

Review

Triazole Resistance in *Aspergillus* spp.: A Worldwide Problem?

Olga Rivero-Menendez ^{1,2}, Ana Alastruey-Izquierdo ^{1,2,*}, Emilia Mellado ^{1,2} and Manuel Cuenca-Estrella ^{1,2}

¹ Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III., Carretera de Majadahonda a Pozuelo Km. 2, Majadahonda, 28220 Madrid, Spain; orivero@isciii.es (O.R.-M.); emellado@isciii.es (E.M.); mcuenca-estrella@isciii.es (M.-C.E.)

² Spanish Network for Research in Infectious Diseases (REIPI RD12/0015)—co-financed by European Development Regional Fund “A way to achieve Europe” ERDF, Madrid, Spain

* Correspondence: anaalastruey@isciii.es; Tel.: +34-918223784

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Abstract: Since the first description of an azole-resistant *A. fumigatus* strain in 1997, there has been an increasing number of papers describing the emergence of azole resistance. Firstly reported in the USA and soon after in Europe, it has now been described worldwide, challenging the management of human aspergillosis. The main mechanism of resistance is the modification of the azole target enzyme: 14- α sterol demethylase, encoded by the *cyp51A* gene; although recently, other resistance mechanisms have also been implicated. In addition, a shift in the epidemiology has been noted with other *Aspergillus* species (mostly azole resistant) increasingly being reported as causative agents of human disease. This paper reviews the current situation of *Aspergillus* azole resistance and its implications in the clinical setting.

Keywords: *Aspergillus fumigatus*; aspergillosis; azole drug resistance; *cyp51A*; mutations

1. Introduction

Invasive aspergillosis (IA) is a life-threatening infection caused by ubiquitous saprophytic *Aspergillus* species, which are the most common cause of invasive mold infections worldwide, especially in immunocompromised patients [1]. *Aspergillus fumigatus* is the leading agent of IA [2] but also of all other forms of aspergillosis, including allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) and aspergilloma [3]. This fungus produces billions of airborne conidia due to an abundant asexual reproduction cycle and has the ability of surviving in very different environments, such as those with temperatures up to 60 °C [4].

Despite the mortality and morbidity of IA remaining high due mainly to difficulties in early diagnosis, the survival rates of these patients have improved due to advances in diagnostics and treatment. The triazoles, itraconazole (ITC), voriconazole (VRC) and posaconazole (POS), are the mainstay of treatment for aspergillosis. Isavuconazole is a new extended-spectrum triazole, and its activity against *Aspergillus* has been proven [5]. Triazoles are the only anti-*Aspergillus* agents that are orally available, making them essential for long-term therapy [6]. Although VRC is recommended as first-line therapy for IA [7,8], ITC is still commonly used for chronic and allergic non-invasive forms of aspergillosis [8,9], and POS was shown to reduce the number of invasive fungal infections in neutropenic patients [10]. Additionally, there are some alternative therapies to triazoles that can function as rescue treatments, such as echinocandins or amphotericin B [8].

2. Antifungal Susceptibility Testing and Azole Resistance within *Aspergillus fumigatus*

The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have developed reference methods to test antifungal susceptibility, which allow the detection of in vitro resistance nowadays. Both committees defined wild-type (WT) MIC (minimum inhibitory concentration) distributions in order to establish epidemiologic cutoff values (ECVs) for *A. fumigatus* and azoles [11,12]. Based on these data and taking into account the clinical outcome, pharmacokinetics and pharmacodynamics, EUCAST defined breakpoints for *A. fumigatus* and azoles (ITC > 2 µg/mL, VRC > 2 µg/mL, POS > 0.25 µg/mL and ISA > 1 µg/mL), which are used to categorize *A. fumigatus* strains as susceptible or resistant [13]. CLSI has also defined ECVs for *A. fumigatus* and azoles: ITC > 1 µg/mL, VRC > 1 µg/mL, POS > 0.5 µg/mL [11]. There are other commercial methods for in vitro susceptibility testing, such as Etest (BioMerieux, Marcy l'Etoile, France) or Sensititre YeastOne (SYO) (Trek Diagnostic Systems Ltd., East Grinstead, UK), that are complementary to EUCAST and CLSI and are easy to perform for routine use.

Since the first reported case in 1997 in clinical *A. fumigatus* isolates collected in the 1980s in the U.S. [14], an ever-growing number of triazole resistance strains have been published [1,6]. The increased description of azole-resistant *A. fumigatus* strains in the last few years may pose a threat to public health because of the lack of alternative treatment [15,16]. In addition, as in vitro antifungal susceptibility testing in *Aspergillus* is not routinely done in non-invasive settings, the prevalence of triazole resistance strains is likely to be underestimated [6].

