

RESEARCH ARTICLE

## Antifungal Resistance Analysis of Environmental Isolates of *Aspergillus* in North India

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### Abstract

Triazoles are the major group of antifungals for treating *Aspergillus* infections. The morbidity and mortality associated with these infections is high and rate of treatment failure is more in patients infected with azole resistant *Aspergillus*. The azole resistant *Aspergillus* isolates have been recovered from both azole treated and azole naive patients. Thus, there may be an environmental route of exposure to azole resistant *Aspergillus*. The present study was envisaged for the isolation and enumeration of environmental isolates of *Aspergillus* resistant to medically available antifungal azole drugs in North Indian environment. A total of 25 soil samples were collected from North Indian agricultural farms where azole pesticides were being used. The soil samples were screened for *Aspergillus* isolates by serial dilution pour plate method. Further, their drug susceptibility testing was performed using disc diffusion, E- strip and micro-broth dilution method against medically available triazoles: itraconazole, ketoconazole, fluconazole and voriconazole. A total of 41 *Aspergillus* species were isolated from the soil samples. Based on conventional microscopic assay, 13 of them were identified as *Aspergillus fumigatus*, 9 as *Aspergillus niger*, 5 as *Aspergillus terreus*, 3 as *Aspergillus nidulans*, 1 as *Aspergillus flavus* and 9 as other *Aspergillus* species. Resistance for all tested antifungal drugs was detected in 7.3% *Aspergillus* isolates and 43.7% isolates were resistant to any of the tested azole drugs. The results demonstrated that *Aspergillus* isolates resistant to medical triazoles are present in the agricultural farms.

**Keywords:** *Aspergillus*, Antifungals, Azole resistance, Antifungal susceptibility test.

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## INTRODUCTION

Azoles are the common synthetic compounds that inhibit sterol biosynthesis. They are used as first choice of treatment against *Aspergillus* infections in humans. Besides, they are also used for preservation and plant protection (Horsfall, 1975; Hof, 2001; Russel, 2005; e Ribas et al., 2016). These have been widely used to treat human mycoses and plant pathogens effectively since 1970s and have similar mode of action in humans and plant fungal infections (Snelders et al., 2009; Cools et al., 2013). It has been reported that among 38 registered fungicides in India (Central Insecticide Board, Govt. of India), 14 are azole pesticides. Majority of these broad range azole fungicides used by farmers have negative impact on environment and human health (Berger et al., 2017). Large scale use of azole fungicide in agriculture is causing mutational resistance in *Aspergillus* species which are present in abundance in environment (Kumar et al., 2013). There have been various reports stating the emergence of cross- and multi- drug resistance of human pathogen *Apergillus* is related to the exposure of fungicides in the agro-ecosystem (Meneau and Sanglard, 2005; Bowyer and Denning, 2013; Lelievre et al., 2013). Azole pesticides have been used in agriculture during pre and post harvesting of crops to avoid the spoilage (Snelders et al., 2012). These pesticides persist in the soil for longer time, accumulate in plants and are transferred to humans through food web (Palazzini et al., 2018). These conditions enhance the development of azole resistant strains.

Azole drug resistance in patients suffering from *Aspergillus* infections has emerged as a global issue in recent years due to failure of drug efficacy, thereby increasing the mortality and morbidity rate. In India, 64%–71% of multi-triazole resistant strains have been isolated from in patients suffering from *Aspergillus* infections who were never been exposed to triazole treatments (Chang et al., 2016). Hence, an environmental origin for triazole resistant *Aspergillus* strains has been proposed. The probable reasons for the emergence of azole resistant strains in the environment include changes in drug target affinity (Perlin et al., 2015) and excessive use of azole pesticides in the agriculture (Verweij et al., 2009; Chowdhary

et al., 2012). The Fungicide Resistance Action Committee (FRAC) fixes the baseline resistance level of fungi prior to commercialization of a fungicide whereas, the failures in disease control and detection of resistant isolates even below the baseline are the clear mark of the hazards of fungicide resistance development (Brent and Hollomon, 2007). The spores of the azole-pesticide resistant environmental strain of *Aspergillus* when inhaled by immuno-compromised individuals cause antifungal azole-resistant diseases, leading to treatment failure and deaths in patients (Pham et al., 2014).

