Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Advances Against Aspergillosis*

Azole resistance in *Aspergillus*: global status in Europe and Asia

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Azole-resistant strains of *Aspergillus* have been reported from European and Asian countries at varying frequencies. Based on the limited rates of isolation of *Aspergillus* from clinical samples in routine practice and the limited number of the screening studies carried out so far, the true prevalence of triazole resistance and the rate of multiazole-resistant strains remain partly unknown. Also, available data are mostly for *A. fumigatus* (complex), thus the situation for non-*fumigatus* Aspergilli is less clear. In general, exposure of *Aspergillus* to antifungal agents via medical or environmental (agricultural) use of these compounds appears to have the possible major impact on acquisition of triazole resistance. Azole resistance in *Aspergillus* remains to be further elucidated by continued surveillance studies. Based on the possible association with agricultural azole use, environmental sampling appears significant as well.

Keywords: *Aspergillus*; azole; resistance; antifungal susceptibility testing

Introduction

Azole resistance in the genus *Aspergillus* initially drew attention following a report of itraconazole-resistant *Aspergillus fumigatus* strains in 1997. This report focused on three clinical isolates (AF72, AF90, AF91) with high itraconazole minimum inhibitory concentrations (MICs) (>16 μg/mL), validation of the *in vitro* resistance by a neutropenic murine model, and investigation of the possible resistance mechanisms (changes in sterol 14α-demethylase and alteration in a membrane transporter). Of note, these strains were from California and isolated in 1980s.[1] Following the identification of these resistant isolates, optimal test parameters have been proposed for both broth and agar dilution methods for generation of reproducible MICs and prediction of clinical outcome.[2]

Proposed breakpoints and epidemiological cut-off values for determination of azole resistance in *Aspergillus*

To facilitate the analysis and interpretation of the MIC data for azoles, CLSI (Clinical and Laboratory Standards Institute) and EUCAST (The European Committee on Antimicrobial Susceptibility Testing) MIC breakpoints for itraconazole, voriconazole, and posaconazole against *A. fumigatus* were initially proposed in 2009. Using these breakpoints, resistance was defined as MICs of >2, >2, >0.5 μg/mL, for itraconazole, voriconazole, and posaconazole, respectively.[3]

The issue of determination and validation of epidemiological cut-off values (ECOFFs/ECVs) and antifungal clinical breakpoints for *Aspergillus* is still dynamic and now includes further analysis by using both CLSI and EUCAST methodologies. ECOFFs/ECVs are the upper MIC values defining the wild-type distributions, and antifungal clinical breakpoints are the MIC values indicating likely clinical response. Using CLSI M38-A2 methodology,[4] ECVs have been proposed for itraconazole, posaconazole, and voriconazole against strains of six *Aspergillus* spp. (*A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, and *A. versicolor*[5]) (Table 1). Likewise, ECOFFs have been proposed for the EUCAST method and for testing *A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans* (Table 1). Based on the available microbiological data and clinical experience, EUCAST species-specific clinical breakpoints have also been proposed for some azole-species combinations[6–9]

doi: 10.1111/j.1749-6632.2012.06815.x

Table 1. ECVs (μg/mL)/ECOFFs (mg/L) for azoles against *Aspergillus* species

Species	Methodology	
Azole	CLSI (ECVs)	EUCAST (ECOFFs)
A. fumigatus		
Itraconazole	1	1
Voriconazole	1	1
Posaconazole	0.5 (0.25)*	0.25
A. flavus		
Itraconazole	1	1
Voriconazole	1	2
Posaconazole	0.25	0.5
A.terreus		
Itraconazole	1	0.5**
Voriconazole	1	2
Posaconazole	0.5	0.25
A.niger		
Itraconazole	2	4
Voriconazole	2	2
Posaconazole	0.5	0.5
A.nidulans		
Itraconazole	1	1
Voriconazole	2	1
Posaconazole	1	0.5
A.versicolor		
Itraconazole	2	ND
Voriconazole	2	ND
Posaconazole	1***	ND

Data from Refs. 5–8.
*Eyeball (statistically calculated) ECVs.
**Has recently been lowered from 1 to 0.5 mg/L since 0.5 appeared to better describe the wild-type distribution.[10]
***Statistically calculated ECVs. ND, not determined.

(Table 2). These ECOFFs and breakpoint values are under current discussion and evaluation.