3. Azole Resistance Development in *Aspergillus fumigatus*

Aspergillus fumigatus is usually susceptible to azoles, but as stated before, secondary resistance is increasingly reported. Since the first azole-resistant isolate detected in 1997 in the U.S., azole resistance has been increasingly reported from many other countries. Particularly in the past few years, there has been an increase in clinical resistant isolates described from the Netherlands [6]. This is well studied at the molecular level and will be further discussed in this review. The development of secondary resistance is thought to be acquired in two possible ways. In patients that suffer chronic aspergillosis and are under long-term azole treatment, resistance can develop through this exposure [17]. These patients are initially infected by a susceptible *A. fumigatus* strain that evolves to a resistant phenotype under azole treatment pressure. These resistant isolates are isogenic to the initial one that caused the infection. Camps et al. reviewed seven cases of acquired resistance during treatment showing an average delay of four months between the latest susceptible and the first resistant isolate [18]. The first reported case of this resistance route was described in 2001 in four isogenic *A. fumigatus* isolates recovered from a patient treated with ITC for a pulmonary *A. fumigatus* infection. Two of them were obtained before treatment with ITC, and two were isolated after treatment finished. The results suggested that the strain acquired resistance to this antifungal during treatment [19]. Alternatively, the use of azole fungicides in the environment that induce cross-resistance to medical triazoles in environmental *A. fumigatus* isolates has been suggested as another source of resistance development [20]. This environmental route was described in a study where a single mechanism of azole resistance was found in 94% of clinical isolates from several hospitals in The Netherlands [20], not being able to relate it to previous antifungal treatment. Finally, intrinsic azole resistance has also been described in other *Aspergillus* spp.

4. Mechanism of Azole Resistance

Since the first report of the *A. fumigatus* azole resistance strain, several studies have been published investigating the underlying molecular mechanisms. In *A. fumigatus*, the main targets of the azoles are Cyp51 proteins, encoded by two different, but related genes sharing 63% sequence identity, *cyp51A* and *cyp51B* [21]. The most frequent resistance mechanism is related to modifications in the azole

target (Cyp51A, a 14 α sterol demethylase), although other mechanisms within *A. fumigatus* have been investigated.

5. Cyp51A Mutations

Up to now, most of the *A. fumigatus* azole resistant strains have been associated with point mutations or overexpression of *cyp51A*. The *cyp51A* encodes a 14 α -sterol-demethylase, a key enzyme in the ergosterol biosynthesis pathway [22]. Ergosterol is the main component of fungal cell membranes. Triazoles bind with one of the nitrogen atoms of the triazole ring to the iron atom in the heme group located at the active site of Cyp51A [22]. This way, demethylation of C-14 of lanosterol is blocked, and ergosterol is not synthesized. Lack of ergosterol alters membrane fluidity and leads to fungal cell death [1]. Several single-nucleotide polymorphisms (SNPs), responsible for *cyp51A* amino acid substitutions, with or without tandem repeats in the promoter region of the gene, have been described. Both mechanisms affect the binding of azoles to the enzyme and lead to the development of resistance.

There are a few point mutations located at hot spot codons, whose link to azole resistance has been corroborated: (i) those associated with glycine 54 (G54), linked to cross-resistance to ITC and POS [23,24]; and (ii) amino acid substitutions at methionine 220 (M220), associated with different patterns of reduced susceptibility for triazoles [25]. Mutations in glycine 138 (G138), causing simultaneous resistance to itraconazole and voriconazole [26], and glycine 448 (G448S), resulting in VRC resistance, with some reduction in ITC and POS susceptibility, have also been reported in several studies [27–29]. Other point mutations, such as P216L, F219C, F219I, A284T, Y431C, G432S and G434C, have been occasionally described related to azole resistance, but further research is needed in order to confirm its role in the development of resistance [17,18,30–36]. In addition, a group of polymorphisms resulting in amino acid changes (F46Y, M172V, N248T, D255E and E427K) is frequently reported, alone or in combination, related to different patterns of susceptibility (they have been detected in azole susceptible and resistant strains), with consistently higher MICs than the wild type strains, although not always exceeding the breakpoint for resistance. More research is needed in order to determine the implication of each amino acid substitution (if any) in the azole profile shown by these strains (Table 1). All of these point mutations are generally described in strains isolated from patients that have been undergoing azole treatment.

A second group of *cyp51A* alterations with different resistance mechanisms has been reported, being normally described as panazole resistant. In *A. fumigatus*, this type of azole cross-resistance depends on specific mutations in *cyp51A* in combination with alterations in the promoter region, leading to multiazole-resistant strains [12,37,38]. These mechanisms are generated by combinations of *cyp51A* modifications: (i) the integration of a 34-bp tandem repeat (TR₃₄) in the promoter region of the gene, leading to an overexpression of *cyp51A* along with a substitution of leucine 98 to histidine (TR₃₄/L98H) [37]; this alteration is the most frequently identified resistance mechanism found in environmental *A. fumigatus* strains [39]; (ii) a 46-bp tandem repeat insertion in the promoter region and substitutions of tyrosine 121 to phenylalanine and threonine 289 to alanine (TR₄₆/Y121F/T289A) [40], which is related to VRC resistance; and (iii) a 53-bp tandem repeat in the promoter region without any *cyp51A* amino acid substitution [41,42].

