation, Resources, Writing - Review & Editing, Supervision. KJ: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

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Since the study was centered around genomic analysis of microbiological isolates and did not involve human participants, exemption from ethical approval was obtained from the Ethics Review Committee, Faculty of Health Sciences at Aga Khan University Hospital (Review reference: 2019-0438-2659).

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