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The impact of azole resistance on aspergillosis guidelines

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Azole resistance in *Aspergillus* species may be on the rise, with significant potential implications for the management of invasive aspergillosis. The main mechanism of azole resistance in *Aspergillus fumigatus* is via alterations of the target enzyme CYP51A. Such azole resistance is either primary or secondary (in the setting of prior azole exposure) and can be derived either from single or multiple mutations. Irrespective of the amino acid substitution type in CYP51A, azole-resistant *Aspergillus* isolates are always itraconazole resistant. There is significant variability among studies and centers in the prevalence of azole resistance, and this is a multifactorial issue. Nevertheless, the exact frequency of azole resistance is unknown, in part because of the low culturability of the fungus in patients with aspergillosis. This work aims to provide an overview of the current knowledge in *Aspergillus* azole resistance and raises questions for future research and practical implications in the management of aspergillosis.

Keywords: azoles; *Aspergillus*, resistance; guidelines

Introduction

Antifungal drug resistance is an emerging but likely underestimated public health concern given the lack of new antifungal agents in the pipeline.[1] The potential implications of azole resistance in the management of aspergillosis could be significant; voriconazole (VRC), a second-generation triazole and the recommended first-line antifungal agent for all types of invasive aspergillosis (IA),[2] would not be the optimal drug for azole-resistant IA, as its use would probably result in high failure rates. Furthermore, as the use of broad spectrum oral triazoles, such as itraconazole (ITC), VRC, and posaconazole (POSA) (given commonly as primary or secondary prophylaxis), is widespread, physicians would have to resort to logistically inconvenient, expensive, and toxic parenteral drugs in patients with azole-resistant IA. In this review, we attempt to describe the magnitude of the problem regarding epidemiology, mechanisms of *Aspergillus* azole resistance, and practical implications in the clinical management of azole-resistant IA, and we raise questions for future research.

Proposed nomenclature and mechanisms of aspergillosis azole resistance

Undoubtedly, proposed standardized nomenclature regarding *Aspergillus in vitro* resistance for clinically licensed azoles, based on both phenotypic and genotypic definitions, has been a significant advance in the field.[3] For example, a pan-azole-resistant *Aspergillus* isolate is characterized by high minimum inhibitory concentrations (MICs) for all azoles.[3] A multiazole-resistant *Aspergillus* isolate is an isolate whose MICs are in the resistance range for more than one but not all mold-active azoles.[3] An *Aspergillus* isolate resistant to an individual azole (e.g., ITC) is one that has MIC within the resistance range for only one azole.[3] Importantly, there is no definition for dose-dependent azole resistance for *Aspergillus* species as has been described for *Candida* spp. [4]

Azole resistance can be viewed either as primary (intrinsic) or secondary resistance (in the setting of prior azole exposure)[5] and can be derived either from single or multiple mutations.[6] The main mechanism of azole resistance in *Aspergillus fumigatus*

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is via alterations, either point mutations or overexpression, of the target enzyme CYP51A.[7–12] Of interest, although there has been no report of azole resistance in IA caused by *A. flavus*, investigators recently described a different mutation of the CYP51C gene for *A. flavus* than the mutations in the CYP51A gene of *A. fumigatus.*[13] Azole resistance due to decreased azole accumulation in cases of increased activity of efflux pumps (ABC, major facilitator superfamily transporters) has been also demonstrated, mainly in laboratory strains;[14] however, the clinical significance of the latter resistance mechanism has yet to be proven. Nevertheless, several azole-resistant *A. fumigatus* isolates do not have any alterations in the target enzyme or in efflux pump activity, suggesting that other uncharacterized mechanisms (e.g., reduced azole uptake) might underlie such resistance.[15]