One of the first studies on azole cross-resistance in *A. fumigatus* was performed in 17 clinical *A. fumigatus* isolates that were ITC resistant. These strains showed cross-resistance between ITC and POS, which have a similar molecule structure, but not with VRC [43,44]. Cross-resistance between azoles was studied by Howard et al. showing that 74% of the ITC resistant isolates studied were cross-resistant to POS and 65% to VRC [17]. The newest triazole isavuconazole has shown higher MICs in strains with reduced susceptibilities to other triazoles and presented a high degree of correlation with VRC susceptibility results [45]. In addition, other azole fungicides are widely used for crop protection (DMIs), which exhibit a related molecule structure to medical triazoles, leading to development of cross-resistance with azole in clinical use [46].

Table 1. Described *Aspergillus fumigatus* *cyp51A* mutations.

<i>cyp51A</i> Amino Acid No./Change	Continents	References
Described in resistant strains with a known mechanism		
G54/W/R/E/V/A	Europe	[12,17,18,23,32,47–53]
	Asia	[3,54–57]
	America	[58]
	Oceania	[59]
M220/T/V/I/K/R/L	Europe	[12,17,20,25,32,33,35,47,48,50,52,60,61]
	Asia	[54,57]
	America	[58,62]
G448S	Europe	[17,27,29,63]
	Asia	[64]
	America	[58]
	Oceania	[59]
Promoter tandem insertion + <i>cyp51A</i> amino acid No./change		
TR ₃₄ /L98H with or without S297T/F497I	Europe	[12,17,20,32,35–38,40,47,48,50–53,60,65–76]
	Asia	[3,77–87]
	America	[58,88,89]
	Africa	[90]
	Oceania	[59]
TR ₄₆ /Y121F/T289A with or without S297T/F497I	Europe	[40,47,51,52,60,66,67,75,76,91–94]
	Asia	[82,95,96]
	America	[58,88,89]
	Africa	[90]
TR53	Europe	[41]
	America	[88]
Described in resistant strains with an unknown mechanism		
G138/C/S	Europe	[17,26,31]
	America	[58]
Described both in resistant and susceptible strains		
F46Y/M172V/N248T/D255E/E42 7K or some other combinations	Europe	[17,33,34,36,53,61,65,71]
	Asia	[3]
	Oceania	[59]
F46Y/M172V/E427K	Europe	[12,17,33,34,74,75]
Occasionally described in susceptible or resistant strains		
P216L		[17,18,53,61,75,97]
F219/S/C/I		[18,32,53,58]
I242V		[12,62]
N248K		[12,34,83]
Y431/S/C		[17,31,35,59]
G432/S/A		[30,83]
G434C		[17,31]

6. Azole Resistance Mechanisms are *cyp51A* Independent

Although triazole resistance in *A. fumigatus* is mainly attributed to *cyp51A* target mutations, a recent survey of resistant isolates in Manchester showed that >50% of resistant isolates had no mutation in *cyp51A* or its promoter [98]. There is also a reported case of a Dutch patient with chronic granulomatous disease treated with azole-echinocandin combination therapy, whose resistant isolate revealed a four-to-five-fold increased expression of *cyp51A* without having any *cyp51A* alterations [2]. Therefore, other mechanisms of resistance in clinical azole-resistant isolates without *cyp51A* mutations need to be explored.

Overexpression of *cyp51B*. In *A. fumigatus*, Cyp51 proteins are encoded by two different, but related genes sharing 63% sequence identity, *cyp51A* and *cyp51B* [21]. As described before, most of the azole-resistant strains have alterations in *cyp51A*; however, the role of *cyp51B* in *A. fumigatus* azole resistance remains unclear. Several *cyp51B* polymorphisms/mutations have been observed, but have never been linked to resistance. Only one study with a clinical azole-resistant isolate without *cyp51A* mutation or over-expression showed an over-expression of *cyp51B* [99]. Further studies are required to clearly define the relationship between this mechanism and azole resistance.

Overexpression of efflux pumps. Fungi have to beat intracellular toxin accumulation in order to successfully colonize human hosts [1]. This is achieved by efflux pumps, of which there are two main categories: ATP-binding cassette (ABC) proteins, primary transporters that take advantage of ATP hydrolysis, and major facilitator superfamily (MFS) pumps, secondary transporters that use the proton-motive force across the plasma membrane [100]. In *A. fumigatus*, at least 49 ABC family transporters and 278 MFS genes have been described, which is more than four-times the number identified in yeasts like *Saccharomyces cerevisiae* [101]. However, in *A. fumigatus*, despite the great number of existing genes encoding transporters, little is known about the connection between ABC or MFS efflux pumps and triazole resistance. To date, only five transporter genes are known to be related to azole resistance: *AfuMDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4* and *AtrF*.

