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ORIGINAL ARTICLE

Azole resistance using agar screening in environmental isolates of *Aspergillus* species from Pakistan

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ABSTRACT

Background: Azole resistance in clinical and environmental *Aspergillus* isolates has been reported globally. The environmental route of resistance follows the use of fungicides in agricultural practices and is known to be more common than the clinical route of resistance. We aimed to screen for azole resistance in *Aspergillus* species from environmental sources in Pakistan.

Material and Methods: This was a cross-sectional, observational study conducted from November 2021 to June 2023 at Aga Khan Aga Khan University Hospital, Karachi. Environmental *Aspergillus* isolates from across Pakistan were screened for azole resistance using previously described methods. Soil samples were collected from different towns and cities of Pakistan using convenience sampling. A suspension of soil in normal saline was cultured on SDA to yield fungal colonies, and onto media containing posaconazole, voriconazole and itraconazole in defined concentrations to screen for resistance. Growth on antifungal containing media was considered to be resistant.

Results: Soil was collected from 171 different sites across Pakistan. A total of 249 *Aspergillus* isolates were recovered from these soil samples. These included 99 (39.7%) *A. niger*, 84 (33.7%) *A. flavus*, 31 (12.4%) *A. fumigatus*, and other species. None of the isolates grew on any of the antifungal containing media, indicating no resistance.

Conclusion: Our study finds no indication of azole resistance in environmental isolates from Pakistan. However, methodologies such as MIC determination and genotypic sequencing are needed for a more robust analysis.

Keywords: *Aspergillus* species, Agar screening, Azole resistance, Environmental, Pakistan

BACKGROUND

Aspergillus is a genus of filamentous molds widespread in the environment and 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 (14α-demethylase) 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 a minimum inhibitory concentration (MIC) to itraconazole greater than 16 µg/ml.[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. While invasive *Aspergillus* infections are responsible for 40-50% of mortality in patients of acute leukemia or hematopoietic stem cell transplants, azole-resistant *Aspergillus* leads to 21% higher mortality compared to infections with azole-susceptible strains.[4,5] In several resource limited countries, including Pakistan, it is not routine practice to determine susceptibilities of *Aspergillus* spp. and treatment of clinical cases is based on empirical therapy recommended by guidelines. These guidelines consider the rates of environmental resistance to recommend empirical options.[6] 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.[7-9]

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Using the agar screening method to determine azole resistance in *Aspergillus* spp. is a relatively inexpensive and easy-to-perform method that can provide a reliable way to rule out resistance.[10,11] Our study aims to bridge the gap related to azole resistance in *Aspergillus* spp. from Pakistan and provide evidence for effective clinical applicability of guidelines for empirical treatment.

MATERIAL AND METHODS

This was a cross-sectional, observational study conducted from November 2021 to June 2023 at Department of Pathology and Laboratory Medicine, Aga Khan University Hospital, Karachi. Soil samples across different cities and towns of Pakistan were collected to recover *Aspergillus* species. and carry out azole resistance screening according to previously described methods with minor modifications.[10,12] For sample collection, convenience sampling was used. Volunteers were handed sterile containers used for routine microbiological specimen collection to collect soil samples from different sites. These volunteers included travelers to different parts of Pakistan. They were instructed to collect soil from sites such as gardens and agricultural fields. For specimen processing, two grams of each sample were suspended in 8 ml of normal saline, vortexed rigorously, allowed to settle, and then re-vortexed. A sterile swab was dipped into the suspension and streaked onto Sabouraud's Dextrose Agar (SDA) in four quadrants, then incubated at 37°C. Initially, 50 µl of the suspension was used for isolation of molds from the soil specimens, however, this led to overgrowth of multiple molds and made it difficult to identify the variety of molds in a sample. After optimization, a swab was found to be the most effective way of obtaining isolated molds that were easy to identify. All samples with growth of *Aspergillus species* were included in the study, whereas those without the growth of *Aspergillus species* were excluded. *Aspergillus* isolates were identified based on the colony morphology and microscopic features. Additionally, 50 µl of the soil suspension was inoculated into each well of a four-well petri dish. Three wells contained SDA-Chloramphenicol (SDAC) supplemented with posaconazole (0.5 µg/ml), voriconazole (1 µg/ml), and itraconazole (4 µg/ml). The fourth well served as a control without any antifungal. The plates were checked for growth at 24, 48 and 72 hours. Growth in any of the antifungal containing wells was considered to screen as positive for resistance. *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. All study data was recorded on a proforma, and proportions were calculated using percentages.