Surveillance studies for detection of azole-resistant *Aspergillus* strains

Nationwide, single-center national, and international surveillance studies have been carried out to explore the possible existence and extent of azole resistance in *Aspergillus* strains. There have been reports of resistant strains in various countries, including France, Spain, Sweden, and the United Kingdom, as well as the United States and Canada.[11] Being the most commonly isolated species in routine practice, *A. fumigatus* has frequently remained as the most commonly tested species as well in *in vitro* susceptibility studies.[12,13]

Nationwide and single-center national studies

The Netherlands

A nationwide multicenter survey carried out in the Netherlands including 170 strains of *A. fumigatus* isolated over 53 years (1945–1998) showed the existence of three strains with high itraconazole MICs (64 μg/mL) and no strains with high voriconazole MICs.[14] The issue gained further importance by the demonstration of the gradual increase in itraconazole resistance (1.7 to 6% within the time period starting after 1999 till 2007) in *A. fumigatus* strains in Nijmegen, the Netherlands.[15] In the noted study by Snelders *et al.*, the isolates growing in presence of 8 μg/mL of itraconazole were tested for their *in vitro* susceptibilities against voriconazole, ravuconazole, and posaconazole as well by using CLSI M38-A microdilution methodology.[4] Importantly and for itraconazole-resistant strains, elevated MICs and reduced susceptibilities were detected for voriconazole, posaconazole, and ravuconazole as well.[15]

Another noteworthy observation in the study by Snelders *et al.*[15] was the spread of a single azole resistance mechanism. As investigated in multicenter strains in the Netherlands, as well as in strains from six other European countries (Belgium, France, Greece, Norway, Sweden, and the United Kingdom), the dominant resistance mechanism was detected to be a combination of a substitution of leucine 98 for histidine in the *cyp51A* gene and two copies of a 34-bp sequence in tandem in the gene promoter (TR/L98H). This observation has verified the existence of the resistance mechanism of TR/L98H in the Netherlands and Norway, in addition to other reports from Belgium, France, Spain, Sweden, and the United Kingdom.[15]

In addition, there were findings suggesting an environmental source rather than a person-to-person transmission for these resistant isolates. The related evidence was the dominance of the resistance mechanism in clinical isolates from epidemiologically unrelated patients, its existence in some isolates from azole-naive patients, and shorter genetic distances in microsatellite typing between the TR/L98H isolate compared to that for other azole-resistant

Table 2. Clinical breakpoints (mg/L) proposed by EUCAST for triazoles against *Aspergillus* spp.

	Species										Non-species related breakpoints	
	A. fumigatus		*A. flavus*		*A. nidulans*		*A. niger*		*A. terreus*			
Antifungal drug	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >
Itraconazole	1	2	1	2	1	2	IE	IE	1	2	IE	IE
Posaconazole	0.12	0.25	IE	IE	IE	IE	IE	IE	0.12	0.25	IE	IE
Voriconazole	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP

Data adapted from Ref. 9.
S, susceptible; R, resistant; IE, insufficient evidence; IP, in preparation.

and azole-susceptible strains. These findings suggested the existence of a spreading azole mechanism among strains of *A. fumigatus* and the need for screening for azole resistance. Also, the question of whether the agricultural use of azole-containing fungicides might have been the source for the development of azole resistance in the environment was raised.[15]

The prospective nationwide multicenter study from the Netherlands (June 2007–January 2009) showed an itraconazole resistance prevalence of 5.3% (range: 0.8–9.5%) for *A. fumigatus*, suggesting widespread multiazole resistance in the country. Importanty, 64% of the patients who harbored an azole-resistant strain were found to be azole-naive.[16]

The United Kingdom

Azole resistance was explored in clinical *A. fumigatus* strains that were received in the Regional Mycology Laboratory in Manchester, UK from 1997 to 2007. The frequency of itraconazole resistance in these strains was reported as 5%; 65% and 74% of them being cross-resistant to voriconazole and posaconazole, respectively (ECOFFs used for analysis: >2, >2, >0.5 μg/mL for itraconazole, voriconazole, and posaconazole, respectively). Importantly, the first itraconazole-resistant strain was detected to be among the strains isolated in 1999.[17]

Japan

Using the epidemiological cut-off values of 1, 0.5, and 1 μg/mL for itraconazole, posaconazole, and voriconazole, respectively, and for the clinical *A. fumigatus* strains isolated in Nagasaki University Hospital, Nagasaki, Japan, the percentages of non-wild-type isolates were found as 7.1, 2.6, and 4.1 for the denoted azoles.[18]

India

Two of 103 clinical *A. fumigatus* strains isolated at University of Delhi, India in years 2005 to 2010 were shown to have high MICs for azoles (rate of resistance: 1.9%). The MICs of these strains for itraconazole, voriconazole, posaconazole, and isavuconazole were > 16, 2, 2, and 8 μg/mL, respectively. The strains were isolated from azole-naive patients with chronic respiratory disease.[19]

International surveillance studies

Two international surveillance studies have so far been carried out for detection of azole resistance in *Aspergillus.* The prospective international surveillance study SCARE included strains from 23 participating centers and 20 countries. The prevalence of azole resistance in *Aspergillus* section *fumigati* was found to be 0–4.2% per center and resistant strains were detected in isolates from Austria, Australia, Belgium, Denmark, France, Italy, the Netherlands, Spain, Sweden, Switzerland, and the United Kingdom.[20]

In the ARTEMIS global surveillance study, isolates from 62 medical centers worldwide were tested. Among the 2008–2009 *A. fumigatus* strains, 5.8% generated elevated MICs to one or more triazoles. Most (82.3%) of these resistant strains were from Hangzhou, China and the rest were from centers in the Czech Republic, Portugal, Brazil, and the United States.[21,22]

Azole resistance in strains of *A. fumigatus* isolated from patients with specific underlying disorders

The azole resistance rates in *Aspergillus* have been explored in specific patient settings, including hematological malignancies, cystic fibrosis, and chronic pulmonary disease due to *Aspergillus*.