AfuMDR1 and *AfuMDR2* ATP-binding cassette transporters were the first described, raising the possibility that these two genes could be directly involved in drug efflux in *A. fumigatus* [102]. Another ABC transporter, *atrF*, was cloned from a clinical isolate of *A. fumigatus* resistant to ITC, and five-fold higher levels of *atrF* mRNA compared to those in susceptible strains were revealed [103]. *AfuMDR3* and *AfuMDR4* were identified to be connected with triazole resistance in a study where resistant *A. fumigatus* mutants showed either constitutive high-level expression of both transporters or induction of expression when exposed to ITC. Two out of 23 mutants seemed to be ITC resistant due to overexpression of these genes, although evidence of a direct relationship between them and an ITC resistant phenotype is lacking. *AfuMDR3* has great similarity to MFS, and *AfuMDR4* is a member of the ABC proteins family [24]. Additionally, *AfuMDR4* has been shown to be induced with VRC in complex *A. fumigatus* biofilm populations and that this contributes to azole resistance [104]. Furthermore, exposure of a clinical azole-susceptible *A. fumigatus* isolate to VRC showed upregulation of five transporters of the ABC superfamily (*abcA-E*) and three of the MFS (*mfsA-C*) [105]. Lastly, a demonstrated link between transporters and azole resistance was the azole-induced expression of *cdr1B*. A *cdr1B* deleted mutant resulted in a four-fold susceptibility reduction in ITC MICs in an *A. fumigatus* clinical resistant isolate [106]. However, further studies are warranted in order to properly understand the relationship between the overexpression of pump efflux and azole resistance mechanisms in *A. fumigatus*.

Cholesterol import. The import of exogenous cholesterol under aerobic conditions, as a substitute for ergosterol after azole treatment, has also been described as a mechanism of resistance. The activity of ITC against *A. fumigatus* is compromised when cholesterol serum in RPMI medium is present [107]. In *A. fumigatus*, a sterol-regulatory element binding protein (*SrbA*) that plays a role in the azole resistance by *erg11* (*cyp51A*) regulation has been characterized [108]. The *srbA* null mutant (Δ *srbA*) was highly susceptible to FLC and VRC, which was explained by a reduction in *erg11A* transcript in response to both azoles. However, further studies on the genetic regulatory network mediated by *SrbA* in *A. fumigatus* and its role in triazole drug interactions need to be carried out [109,110].

Role of Hsp90. Heat shock protein 90 (Hsp90) is a eukaryotic molecular chaperone that helps crucial regulatory proteins in their folding, transport and maturation steps under environmental stress. Its involvement in the resistance of *Candida albicans* to azole and echinocandin antifungals is well established, but the function of Hsp90 in *A. fumigatus* remains unclear [111]. Using *S. cerevisiae* mutants expressing different levels of this chaperone, it was revealed that Hsp90 potentiates the acquisition of azole resistance and plays a key role in its continuance once it has been acquired. In *C. albicans* and

Aspergillus terreus, Hsp90 inhibitors can beat azole and echinocandin resistance in vivo [112]. However, the mechanisms by which Hsp90 controls these functions remain to be fully investigated.

HapE mutation. Another described mechanism is caused by a mutation in *HapE*, a CCAAT-binding transcription factor complex subunit. Two isogenic isolates with the wild-type *cyp51A* genotype, one azole susceptible isolated before treatment and the second with a resistant phenotype isolated post-treatment, were whole-genome sequenced in order to detect the resistance conferring mutation. Six out of a sixty-nine of identified point mutations in protein-coding regions were confirmed, and sexual crossing experiments revealed that a P88L substitution in HapE was the only one leading to resistance in progeny. This mutation in HapE can lead to a resistant phenotype by itself, as it was proven by cloning the mutated *hapE* gene into an azole-susceptible reference strain. This increase in resistance has been suggested to be due to a gain of function mutation if the mutated Hap-complex binds to a CCAAT-box in the promoter region of *cyp51A* and induces its expression [113].