Investigators have shown in animal models that azole-resistant *A. fumigatus* strains retain their fitness and infected animals have high fungal burden, suboptimal protection by azoles, and increased mortality.[16] In contrast, *A. fumigatus* resistance to other antifungals such as echinocandins or amphotericin B is rare, probably reflecting a fitness loss of those isolates.[17–19] Furthermore, there are specific non-*fumigatus Aspergillus* species, such as *A. terreus*[20] and *A. nidulans,*[21] that have tolerance or even intrinsic resistance to polyenes. It is important to realize that some of the azole-resistant *A. fumigatus* isolates might be "cryptic" rare strains that are erroneously identified as *A. fumigatus* in routine clinical laboratory testing. Some such isolates have been shown to belong to *A. ustus,*[22] *A. lentulus*,[23] and *A. calidoustus.*[24] Similarly, cryptic species were recently found in azole-resistant clinical isolates of the *A. niger* complex.[25] ITC resistance was high, but azole cross-resistance was unusual and the mechanism of resistance remains obscure.[25]

The association between genotype and phenotype in azole resistance for *A. fumigatus* is complex in the setting of the target enzyme CYP51A alterations. Cross-resistance between azoles is related to the specific amino acid substitution.[3] Importantly, the majority of studies have shown that irrespective of the substitution type, these isolates are always ITC resistant.[26] Therefore, ITC resistance may serve as a practical screening marker for azole-resistant *Aspergillus* isolates that would ultimately need further evaluation regarding the genotypic alteration and the degree of cross-resistance or tolerance to other triazoles. Of interest, VRC-only resistance (i.e., without cross-resistance to ITC/POSA) has been reported in the literature, albeit without a causative mechanism of resistance.[27,28]

Issues with current *Aspergillus* azole resistance *in vitro* testing

Optimal *in vitro* testing for azole resistance in *Aspergillus* spp. remains a challenge. Of note, current *in vitro* susceptibility testing is culture based and this is a major limitation in view of the suboptimal culturability of the organism. For example, the diagnostic culture yield of bronchoalveolar lavage in patients with IA is no more than 30%.[29] Furthermore, cultures might take up to five to seven days for completion, so clinicians do not have real time information for the presence of azole resistance.[30] The *in vitro* conditions employed in susceptibility testing are also artificial. Specifically, the methods used in the setting of high inoculum of conidiae in an aerobic, high-glucose environment do not simulate *in vivo* growth of the fungus, which is characterized by a low inoculum, hyphal growth, poor nutrient substrates, semianaerobic environment, and possibly biofilm formation.[31] For example, *Aspergillus* isolates that reside in cavities could develop azole tolerances in the setting of hypoxia.[32] To complicate things further, in a recent study researchers observed diverse microevolutionary alterations in *Aspergillus* isolates residing in cavitary lesions, revealing differences in azole susceptibility, mechanisms of resistance, and genetic type even within the same aspergilloma lesion.[33] In addition, *in vitro* susceptibility tests of *Aspergillus* spp. do not address the heteroresistance phenomenon or fitness cost that renders resistant strains less pathogenic. Most if not all routine microbiological laboratories do not routinely screen for cryptic *Aspergillus* spp., such as *A. lentulus*. Finally, current *in vitro* susceptibility tests do not address the correlation of the genotype derived from either a single nucleotide alteration or multiple mutations of CYP51 with *in vivo* azole resistance and azole cross-resistance.

Epidemiology of azole-resistant aspergillosis

During the past five years, there has been an increasing awareness that azole resistance in *A. fumigatus* might be on the rise, based mainly on the

observations of two European medical centers that care for different types of patient populations: Manchester Reference Center (UK), where researchers reported the presence of azole resistance mainly in azole preexposed patients with chronic pulmonary aspergillosis in the setting of underlying structural lung disease,[34,35] and the Nijmegen Medical Center (the Netherlands), where researchers reported the presence of azole resistance in patients with intrinsic *Aspergillus* resistance (no prior azole use), even in patients without underlying chronic pulmonary disease.[11,36,37] Epidemiological exposures such as agricultural fungicides have been also implicated as considerable contributors to azole resistance in the latter center.[38–42] Azole resistance seems to be a relatively recent phenomenon in several other European medical centers as well,[43,44] and its frequency (of both azole and multiazole resistance) seems to be increasing in *A. fumigatus*.[35]