RESULTS

Soil was collected from 171 sites across Pakistan. These included sites from each of the 4 provinces and the federal territories. Out of a total of 249 *Aspergillus* isolates recovered from these samples, 99 (39.7%) were *A. niger*, 84 (33.7%) *A. flavus*, 31 (12.4%) *A. fumigatus*, and the remaining other *Aspergillus* species. *A. flavus* was the most frequently isolated species from Islamabad (same frequency as *A. niger*), Punjab and Baluchistan, while *A. niger* was the most frequently isolated species from Sindh and Khyber Pakhtunkhwa (KPK) (Table-I). None of the soil samples screened positive for azole resistance.

Table-I: Distribution of Aspergillus species in soil specimens collected across various location of Pakistan. The data shows the number of sites (n) from each province/territory that yielded Aspergillus species, to illustrate the most common environmental species by province/ territory.

Number of sites screened from each province/territory with growth of *Aspergillus* species (%)								
	No. of sites screened (n)	***A. flavus* n (%)**	***A. fumigatus* n (%)**	***A. niger* n (%)**	***A. terreus* n (%)**	***A. versicolor* n (%)**	***A. nidulans* n (%)**	***A. species* n (%)**
KPK	6	1 (17)	1 (17)	2 (33)	0 (0)	0 (0)	0 (0)	0 (0)
Islamabad	10	3 (30)	2 (20)	3 (30)	3 (30)	0 (0)	0 (0)	0 (0)
Punjab	38	25 (66)	13 (34)	23 (61)	11 (29)	0 (0)	0 (0)	0 (0)
Baluchistan	4	4 (100)	3 (75)	3 (75)	0 (0)	0 (0)	0 (0)	0 (0)
Sindh	113	51 (45)	12 (11)	68 (60)	15 (13)	2 (2)	1 (1)	3 (3)

DISCUSSION

Azoles continue to be used as first line therapy for Aspergillosis in Pakistan, and our study presents evidence to suggest that there is no resistance to these agents in *Aspergillus* species in Pakistan. Previously, our study group had shown similar results among a

smaller sample of environmental isolates where 25 soil samples were analyzed using agar screening.[13] Among clinical isolates, another previous study by this group had shown no resistance using agar screening in 114 isolates.[14] The current study builds upon that work to ensure continued surveillance for any emerging resistance, with the results providing a certain solace for physicians.

Aspergillus niger turned out to be the most frequent *Aspergillus* isolate recovered from soil specimens across Pakistan. Regionally, in Balochistan and Punjab, *A. flavus* was the most common species followed closely by *A. niger*. *A. fumigatus* remains the third most frequently isolated *Aspergillus* species from soil specimens. Despite *A. niger* being the most common species in soil, *A. flavus* and *A. fumigatus* both are more common in clinical cases of Chronic Pulmonary Aspergillosis in Pakistan.[15] The World Health Organization (WHO) also places *A. fumigatus* in the 'Critical group' of priority fungal pathogens, the only *Aspergillus* species in the group.[16] This emphasizes the need for continued and enhanced surveillance for azole resistance, especially in *A. fumigatus*.

Agar screening remains an important and relatively inexpensive method to carry out surveillance for azole resistance. EUCAST also provides a methodology to carry this out specifically for *A. fumigatus*, since it is the only species with clinical breakpoints. It recommends reporting isolates susceptible based on this method. Only for isolates that test non-susceptible does EUCAST recommend further broth microdilution (BMD) testing.[11] This is supported by studies where the sensitivity and specificity of this technique has been found to be 99% each when compared to genotypic methods as the reference.[17] For other species however, guidance is lacking. Also, despite these assurances, clinical breakpoints for azoles against *A. fumigatus* have been revised following the introduction of the agar screening technique, which incorporated the breakpoints available at that time for the antifungal concentration in the agar. This, along with the fact that azole resistance has been documented in our neighboring regions Iran (Nabili *et al*: 6.6 %), India (Chaudhary *et al*: 1.7%) and China (Chen *et al*: 10.2%) [7,9,18], implies that it is imperative to carry out more extensive efforts by having a larger sample size and using techniques such as MIC determination and genotypic analysis.

CONCLUSION

Our study presents evidence for lack of azole resistance in *Aspergillus* species in environmental samples, based on agar screening. However, methodologies such as MIC determination and genotypic sequencing are needed for a more robust analysis.

CONFLICT OF INTEREST

None

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHOR CONTRIBUTION

Syed Ali Raza Nasir, Sadaf Zaka: Acquisition of data, interpretation of data, manuscript drafting, final approval, accountable for all aspects of publication.

Joveria Farooqi: Study conception, acquisition, analysis and interpretation of data, manuscript drafting, final approval, accountable for all aspects of publication.

Adan Zubair: Acquisition of data, interpretation of data, manuscript drafting, final approval, accountable for all aspects of publication.

Kauser Jabeen: Study conception, acquisition, analysis and interpretation of data, manuscript drafting, final approval, accountable for all aspects of publication.

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