The proposition of emergence of azole-resistant *Aspergillus* species due to azole fungicides in agri-ecosystem was initially suggested by studies conducted in the Netherland, where the triazole resistant *Aspergillus* was reported for the first time (Snelders et al., 2008). Later corroborated studies were conducted in Spain, Belgium, Norway, Great Britain, Denmark France, China, Italy, Austria and India (Rodriguez-Tudela et al., 2008; Howard et al., 2010; Mortensen et al., 2010; Lockhart et al., 2011; Burgel et al., 2012; Van der Linden et al., 2015). The varying rates of azole resistant *Aspergillus* has been documented across the world ranging from 0.6% to 38% (Snelders et al., 2008; Howard et al., 2009; Bueid et al., 2010; Mortensen et al., 2010; van der Linden et al., 2011; Burgel et al., 2012; Chowdhary et al., 2012; Badali et al., 2013; Bader et al., 2013; Pham et al., 2014; van Ingen et al., 2015). In India, 7% environmental isolates and 1.75% Clinical isolates of *Aspergillus* strains have been reported to be multi-triazole resistant (Chowdhary et al., 2012; Chowdhary et al., 2015). All these epidemiological studies revealed that there is the link between triazole resistant clinical isolates and the widespread use of azole fungicides. Thus, continued surveillance of resistance in environmental *Aspergillus* strains is crucial. The present study was envisaged to study the prevalence of azole resistant *Aspergillus* species in agricultural fields of north western India.

## MATERIALS AND METHODS

### Soil sample collection

A total of 25 soil samples from various regions of North Indian farms were collected (Fig 1): Amritsar (n = 2), Chandigarh (n = 3), Hisar (n

=2), Rohtak (n = 2), Ghevra (n = 2), Bawana (n = 4), Noida (n = 2), Greater Noida (n = 2), Gonda (n = 2), Varanasi (n = 2) and Singrauli (n = 2). The soil samples were coded as NIS-001 to NIS-025.

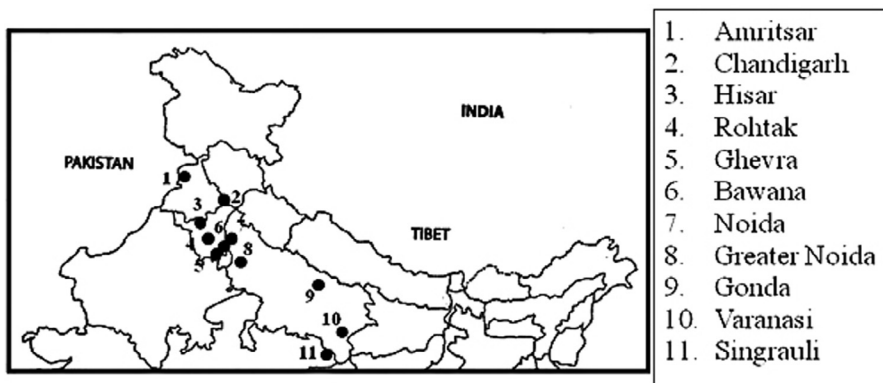


Fig. 1. Sites of sample collection in North India

#### Screening and isolation of environmental *A. fumigatus* from soil samples

Soil samples were processed for isolation of *Aspergillus* colonies using serial dilution method (Chowdhary et al., 2012). Briefly, 1 g of soil was dissolved in 10 ml of 0.9% normal saline, vortexed and allowed to settle for 30 seconds. From this stock suspension,  $10^{-1}$ -  $10^{-4}$  dilutions were prepared and 50 $\mu$ l of each dilution were spread on potato dextrose agar (PDA) plates supplemented with 0.5 mg/ml chloramphenicol. The plates were incubated for 5 days at 28°C. Each experiment was conducted in triplicates.

The macroscopic features like colony colour, texture were studied. The fungal colonies were identified by conventional microscopy and then by following mycological literature (de Hoog et al., 2000; Klich, 2002; McClenny, 2005). The filamentous colonies on PDA plates were teased apart and stained with lacto-phenol cotton blue and conidial heads, vesicle shape, seriation and conidia shape were investigated under light microscope at 40X magnification for identification of *Aspergillus* species (Nugent et al., 2006).

#### Procurement of azole-susceptible *A. fumigatus*, antifungal azole discs and e-strips

*A. fumigatus* (NAIMCC-F-02473) was procured from National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, India. The fungal strain was maintained by sub-culturing on PDA slants at  $28 \pm 2^\circ\text{C}$  for 5 days to obtain conidial growth. The organism was maintained at

4°C and sub cultured every 15 days.

