ORIGINAL ARTICLE

Triazole Resistance Screening in Environmental *Aspergillus* Species from Pakistan

Safia Moin, Fatima Syed Amanullah, Manzar Abbas, Sidra Laiq, Joveria Farooqi, Afia Zafar, Kauser Jabeen

Department of Pathology and Laboratory Medicine,
Aga Khan University, Karachi, Pakistan

Abstract

Background
Globally resistance to azoles is increasingly being recognized and reported in *Aspergillus* species isolated from environment. Major risk factor for this resistance is the use of azole and related compounds as fungicides in the agriculture. Data from Pakistan in this regard is not available and is needed.

Objective
This study aimed to estimate azole resistance in *Aspergillus* species isolated from soil specimens.

Methods
Soil specimens were collected from four cities of Pakistan and was cultured by dissolving in normal saline and further inoculation on azole resistance screening agar. *Aspergillus* species was identified using conventional method. Screening plates were incubated and interpreted using recommended methodology.

Results
Twenty-five soil specimens were screened, and all grew *Aspergillus* species. *Aspergillus niger* grew in 24/25 specimens. Azole resistance could not be detected in any of these specimens.

Conclusion
Triazole resistance was not detected amongst environmental *Aspergillus* isolates from Pakistan. This small study provides baseline for further large scale studies in Pakistan.

Background
Aspergillus species is an important pathogen in immunocompromised patients with a wide disease spectrum requiring systemic antifungal therapy.[1] Azoles are the drug of choice for *Aspergillus*; however, azole resistance is increasingly recognized in *Aspergillus* disease and has been reported worldwide.[1] Recent data from Asia reports 1.7% resistance rate to azoles in clinical *Aspergillus* strains with concomitant treatment failure and poor outcomes.[2] Acquired resistance in *Aspergillus* species especially in *A. fumigatus* to azoles in environmental isolates has been identified as a major public health challenge. Major risk factor for this resistance has been postulated to be use of azole and related compounds as fungicides in the agriculture.[1] These fungicides are structurally similar to triazoles used in treatment of patients and hence lead to azole resistance. In regions which are endemic for *Aspergillus*, more than 90% of azole resistance in clinical aspergillosis could be attributed to environmental route of resistance selection.[3-5] This is of great importance to an agricultural country like Pakistan. However, resistance detection for *Aspergillus* species from environment has not been conducted before in the country. It has therefore also become imperative to determine the extent of resistance not only in clinical but also in environmental isolates as guidelines recommend using resistance in environmental isolates to guide empirical therapy.[6] This knowledge is essential for clinicians to make informed decisions in clinical management and also for policy makers to develop prevention and control strategies.

Hence, in this study, we evaluated *Aspergillus* species isolated from soil specimens for triazole resistance.

Materials and Methods
Collection of samples: Twenty-five soil specimens from four cities of Pakistan (Karachi, Quetta, Muzaffargarh and Multan) were collected during the month of August. These specimens mainly included soil, leaves and other organic material from gardens and agricultural fields. To recover *Aspergillus species* from soil, leaves, or other organic material, two grams of each sample was suspended in 8 ml of normal saline and vortexed rigorously. This suspension was allowed to settle and then re-vortexed.[7,8] Subsequently, 100 µl of this suspension was plated on Sabouraud's dextrose agar (SDA), and incubated at 37°C. *Aspergillus* isolates were identified based on the colony morphology and microscopic morphology.[9]

Azole resistance screening: Azole resistance was determined using azole resistance screening agar by antifungal agar screening method.[8] Itraconazole, voriconazole and posaconazole, powders from the Sigma-Aldrich Company (St. Louis, MO, USA) were used to prepare the agar screening plates. Subsequently 50µl was inoculated in each well of a four-well petri plate containing Roswell Park Memorial Institute (RPMI) 1640 agar with 2% glucose supplemented with itraconazole (4 mg/L), voriconazole

Corresponding author: Kauser Jabeen,
Department of Pathology and Laboratory Medicine,
Aga Khan University,
National Satdium Road, Karachi, Pakistan
Email: kausar.jabeen@ aku.edu

(1 mg/L), and posaconazole (0.5 mg/L), and no antifungal (positive-control well). Plates were incubated at 35°C and read at 24, 48, and 72 h. The isolates were categorized as azole susceptible if *Aspergillus* colonies were observed in the control well with no growth in azole containing wells and azole-non-susceptible if *Aspergillus* colonies were seen on both control and azole containing wells. American Type Culture Collection (ATCC) strains used as controls in the susceptibility testing were *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258 and *A. flavus* ATCC 204304. These isolates are triazole susceptible, and were found susceptible according to the agar screening method. As a resistant control, a known triazole resistant *Candida auris* isolate, which was previously tested by the Centers for Disease Control and prevention (CDC), was tested which was found resistant to the triazoles on the agar screening.

Results

25 samples of soil from different provinces of Pakistan were included in the study. All specimens had growth of *Aspergillus* species. Except for one, all soil specimens had growth of *A. niger.* Six specimens had growth of *A. flavus.* One specimen grew *A. fumigatus*. Four specimens had growth of two *Aspergillus* species. One specimen grew three *Aspergillus* species. Azole resistance was not detected in any of the *Aspergillus* isolates based on the triazole antifungal agar screening (Table 1).