7. Prevalence of Azole Resistance in *Aspergillus fumigatus* throughout the World

To date, Europe is the continent with the highest reported azole resistance in *A. fumigatus* (Table 2). Two reports in the late 2000s in the Netherlands and UK raised the alarm about an increase of azole resistance cases. The first one, in 2007, involved a series of Dutch patients suffering IA caused by panazole resistant strains, even those who had not been under azole treatment. One new resistance mechanism was found in these strains, TR₃₄/L98H [37,38]. The second study, in 2009, described a wide range of *cyp51A* mutations found in patients in the U.K., becoming clear that a dramatic increase in azole resistance in *A. fumigatus* was occurring [17]. Since then, azole resistant cases in clinical samples have been reported in almost every European country, including Austria [70], Belgium [68,76,92,94], Denmark [35,61,66,70], France [19,27,30,48,50,73,91,114], Germany [32,47,51,60,72], Greece [115], Italy [36], The Netherlands [18,20,37,38,40,41,53,65,67,74–76], Poland [69,116], Portugal [117], Romania [118], Spain [12,23,25,29,34,37,49,63,70,93,119], Sweden [120], Turkey [71] and the UK [17,26,31,33,65]. Even though G54 and M220 point mutations have been occasionally reported in Europe since they were described [12,17,18,20,23,25,32,35,48,50,51,60,71], the TR₃₄/L98H is by far the most common mutation found, both in environmental and clinical samples. Since its first report in 2007 in Spanish and Dutch isolates [37], TR₃₄/L98H has been detected across Europe (Figure 1) [12,32,35,38,41,48,50,51,53,60,67,69,71,75]. In 2009 a new resistance mechanism, TR₄₆/Y121F/T289A, was identified in The Netherlands [40]. Since then, it has also been reported in several countries [39,51,60,66,67,75,76,91–93]. Azole resistance in environmental strains in Europe has been commonly detected, with TR₃₄/L98H and TR₄₆/Y121F/T289A being the most often described mechanisms (Figure 1), and therefore, their emergence has been related with the extensive use of agricultural fungicides. Van der Linden et al. found that out of 140 environmental resistant strains, 14 had the TR₄₆/Y121F/T289A mechanism, while 126 had TR₃₄/L98H [40]. In Germany, an analysis of 455 environmental isolates revealed 45 that harbored the TR₃₄/L98H mutation and six TR₄₆/Y121F/T289A [47]. Another analysis reported 16% resistance (to ITC and POS) in environmental *A. fumigatus* isolates in Italy [36]. Other, less frequent point mutations have been described as related to the azole-resistant phenotype, but further research is needed in order to confirm it.

Table 2. Azole resistance prevalence in *A. fumigatus* by continent and/or country. Only significant publications with more than 50 isolates tested are reported.

Continent/Country	% Resistance	Source of the Isolates	References
Europe			
Belgium	5.7	C	[76]
France	0.85–10.6	C	[30,48,50]
Germany	1.1–12	C and E	[32,47,60]
Netherlands	2.1–20	C and E	[20,53,67,74]
Poland	2.25	C	[69]
Spain	1.8	C	[63]
Turkey	10.2	C	[71]
United Kingdom	6.6–28	C	[17,33]
Other continents			
Asia *	1.9–11.1	C and E	[55,77,78,80–86,121]
Africa (Tanzania)	13.9	E	[90]
America (USA)	0.6–11.8	C	[58,122]
Oceania (Australia)	2.6	C	[59]
International surveillance studies			
America-Asia-Australia-Europe	1.4–5.8	C and E	[52,70,123,124]

C = clinical strains, E = environmental strains; * including China, India, Iran, Japan, Kuwait and Pakistan.