Antifungal discs and e-strips of itraconazole, ketoconazole, fluconazole and voriconazole were procured from Himedia Chemicals.

#### Disc diffusion assay

*Aspergillus* conidia were harvested in sterile 0.85 % saline, conidial suspension was adjusted to  $10^6$  conidia per ml and 50 $\mu$ l of this suspension was inoculated on PDA plate. Discs containing the itraconazole (10 $\mu$ g/disc), ketoconazole (10  $\mu$ g/disc), fluconazole (10 $\mu$ g/disc) and voriconazole (1 $\mu$ g/disc) were impregnated to the surfaces of inoculated plates. Plates were incubated at 28°C for 5 days to allow fungal growth. Zone of inhibition diameters were measured in millimetres (Nweze et al., 2010). An azole-susceptible *A. fumigatus* (NAIMCC-F-02473) was used as control. All the experiments were run in triplicates.

#### E-strip test

Susceptibility to different azoles in *Aspergillus* isolates was determined by E-strip test as described by Pfaller et al., 2003 with minor modifications. Isolates of *Aspergillus* species were allowed to sporulate on PDA slants for 5 days. Conidial suspensions were prepared in 0.9% normal saline supplemented with 0.1% Tween-20. The concentration was adjusted to an optical density of 0.09–0.13 at 530 nm. The PDA culture plates were inoculated with 50 $\mu$ l of prepared conidial suspension. The E-strip of itraconazole

(0.002-32 µg/ml), ketoconazole (0.002-32 µg/ml), fluconazole (0.016-256 µg/ml) and voriconazole (0.002-32 µg/ml) was placed across the central surface of the freshly inoculated PDA plate. An azole-susceptible control strain *A. fumigatus* (NAIMCC-F-02473) was used. The plates were incubated at 28°C and results were recorded for 5 days post inoculation.

#### Antifungal susceptibility testing (AFST)

*Aspergillus* isolates resistant to all tested azole drugs on agar plates were subjected to AFST using CLSI M38-A2 broth micro-dilution method (Clinical and Laboratory Standards Institute, 2008) for filamentous fungi. Briefly,  $0.4 \times 10^4$  to  $5 \times 10^4$  *Aspergillus* conidia/ml were added to 96-well micro-titre flat-bottom plate (Tarson) consisting of media with azole drugs ranging from 500 µg/ml to 0.97 µg/ml. Minimum inhibitory concentration ( $MIC_{100}$ ) was read after 48 h as the concentration of drug at which no visible fungal growth was observed. The AFST results were analyzed by using epidemiological cut-off values (ECVs) proposed by Espinel-Ingroff et al., 2011a. For itraconazole, voriconazole and ketoconazole  $MIC_{100} > 1$  µg/ml were considered as ECVs for resistant isolates. However, fluconazole  $MIC_{100} > 64$  µg/ml were ECVs for resistant isolates of *A. fumigatus*, *Aspergillus flavus* and *Aspergillus nidulans*.  $MIC_{100} > 4$  µg/ml was ECV for *Aspergillus terreus*. Clinical breakpoints  $> 2$  µg/ml have been considered for resistant *A. fumigatus* (Lass-Flori, 2014; Alastruey-Izquierdo et al., 2015).

## RESULTS AND DISCUSSION

The emergence of drug-resistant strains has become a challenge for treating *Aspergillus* diseases especially among immune-compromised patients. The majority of cases of azole-resistant diseases are due to resistant *Aspergillus* originating from the environment (Verweij et al., 2016). In the present epidemiological study, we have studied the presence of azole resistant *Aspergillus* species in agricultural fields of North India. A total of 165 fungal isolates having different morphological characteristics were recovered from 25 soil samples collected from different sites. Conidial head and shape is important feature for fungal identification (Khalil and Hashem 2018). On the basis of microscopic and morphological examination, 24.8 % were *Aspergillus* isolates.