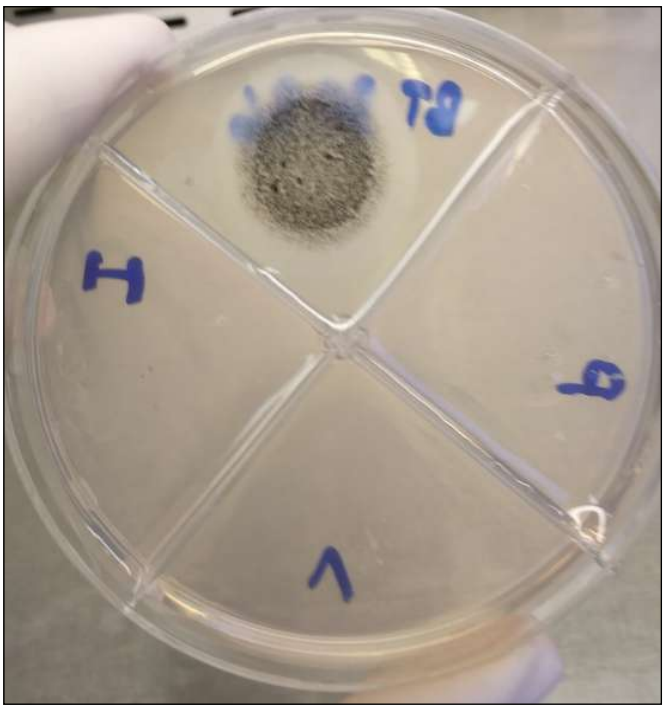

Fig 1. *Aspergillus niger* **isolate recovered from soil sample, found susceptible to the triazoles on agar screening. Itraconazole 4 mg/L in left quadrant, Voriconazole 1 mg/L below and on the right, Posaconazole 0.5 mg/L. Growth control is on the top quadrant.**

Table 1: Susceptibility of the environmental isolates to triazoles.

Sample no. (n=25)	Location	*Aspergillus* spp.	Susceptiblity to Itraconazole, Voriconazole, Posaconazole
1	Karachi	*A. niger*	Susceptible
2	Karachi	*A. niger*	Susceptible
3	Karachi	*A. niger*	Susceptible
4	Karachi	*A. niger*	Susceptible
5	Karachi	*A. niger*	Susceptible
6	Karachi	*A. niger*	Susceptible
7	Karachi	*A. niger*	Susceptible
8	Karachi	*A. niger*	Susceptible
9	Karachi	*A. niger*	Susceptible
10	Karachi	*A. flavus*	Susceptible
11	Multan	*A. niger, A. flavus*	Susceptible
12	Multan	*A. niger, A. flavus*	Susceptible
13	Multan	*A. niger, A. flavus*	Susceptible
14	Multan	*A. niger, A. flavus*	Susceptible
15	Multan	*A. niger*	Susceptible
16	Quetta	*A. niger*	Susceptible
17	Quetta	*A. niger*	Susceptible
18	Quetta	*A. niger*	Susceptible
19	Quetta	*A. niger*	Susceptible
20	Quetta	*A. niger, A. flavus, A. fumigatus*	Susceptible
21	Muzaffargarh	*A. niger*	Susceptible
22	Muzaffargarh	*A. niger*	Susceptible
23	Muzaffargarh	*A. niger*	Susceptible
24	Muzaffargarh	*A. niger*	Susceptible
25	Muzaffargarh	*A. niger*	Susceptible

Discussion

We examined itraconazole, voriconazole and posaconazole susceptibility in a small number of soil specimens collected from four cities of Pakistan, two from Punjab, one from Baluchistan and one from Sindh province. Azole resistance could not be detected in any of the environmental *Aspergillus* isolate. We have previously screened and did not detect triazole resistance in 114 clinically significant *Aspergillus* isolates.[10]

Although limited in numbers, our data can nevertheless serve as baseline for future surveillance of triazole susceptibility in environmental *Aspergillus* species in our country. Data from several countries reports existence of azole resistant *Aspergillus* isolates with either TR34/L98H or TR46/Y121F/T289A mutations in both environmental and clinical sources.[11] Triazole resistance has been reported in 7 % of 630 *A. fumigatus* isolates from India with TR34/L98H mutation in the *cyp*51 gene. In this study, cross-resistance to itraconazole, voriconazole and posaconazole, and to other six

triazole fungicides used in agriculture was observed.[12] These samples originated primarily from Northern India, some from New Delhi which is close to Pakistan's Punjab province.

A study from Iran conducted over a period of three years reported a 6.6% prevalence of azole-resistant *A. fumigatus* in clinical and environmental isolates amongst 213 clinical and 300 environmental isolates.[13]

Although this study has a small sample size, it is the first from the country and provides a baseline data for future large studies. Triazole resistance was not detected in this small study and highlights the need for large scale investigation to include increased sample size and geographical locations.

Conclusion

Triazole resistance was not detected amongst environmental *Aspergillus* isolates from Pakistan.

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