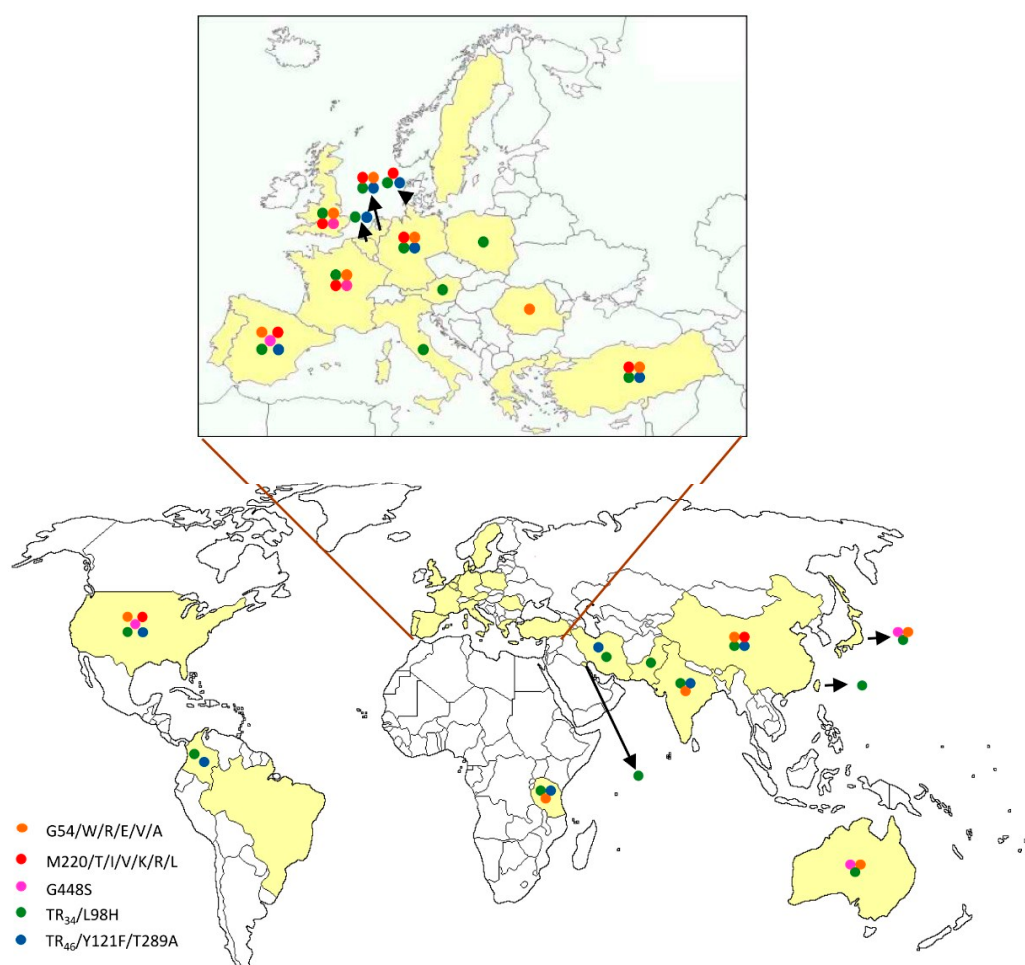


Figure 1. Worldwide distribution of azole resistance in *A. fumigatus* by mechanisms.

Reports from Asiatic countries suggest that triazole resistance rates in Asia are lower than in Europe (Table 2). The first two reports describing azole resistance in *A. fumigatus* in this area were

published in 2005. One was from clinical strains from Taiwan, where two out of 40 isolates showed azole resistance, but mutations in *cyp51A* were not investigated [125]; and the second one was based on six isogenic isolates obtained from a Chinese patient treated with azoles and suffering from lung aspergilloma. ITC resistance was found in four post-treatment isolates, one of them with a M220I mutation and the rest with G54R [54]. Several other cases have been reported since then. The ARTEMIS global antifungal susceptibility program included more than 100 medical centers worldwide and detected several clinical isolates from China that had a TR₃₄/L98H resistance mechanism [123]. This alteration has also been reported in 7.9% of the multi-azole resistant strains isolated from azole-naïve patients in Taiwan [87] and in three out of fourteen resistant clinical isolates in Pakistan [85]. In contrast, TR₃₄/L98H has not been described in Japan, with reports showing a low azole resistant strains rate. Kikuchi et al. found three resistant isolates out of 171 *A. fumigatus* clinical strains isolated between 1987 and 2008 [121]. Some novel mutations have been reported in this country, such as the P216L [97] or F332K [126], and the G448S and TR₄₆/Y121F/T289A mechanisms were recently identified in Japan for the first time [64,95]. Azole resistance prevalence in *A. fumigatus* is also low in India, where three studies revealed the presence of TR₃₄/L98H as the resistance mechanism in clinical isolates: 44 out of 630 (6.9%), two out of 103 (1.9%) and 10 out of 685 (1.5%) [3,80,81]. Similar findings have been observed in Middle East countries, like Iran (3.5% of clinical samples) [84] or Kuwait (two out of 16 clinical isolates and one out of 50 environmental isolates) [77]. Azole resistance in environmental strains in Asia is also lower than in Europe (Table 2). In fact, a recent report on the use of azole fungicides on a pumpkin farm revealed no azole resistance in 50 *A. fumigatus* isolates [127]. Several environmental studies have been performed in India, describing the TR₄₆/Y121F/T289A mechanism for the first time in Asia in isolates from agricultural fields [82] and showing that 44 out of 630 *A. fumigatus* sampled from the soil of paddy fields, tea gardens, cotton trees, flower pots and indoor air of hospitals were resistant and harbored the TR₃₄/L98H resistance mechanism [81]. A report from Iran described 12.2% of environmental resistant strains [79], and in Kuwait, 7% of environmental samples were also resistant [78], all of them carrying TR₃₄/L98H. This difference in environmental azole resistance rates between Asia and Europe could be due to the lower use of azole fungicides in Asian countries [128].

The first study involving a large number of isolates in the U.S. included 181 *A. fumigatus* isolates from transplant patients with proven IA from 2001–2006 (multicenter prospective study). Only one of these isolates was triazole resistant [122] and indicates a low azole resistance prevalence in this country. Similarly, 1096 *A. fumigatus* clinical strains from all over the U.S. collected between 2011 and 2013 were studied; 51 of them were sequenced for *cyp51A* mutations. One isolate possessed the M220I mutation in *cyp51A*, and 13 isolates had another mutation, I242V; TR₃₄/L98H was not identified [62]. A recent comprehensive study in the U.S. included 220 clinical *A. fumigatus* isolates obtained from 2001–2014, with the description of two isolates harboring TR₃₄/L98H mutations and the other two with TR₄₆/Y121F/T289A. This was the first report of both resistance mechanisms in *A. fumigatus* isolates in the United States. Other point mutations detected in the 26 azole resistant strains were G54R/W/E, M220I/K/V, G138S/C, G448S and F219S [58]. To our knowledge, no environmental sample studies have been reported in this country yet, but there is also lower use of fungicides in the U.S. as compared to Europe [128].