Further, out of these isolates, 31.7% (n=13) were *A. fumigatus*, 21.9% (n=9) were *Aspergillus niger*, 12.1% (n=5) were *A. terreus*, 7.3% (n=3) were *A. nidulans*, 2.4% (n=1) were *A. flavus* and 21.9% (n=9) were identified as other *Aspergillus* species microscopically. All these isolates were screened for their azole resistance using Disc diffusion assay, E-strip test and broth micro-dilution method. In an investigation during 2012-13, 27 soil samples from Delhi and 10 soil samples from Varanasi yielded 88 and 38 *A. fumigatus* strains respectively, which were later screened for their triazole resistance (Chowdhary et al., 2014).

#### Disc diffusion method

Disc tests are inexpensive and easy to set up, and provide an ideal screening test (Alastruey-Izquierdo et al., 2015). Clinically used triazole antifungals are derivatives of either fluconazole (voriconazole and isavuconazole) or ketoconazole (itraconazole and posaconazole) as the lead compound (Dudakova et al., 2017). Therefore, in the present study voriconazole, itraconazole, ketoconazole and fluconazole were used as screening drugs. Among 13 isolates of *A. fumigatus*, 3 isolates had no inhibition zone against any azole antifungal drugs i.e. resistant to triazoles. The other isolates were resistant to atleast one azole drugs. The inhibition zones of *Aspergillus* isolates are presented in Table 1. The inhibition zone ranges reported for *A. fumigatus* susceptible strains against itraconazole and voriconazole were 11 to 21 mm and 25 to 33 mm, respectively (Espinel-Ingroff et al., 2011b, Al-Wathiqi et al., 2013). Similar results were observed in the present study.

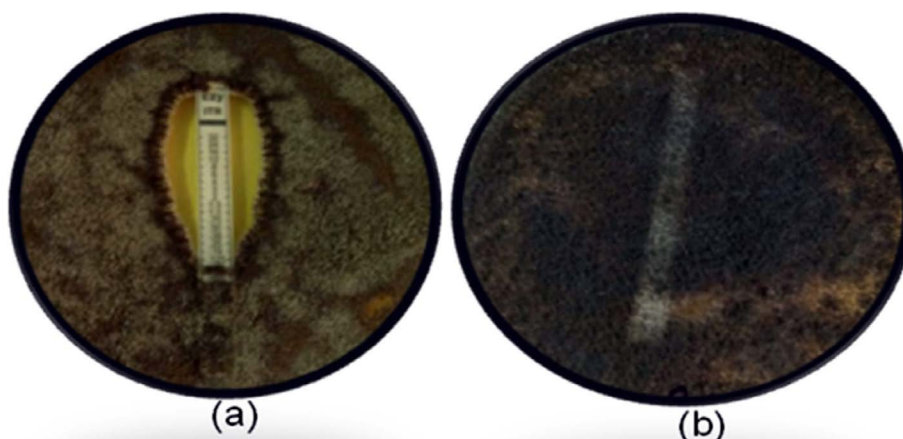
#### E-strip test

Agar based E-strip test is the modification of the CLSI reference method and is convenient and flexible (Posteraro and Sanguinetti, 2014). The strains AF-003, AF-006 and AF-008 were selected for E-strip test on the basis of disc diffusion assay, showing no inhibition zone for any of the tested azoles (Fig 2). E-strip test results were also consistent with the results of disc diffusion assay, where the drugs strips had no/negligible inhibitory effect on resistant isolates. According to Alastruey-Izquierdo et al., 2015, the E-strip test of itraconazole reveals a good correlation with the CLSI M38-AFST to detect *Aspergillus* resistance than other antifungal drugs.