In a recent study, investigators tried to address the regional magnitude of azole resistance;[36] seven university medical centers from the Netherlands participated over a 20-month period, during which all *Aspergillus* isolates were routinely tested for azole resistance in ITC plates (4 mg/L) and were incubated at 35–37 °C. *Aspergillus* isolates were identified by routine macroscopic and microscopic methods. For azole-resistant *Aspergillus* isolates identified after plating in ITC plates, broth microdilution MICs for ITC, VRC, and POSA were then performed, as well as sequencing of the target enzyme CYP51A gene. In this particular study, resistance prevalence was 5.3% per patient and 4.5% per sample; nevertheless, the prevalence varied widely (0.8–95%) between the participant medical centers, even at centers in close geographical proximity to each other. Over 80% of the *Aspergillus* spp. isolates were derived from respiratory samples, and not many cryptic *A. fumigatus* spp. were found (only 2% of isolates). The dominant mechanism of resistance was a TR/L98H mutation in the CYP51A gene that had been previously described by the same investigators.[35,45] Eighty percent of those isolates were cross-resistant to VRC; notably, the degree of resistance to VRC varied widely from 1 mg/L to 16 mg/L. In addition, 17% of ITC-resistant *A. fumigatus* isolates were also resistant to POSA. Interestingly, 64% of the patients were azole naive at the time the resistant *Aspergillus* isolate was cultured. There was no difference in the frequency of underlying diseases between patients with azole-resistant compared with susceptible *A. fumigatus* isolates; nevertheless there was a trend for patients with hematologic malignancies to harbor resistant *Aspergillus* isolates. Overall, the outcome for patients with azole-resistant IA was poor, with over 88% crude mortality,[36] although IA-attributable mortality was not estimated.

Furthermore, attempts have been made recently to evaluate the global distribution of azole resistance in *Aspergillus* species. A global network (SCARE Network) has been created in 20 participating countries from four different continents, based on a culture-based prospective surveillance.[46] Of the 2,449 unselected *A. fumigatus* isolates screened on ITC plates, 51 isolates (5.3%) were found to be ITC resistant. Similar to findings in the Netherlands study,[36] significant prevalence variability was described among the different centers (0–4.2%). Azole resistance was demonstrated in many but not all countries. In this study, 11 of the 51 (22%) ITC-resistant *A. fumigatus*-like isolates were identified as "sibling" species; of these 11 isolates, 5 (45%) were *A. lentulus*. Moreover, in agreement with most of the European studies, the main mechanism of resistance was due to the TR/L98H mutation, and 82% of the patients were azole naive. In contrast, in a recent multicenter study in which 1789 *Aspergillus* isolates from 63 medical centers worldwide were tested using an epidemiological cut-off value, *in vitro* VRC resistance was low (less than 2%) and there was no consistent trend toward decreased susceptibility for any broad spectrum triazole during a 9-year period (2001–2009).[47] Of interest, geographical niches of presumably nonwild-type *Aspergillus* isolates having high MICs to azoles were identified outside Europe, especially in China.[47,48]

Prevalence of aspergillosis azole resistance: a multifactorial issue

Undoubtedly, the tremendous variability of azole resistance prevalence among previous studies is multifactorial (Table 1): patient referral patterns, selection versus random testing of specimens, time of the study (older versus recent studies), number of isolates tested (studies examining more versus fewer than 100 isolates), differences in the screening methods (e.g., plate-based versus broth-based), work-up to exclude cryptic *Aspergillus* spp. (e.g., *A. lentulus*), differences in the definitions of *in vitro* resistance, and the testing methods or inocula

Table 1. Variability of azole resistance prevalence in *Aspergillus* isolates among medical centers and published studies: a multifactorial issue

Parameters
Patient referral patterns (acute versus chronic aspergillosis)
Selected versus random specimens
Time factors (recent versus older series)
Small collections of isolates (e.g., <100 isolates)
Differences in screening methods (microdilution versus plate method)
Exclusion of cryptic *Aspergillus* species
Rates using different denominators (patient versus isolates)
Different susceptibility methods and inocula
Different breakpoints[a] and definitions of resistance
Differences in geographic location of the center