Some investigations have been carried out in South American countries, such as Brazil, where six out of 170 clinical *A. fumigatus* collected between 2000 and 2012 showed azole resistance, but neither the TR₃₄/L98H nor the TR₄₆/Y121F/T289A mechanisms were found [129]. An environmental study has been carried out in Colombia, known to be the fourth country in the world for pesticide use, 30% of which are fungicides. Sixty soil samples from flower beds and flower fields were analyzed, describing one TR₃₄/L98H, 17 TR₄₆/Y121F/T289A and one TR53 isolates [88]. Colombia is the second biggest flower exporter after The Netherlands, which could explain the high environmental azole resistance rate in *A. fumigatus* [129].

Azole resistance has also been reported in Africa; 15 out of 108 environmental samples taken in Tanzania were azole resistant, 11 of them with the TR₃₄/L98H mutation and four with

TR₄₆/Y121F/T289A [90]. Another study in the same country describes G54E as responsible for 46.4% of resistant environmental *A. fumigatus* isolates from this country [118]. To our knowledge, no reports from clinical samples have been published in Africa yet.

In Australia, 418 *A. fumigatus* clinical strains were collected from 2000–2013, revealing nine isolates with reduced susceptibility to ITC, VRC and POS. All of them had between two and five amino acid substitutions, including G54R, F46Y, Y431S, G448S, M172V, N248T, D255E, E427K and TR₃₄/L98H, the latter being identified in two isolates. The first TR₃₄/L98H *A. fumigatus* was recovered in 2004, and it is believed to be Australian-acquired in a patient on long-term ITC therapy, while the second isolate was suspected to have been acquired in Europe while the patient was travelling in 2012 [59].

8. Azole Resistance in Other *Aspergillus* Species

A shift in epidemiology of fungal infections towards a greater number of species able to cause disease in humans has occurred [130]. The leading cause of IA is *A. fumigatus* (85%), followed by *A. flavus* (5%–10%), *A. terreus* (2%–10%) and *A. niger* (2%–3%) [100]. However, the use of molecular tools has led to the description of new species within the genus *Aspergillus*. Some of these species are considered cryptic or sibling because they are difficult to differentiate by classical methods, and they have been frequently misidentified. Their prevalence in the clinical setting has been reported to be between 10% and 15% in two studies. The TRANSNET (Transplant-Associated Infection Surveillance Network) study included 218 *Aspergillus* isolates from transplant recipients with proven or probable IA from 2001–2006 from the U.S. and documented an 11% cryptic species [131]. The FILPOP study (population-based survey of filamentous fungi) from Spain described 15% cryptic species among 323 isolates analyzed [119]. The importance of these cryptic species in the clinical setting is based on their different susceptibility profile, as they are frequently more resistant to the antifungals available [132]. As these cryptic species are difficult to differentiate, it has been recommended that when using classical identification methods in the clinical setting, an *Aspergillus* isolate should be classified to the “species complex” level, thereby accounting for gathering all closely-related cryptic species.

The *Aspergillus fumigatus* complex includes several species that have been reported in human infections: *Aspergillus lentulus*, *A. udagawae* (syn. *Neosartorya udagawae*), *A. pseudofischeri* (syn. *Neosartorya pseudofischeri*), *A. viridinutans*, *A. fumigatiaffinis*, *A. fumisynnematus* and *A. hiratsukae* (syn. *Neosartorya hiratsukae*) [119,131,133]. Antifungal susceptibility testing of these species revealed heterogeneous patterns. *Aspergillus lentulus*, *A. fumigatiaffinis* and *A. udagawae* show high MICs for AmB, with the first two of these also having high MICs for azoles, but *A. udagawae* has intermediate values for VRC and low MICs for ITC or PCZ. *Aspergillus viridinutans* and *A. pseudofischeri* have reduced susceptibility for azoles, but not for AmB, and *A. hiratsukae* and *A. fumisynnematus* are susceptible to all drugs [132–137].

The *A. niger* includes *A. tubingensis*, the second most frequent species of the complex in clinical isolates, and has been found with similar prevalence as *A. niger* in some studies [76,119]. *Aspergillus awamori* and *A. foetidus* have also been described in clinical samples, although there is debate about their classification as new species or subspecies of *A. niger* [138]. The susceptibility profile of these species is isolate dependent, and three patterns have been described regarding ITC: low MICs, high MICs and isolates that show a paradoxical effect (which are able to grow in the presence of high antifungal concentrations, but remain fully susceptible at intermediate-to-low concentrations [139]) for this antifungal [140]. *Aspergillus niger* and *A. awamori* have been reported to have higher MICs to azoles than *A. tubingensis* [141].