A prospective 4-year study, including *A. fumigatus* strains isolated from a French cohort of patients treated for hematological malignancies, revealed itraconazole resistance in only 1 of 118 strains (0.85%).[23] Azole resistance in *A. fumigatus* strains isolated from patients with csytic fibrosis was reported from France and Denmark. Itraconazole MICs of $\geq$ 2 μg/mL were detected in 4.6% of patients with cystic fibrosis at Cochin University Hospital, France. Importantly, azole-resistant *A. fumigatus* strains were detected in 20% of subjects who received itraconazole within the previous three years.[24] The report from Denmark, on the other hand, revealed the existence of azole-nonsusceptible or azole-resistant *A. fumigatus* strains in 6 of 133 cystic fibrosis patients (4.5%). All six patients had been exposed to an azole previously—46–278 weeks prior to detection of the resistant isolate and longer than that for patients with no mold isolation or those who harbored azole-susceptible *Aspergillus* isolates.[25] These findings further emphasized the well-known association between azole resistance and previous exposure.

Based on the common occurrence of very low organism burden and a negative culture, molecular studies have been carried out for detection of azole resistance in patients with chronic pulmonary aspergillosis. Triazole resistance in *A. fumigatus* strains from lungs of patients with chronic fungal disease was studied in samples that were culture negative but polymerase chain reaction (PCR) positive. Importantly, triazole-resistance mutations (L98H/TR and M220) were detected in as high as 55% of these samples.[26]

Is azole resistance increasing in strains of *A. fumigatus*?

Increase in rate of azole resistance in *A. fumigatus* strains has so far been reported only from some centers. The single-center surveillance study in the Netherlands (screening of 1912 strains isolated from 1994 to 2007) showed an increase in itraconazole resistance after 1999.[15] Similarly, among the isolates submitted to Regional Mycology Laboratory, Manchester, UK, an increase in azole resistance has been detected since 2004,[17] and the continuation of this surveillance study in 2008 and 2009 showed a continued increase in azole resistance after 2007 as well.[27]

In contrast to the reports from the Netherlands and United Kingdom, no increase has been observed in voriconazole MICs in the post- versus pre-voriconazole era in Spain.[28] Similarly, a part of the ARTEMIS study that included isolates of nine years (from 2001 to 2009) showed no consistent trend toward decreased susceptibility to any triazole by *A. fumigatus*.[29] These results indicate that an increase in azole resistance in *A. fumigatus* may constitute a current problem at least in some centers, and surveillance studies need to be carried out in each center to reveal a possible trend toward an increase in resistance.

Data available for non-*fumigatus Aspergillus*

As noted previously, most available data on azole resistance cover only the *A. fumigatus* complex. Except for these studies, there is a published report on azole resistance in the *A. niger* complex. Interestingly and overall, 52% of the isolates included in this study were itraconazole resistant. Importantly, the rates of itraconazole resistance varied remarkably from one clade to other (100, 90, 36, and 33% in *A. acidus*, *A. tubingensis*, *A. awamori*, and *A. niger*, respectively). Data obtained in this study indicated that itraconazole resistance was common in the *A. niger* complex but cyp51A mutations might not be playing an important role. Azole cross-resistance, on the other hand, was unusual among the strains of the *A. niger* complex.[30] Further data are awaited on rates of azole resistance among strains belonging to *A. fumigatus* and *A. niger*, as well as other *Aspergillus* species complexes.

Environmental studies

The possible role of agricultural use of azole-containing fungicides in emergence of azole resistance in *Aspergillus* strains evoked studies on environmental sources of resistance. In one of these environmental studies in the Netherlands, itraconazole-resistant *A. fumigatus* strains were cultivated from indoor hospital environment, soil

obtained from flower beds, commercial compost, leaves, and seeds. Remarkably, cross-resistance was observed to voriconazole and posaconazole, as to well as to azole fungicides (metcunazole, tebuconazole). Also, resistant environmental and clinical isolates were found to be genetically clustered and apart from nonresistant strains. These results suggest that colonization with azole-resistant isolates from the environment is possible.[31]

The existence of azole-resistant *Aspergillus* in environmental samples was explored in Austria, Denmark, and Spain. Multi-azole-resistant *A. fumigatus* strains were isolated from soil samples in Denmark, and an *A. lentulus* strain with high voriconazole MIC was isolated from the soil sample in Spain.[32]

Conclusions

Azole resistance in *A. fumigatus* appears to have emerged at varying rates in European and Asian countries. Further studies on clinical and environmental isolates belonging to various *Aspergillus* species complices may provide more insight and elucidation for the true prevalence, mechanism, and clinical impact of azole resistance in *Aspergillus*.

Conflicts of interest

The author does not have any potential conflicts of interests related particularly to this paper.

Otherwise, she has received investigator initiated research grant support from Pfizer and speaker honoraria from Merck and Pfizer.

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