**Table 1.** Zone of inhibition (in mm) of all *Aspergillus* isolates against tested azole antifungals

Isolates	Itraconazole	Ketaconazole	Fluconazole	Voriconazole
Reference strain				
AF <sup>#</sup>	19.1	25.1	8.2	26
<i>A. fumigatus</i>				
AF-001	10	18.2	-	-
AF-002	13	-	-	18
AF-003*	-	-	-	-
AF-004	21.2	-	-	14.5
AF-005	12.3	19.8	4.4	6.8
AF-006*	-	-	-	-
AF-007	15.2	17.4	-	10
AF-008*	-	-	-	-
AF-009	13.6	18	-	-
AF-010	9.2	-	-	4.2
AF-011	8.4	17.2	-	2.2
AF-012	11.4	-	-	-
AF-012	21	19.3	-	-
AF-013	10.8	14.2	1.5	3.4
<i>A. nidulans</i>				
ANd-001	50	40	12	22.4
ANd-002	35	36.4	-	40
ANd-003	50	32	14	31.8
<i>A. niger</i>				
AN-001	11	-	-	-
AN-002	11.8	-	-	-
AN-003	38	20	30	31
AN-004	40	21	35	38
AN-005	36	23	40	38
AN-006	12	-	-	-
AN-007	36	23	33	-
AN-008	13	-	10	-
AN-009	25	-	-	-
<i>A. terreus</i>				
AT-001	33	11	-	21
AT-002	31	21	14	26.4
AT-003	33	10	10	15.8
AT-004	50	20	-	-
AT-005	45	18	15	20.3
<i>A. flavus</i>	41	-	20	-
<i>Aspergillus</i> species				
ASp-001	35	20	27	26.8
ASp-002	54.8	50.4	48.6	50
ASp-003	52.6	44.7	38.2	50
ASp-004	40	-	-	-
ASp-005	5	-	-	-
ASp-006	48.9	34.1	33.5	45.7
ASp-007	40	35	37	40
ASp-008	45	30	40	36.5
ASp-009	9	-	-	-

<sup>#</sup>AF is the susceptible strain obtained from NBAIM, Mau, India, \* signifies isolates with no inhibition zone for any of the azoles used in present study, (-) signifies no zone of inhibition observed.



**Fig. 2.** E-strip result for susceptible and resistant strains. (a): Inhibition zone by itraconazole-strip against susceptible control strain, (b): no inhibition in resistant isolates.

### Antifungal susceptibility testing (AFST)

At present, CLSI is one of the universally recognized standard methods to perform *in vitro* AFST for either yeasts or filamentous fungi (Sanguinetti and Posteraro, 2018) which apply broth micro-dilution method. The antifungal activity is expressed as MIC of antifungal drug indicating the minimal drug concentration that inhibits fungal growth. In the present study, resistant and susceptible isolates were

distinguished using broth micro-dilution and were confirmed resistant on the basis of having MICs above ECVs and breakpoints. AF-003, AF-006, AF-008 had high MIC<sub>100</sub> in comparison to ECV against azole antifungals, where no hyphal growth was observed in 96 well micro-plate. No MIC<sub>100</sub> was observed for fluconazole in all the resistant isolates. Susceptible strain had MIC<sub>100</sub> < ECV against azole antifungals (Table 2).

**Table 2.** MIC<sub>100</sub> of *Aspergillus* isolates

Isolates	Itraconazole		Voriconazole		Ketoconazole		Fluconazole	
	ECV (µg/ml)	MIC <sub>100</sub> (µg/ml)	ECV (µg/ml)	MIC <sub>100</sub> (µg/ml)	ECV (µg/ml)	MIC <sub>100</sub> (µg/ml)	ECV (µg/ml)	MIC <sub>100</sub> (µg/ml)
#AF	1	0.39	1	0.78	1	0.78	64	50
AF-003	1	3.12	1	-	1	3.12	64	-
AF-006	1	3.12	1	3.12	1	3.12	64	-
AF-008	1	6.25	1	12.5	1	6.25	64	-

MIC<sub>100</sub>: Minimum Inhibitory Concentration, #AF is the susceptible strain obtained from NBAIM, Mau, India, (-) signifies no MIC<sub>100</sub>, ECV: Epidemiological cut-off values.

Hence, among 41 environmental *Aspergillus* isolates from various parts of North India, 7.3% (n=3) isolates were resistant to all the tested azole drugs, 19.5% (n=8) were diazole resistant and 17.07% (n= 7) were resistant to itraconazole only. Multi-triazole resistance was only found in *A. fumigatus* which is similar to the results observed by Chowdhary et al., 2015. The

correlation between azole-based fungicides and resistant strains has been long argued; however, in recent years resistant isolates have also been found in environmental samples in several countries (Snelders et al., 2009; Mortensen et al., 2010; Berger et al., 2017). Among all *Aspergillus* species, the azole resistance was observed maximally in *A. fumigatus*.

## CONCLUSION

Environmental azole resistance *Aspergillus* strains are present in environment of North India. This indicates the possible exposure of the population to these resistant strains. The regular screening of environmental azole resistant *Aspergillus* strains is crucial to analyze development of resistance in human pathogenic fungi and developing mitigation strategies.

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## CONFLICTS OF INTEREST

The authors declare that there are no conflict of interest.

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