[a]In the absence of clinical breakpoints, epidemiological cutoff values have been established as a means of distinguishing wild-type isolates of *Aspergillus* spp. by the Clinical and Laboratory Standards Institute (CLSI).[65]

account for this variability. Importantly, it is possible that the prevalence of *A. fumigatus* azole resistance is underestimated if culture-independent, molecular methods are employed. In a recent study, in which investigators used an ultrasensitive PCR amplification of the CYP51A gene in sputum specimens of both patients with different aspergillosis syndromes and healthy volunteers, it was found that PCR was significantly more sensitive than culture-based methods.[34] Specifically, PCR was positive for *Aspergillus* spp. in 79% and 71% of patients with allergic and chronic pulmonary aspergillosis, compared with 0% and 16% by culture, respectively. In culture-negative PCR-positive cases, resistance mutations were found in 55% of tested samples, which raises the question whether those isolates have a fitness cost and do not grow easily in cultures. Resistance markers were seen even in patients without prior azole exposure, as well as in patients with concurrent azole therapy and adequate serum azole levels, although the data were too limited to draw valid conclusions. Nevertheless, this study raises the question whether PCR is a better way to detect both *Aspergillus* spp. and azole resistance markers, at least in aspergillosis syndromes that are associated with low inoculum disease.

However, the controversy regarding the impact of *in vitro* azole resistance in *A. fumigatus* is enhanced by the fact that clinical failure in IA syndromes is multifactorial and associated not only with the fungal isolate and its possible biological fitness, but also with the pharmacology of the antifungal agent used and with a variety of host factors (Table 2). For example, the mode of action of antifungal drugs (fungistatic versus fungicidal) and their pharmacokinetics (absorption, tissue distribution, metabolism, drug–drug interactions) are significant factors in prognosis.[49] In the big picture, host factors continue to be the most important determinants of clinical failure of azoles in IA, due to poor immune status, site of infection, presence of tissue sequestration, severity of infection, and patient noncompliance with the drug regimen (especially in chronic IA syndromes that require several months of antifungal treatment).

Unresolved issues of azole resistance for the clinician

Whether *in vitro* azole resistance is an *independent* risk factor for clinical failure remains uncertain. Notably, previous studies regarding azole-resistant IA usually did not report on the time of onset of antifungals from the IA diagnosis, the activity of the underlying disease, whether antifungals were given intermittently or continuously or whether antifungals were given as monotherapy or in combination treatment, and the effect of mixed or subsequent infections.[11,34–36] This controversy is further enhanced by the fact that the recently described transplant-associated infection surveillance network (TRANSNET) did not report any mortality difference between patients with IA caused by either VRC-resistant or VRC-sensitive *Aspergillus* isolates.[50] The effect of pharmacokinetic parameters such as the area under the curve (AUC) and AUC/MIC of each azole, and the importance of fluctuations of azole serum levels in relationship to the development and outcome of IA caused by azole-resistant *Aspergillus* isolates, are obscure. Interestingly, although most patients with chronic aspergillosis symptoms fail to improve and remain stable, not all actually progress.[35] This observation suggests that in at least that patient population, structural underlying lung disease,

Table 2. Azole failure in invasive aspergillosis: a multifactorial issue

Host	Azole	Pathogen
Prior exposure to azoles	Intermittent versus constant exposure	Species-specific variability
Cavitary lung lesions[a]	Decreased AUC/MIC	Isolate-specific variability
Compliance	Volume of distribution	Fungal burden
Poor systemic and infection site–specific immune responses[a]	Lipophilicity	
Delayed diagnosis[a]	Sequence of azole use (e.g., FLU → POSA, ITC → POSA)	

[a]Predisposing factors for high fungal burden.
AUC, area under the curve; FLU, fluconazole; ITC, itraconazole; MIC, minimal inhibitory concentration; POSA, posaconazole.

fitness loss of resistant *Aspergillus* strains, and azole resistance may induce competing scenarios. Similarly, the suitability of using another azole sequentially and the optimal sequence of different classes of antifungal agents (either alone or in combination), as well as the "threshold" of resistance in order to start a non-VRC-containing regimen in patients with IA in each center, are still unknown. Finally, there are no studies regarding the potential effect on *Aspergillus* galactomannan kinetics in azole-resistant *Aspergillus* isolates under various treatments.