Aspergillus flavus is the second most common *Aspergillus* causing IA, and it is reported as the most prevalent in countries with arid climates, such as those in the Middle East, Africa and Southeast Asia, as it is capable of surviving in extreme conditions [142]. *Aspergillus alliaceus* is part of the *A. flavus* complex. This species has elevated MICs to AmB and echinocandins, but is variable regarding azoles. The first report describing *A. alliaceus* stated that ITC was the most active antifungal in vitro against this mold [143], but the first study reporting IA caused by *A. alliaceus* (together with *A. flavus*) defined

VRC as the best option for treatment, as the isolate tested was resistant to ITC and POS [144]. VRC resistance has also been reported in clinical strains of *A. flavus*, and T788G and Y319H mutations in the *cyp51C* gene have been found to be associated with these high MICs to VRC [145,146].

Aspergillus terreus shows high MICs to AmB both in vitro [147,148] and in vivo [149], and reduced susceptibility to azoles has also been described. A study from 2012 reports a *cyp51A* mutation, M217I, in some clinical *A. terreus* isogenic isolates causing ITC resistance [150]. The *A. terreus* complex includes *Aspergillus alabamensis*, *A. floccosus*, *A. neoafrikanus*, *A. aureoterreus*, *A. hortai*, *A. pseudoterreus* [151] and *Aspergillus citrinoterreus*. They all have high MICs to AmB, but *A. hortai* and *A. citrinoterreus* are more susceptible to azoles than *A. terreus* [152,153].

The *Aspergillus ustus* complex is known for its elevated MICs to most drugs. *Aspergillus calidoustus* was described in 2008 as being able to grow at 37 °C, in contrast to *A. ustus*, and has been isolated from human infections [154]. Triazoles have been reported to be inactive in vitro against *A. calidoustus* [122], and the same has been reported of other antifungal classes, so it is considered a multiresistant species. Other cryptic species with high MICs to all antifungals in this complex are *A. keveii* and *A. insuetus*, also isolated from clinical samples [155].

9. Treatment Options

Mortality rates in patients infected with azole-resistant strains (ITC > 2 µg/mL, VRC > 2 µg/mL, POS > 0.5 µg/mL, determined by the CLSI reference method) are higher than those affected with azole-susceptible ones (88% vs. 30%–50%) [53]. As mentioned above, VRC is the primary treatment for IA, but liposomal amphotericin B (L-AMB) is recommended as an alternative therapy [156]. L-AMB was demonstrated to develop no cross-resistance in a murine model of disseminated azole-resistant aspergillosis, being either active against azole-susceptible or azole-resistant strains [157]. However, this drug is not recommended to treat infections caused by *A. terreus* or other AMB-resistant species. Another approach to consider is an antifungal combination therapy that leads to a synergistic response. A great number of in vitro, in vivo and clinical studies have tested various antifungal combinations and found some of them effective against *A. fumigatus* [158]. Recent studies have focused on the combination of an azole, normally VRC, with an echinocandin, both for azole-susceptible and azole-resistant *A. fumigatus* strains. The efficacy of this combined therapy mainly relies on anidulafungin (AND) [159], which is currently not licensed for the treatment of IA. In one clinical study, mortality rates were 27.5% for monotherapy and 19.3% for combined therapy of VRC and AND [160]. In a murine model, AND was successful against 45% of VRC-resistant strains when used as monotherapy [161]. Further studies for combined therapy are warranted in order to find alternative treatment options, given the limitations of current monotherapy. Although azole-resistant strains have been present in clinical samples for more than two decades, it has been suggested that first-line therapy should remain as azoles whilst local azole resistance prevalence remains below 10% [162]. Still, therapeutic options for IA should be revised taking this issue into account.

10. Conclusions and Recommendations for Clinical Practice

Clinical and environmental triazole resistance in *Aspergillus* species is a growing public health concern that has become a worldwide problem. Even though the highest rates of triazole resistance have been described in Europe, several cases have been reported in every continent, and new resistance mechanisms are being described. Despite *A. fumigatus* being the most common *Aspergillus* species, triazole resistance has also been identified in many cryptic species of *Aspergillus*. Therefore, the morphological identification of an isolate cannot always drive the treatment strategy. We recommend performing antifungal susceptibility testing on every *Aspergillus* isolate associated with IA in order to select the best antifungal treatment. In addition, the prevalence of resistant strains should be investigated in every country to understand the prevalence of resistance and to adjust therapeutic options where high rates of resistant isolates are present. Moreover, the development of

molecular methods to detect azole resistance in culture-negative infections could be very useful in laboratory practice.

It is important to investigate more extensively the origin of environmental samples that are resistant to triazoles, since measures to reduce the use of agricultural azoles could be an important step in reducing resistance rates in the clinical setting, as stated in the technical report published by the European Centre for Disease prevention and Control (ECDC) [163].

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