Future perspective and practical considerations

Undoubtedly, there is an urgent need for continuation of global surveillance of both clinical and environmental *Aspergillus* spp. isolates that should be based on standardized nomenclature and laboratory detection methods. A practical way to screen for azole resistance could be based on plating on ITC plates, as well as on the regular performance of thermotolerance tests to look for *A. lentulus*.[23] Furthermore, as azole resistance has been described even in patients who received antifungal treatment for less than one month,[36] it might be reasonable to screen for azole resistance all patients with culture-documented IA who are to proceed to hematopoietic stem cell transplantation or reinduction cytotoxic chemotherapy for acute leukemia, in order to estimate whether different therapeutic antifungal strategies should be adopted. We believe that in centers where the prevalence of azole resistance is known to be high (e.g., over 5%), initial treatment with different classes of antifungal agents such as an echinocandin with liposomal amphotericin B (each alone or in combination) might be prudent, although this is yet to be demonstrated. Of note, previous *in vitro* studies on the synergistic effect of combination of VRC with an echinocandin against VRC-resistant *Aspegillus* spp. showed conflicting results.[51–53] In addition, there is an urgent necessity to expand on research in PCR detection methods both in bronchoalveolar lavage and tissues, in order to employ more sensitive tests than cultures for IA diagnosis, as well as azole-resistance mutation(s) identification.[54,55] Finally, as *Aspergillus* galactomannan (GM) is increasingly considered a surrogate marker for response,[56,57] a rising GM titer in a patient with documented IA who is on triazole-based therapy might be indicative of azole-resistant IA, especially if there is documentation of adequate serum triazole levels. Further preclinical and clinical research is needed on the role of GM as a marker of azole-resistant aspergillosis.

However, as we enter the era of genomic medicine and the cost of DNA sequencing is becoming affordable (sequencing the whole human genome currently costs about $1,000), we wonder whether deep-sequencing on a large scale of *Aspergillus* isolates, and not only of the CYP51A gene, might be the answer needed to address the complex issues of compensatory mutations, multiple targets, etc. This approach proved to be valid when large-scale phenotypic and genotypic data were correlated with patient-level clinical data leading to the

establishment of compensatory mutation roles in the management of human immunodeficiency virus infection.[58] Disturbingly, autopsy rates are very low in cancer care hospitals in the Western world (e.g., at MD Anderson Cancer Center the current autopsy rate is less than 5%); therefore, we might not be aware of the true magnitude of this problem. However, the little available data we have so far suggest that azoles might have had a major positive effect in the prevalence of IA at autopsy; for example, data from our tertiary care cancer suggest that the incidence of IA in the era of widespread use of *Aspergillus*-active triazoles has gradually decreased (Kontoyiannis, submitted).

Nonetheless, it should not be extrapolated that azole resistance in *Aspergillus* will eventually result in a worldwide pandemic. The roles of the unique host and geographic niches of resistant opportunistic fungal infections are reminders of the fact that fungal resistance is a complex multifaceted issue. For example, fungal infections with *Candida krusei*[59] or *Fusarium solani*[60] did not spread beyond the niches of leukemia and hematopoietic stem cell transplantation patient populations. The same observation applies for *Scedosporium prolificans* infections that have been both geographically restricted (e.g., Australia and Spain) and typically reported in patients with underlying cystic fibrosis.[61] Likewise, *A. nidulans* is a typical pathogen only in patients with chronic granulomatous disease,[62] and *A. terreus* has been described mainly in isolated centers of Austria[63] and Texas.[64]

Conclusions

Resistance of *Aspergillus* spp. to azoles might be on the rise; nevertheless, inferences are drawn from incomplete observational data, and it is rather early to consider that azole resistance is becoming a menacing worldwide phenomenon. A better understanding of the epidemiology and the biologic plausibility of resistance would assist in the development of better diagnostic strategies (probably PCR-based assays) for both prevention and treatment. Currently, we need better field studies but not necessarily modification of guidelines.

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Conflicts of interest

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