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Original Article

Triazole resistance in *Aspergillus fumigatus* isolates from patients with cystic fibrosis in Italy

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Abstract

Background: Aspergillus fumigatus is frequently recovered from respiratory secretions of cystic fibrosis (CF) patients. Azole resistance has been increasingly reported.
Objectives: To assess the prevalence of azole resistance in *A. fumigatus* isolates from patients followed by two CF centers of northern Italy.
Methods: 423 isolates (220 patients) were screened for azole resistance. Resistance was confirmed with the EUCAST method and *cyp51A* gene sequencing. Microsatellite genotyping was performed and results were compared with those of environmental resistant isolates.
Results: No resistance was detected in one center, while 8.2% of the patients of the other center harbored resistant isolates. The TR_{34}/L98H alteration in the *cyp51A* gene, present in seven cases, resulted associated with poor in-vitro activity of all tested azoles.
Conclusions: The environmental origin of the resistance seems to be probable since azole resistance was found also in naïve patients and an identical microsatellite genotype in clinical and environmental isolates was observed.
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Keywords: Aspergillus fumigatus; Azole resistance; Italy; Cystic fibrosis

1. Background

Cystic Fibrosis (CF) is the most common severe genetic disease in the Caucasian people, affecting around a newborn on 3000 in Italy (data from Italian National Registry). CF is a

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chronic and progressive disease caused by mutations in the Cystic Fibrosis Transmembrane Regulator (CFTR) gene encoding a chloride channel present on the membrane of epithelial cells. These mutations result in alteration of electrolyte transport and production of abnormally thickened secretions with progressive damage of various organs. Pulmonary involvement is present in more than 90% of the patients and it is the leading cause of morbidity and mortality. Abnormal viscous bronchial secretions impair mucociliary clearance and facilitate the entrapment and proliferation of inhaled bacteria and fungi.

Aspergillus species, mainly *Aspergillus fumigatus*, are the predominant filamentous fungi recovered from respiratory secretions of CF patients. In these patients, the fungal colonization of the respiratory tract may lead to exacerbation of respiratory symptoms that improve with antifungal treatment and in some patient colonization is complicated by allergic bronchopulmonary aspergillosis (ABPA), a condition that occurs mainly in older children and adults with a prevalence ranging from 6% up to 25% [1]. Invasive aspergillosis and aspergilloma have been rarely reported [2]. Invasive aspergillosis is more frequently observed in the setting of lung transplantation.

Orally administrable azole antifungals, such as itraconazole and voriconazole, are the cornerstone in the treatment of aspergillosis in such patient population. Itraconazole is the primary choice to treat allergic and chronic aspergillosis, while voriconazole is the first-line agent for invasive infection.

In the last decade, azole-resistance has been increasingly reported as a result of both in vivo selection during long-lasting azole therapy and acquisition from the environment linked to the widespread use of azole fungicides in agriculture [3]. Five specific azoles fungicides (propiconazole, bromuconazole, epoxiconazole, difenoconazole and tebuconazole) show a molecular structure very similar to the medical azoles and West-Europe represents the region with the highest fungicide use in the world [3]. The isolation of azole-resistant *A. fumigatus* strains from the environment has been recently reported also in Italy [4].

Azole resistance in *A. fumigatus* is mainly linked to mutations in the *cyp51A* gene, encoding lanosterol 14α-demethylase, target of azole antifungals [5]. The dominant resistance mechanism in environmental isolates consists in a point mutation leading to an amino-acid substitution at codon 98 coupled with the alteration consisting in a 34-base pair tandem repeat in the promoter (TR_{34}/L98H) and it is often associated with a multi-azole-resistant phenotype. Recently, TR_{46}/Y121F/T289A mutation has been identified in both environmental and clinical isolates [6]. Resistant isolates from patients under chronic azole therapy are reported to harbor also different mutations in hot spot codons such as G54, G138, M220, Y431, and G448 [3].

The aim of this study was to evaluate prospectively the prevalence of azole resistance among *A. fumigatus* isolates from patients followed in two large CF centers of northern Italy.

2. Methods

A prospective study was conducted to assess the prevalence of azole resistance in *A. fumigatus* isolates in patients followed by the two main CF centers of northern Italy, one located in Milano (Fondazione IRCCS Ca′ Granda-Ospedale Maggiore Policlinico) and the other in Verona (Azienda Ospedaliera Universitaria Integrata di Verona), each following more than 800 patients per year.

2.1. Isolates

Isolates from respiratory tract samples (420 sputum and 3 bronchial secretions) cultured during routine diagnostic procedures from December 2013 to May 2015 were collected, namely 196 from 97 patients of the CF center of Milano, one of which was a lung transplant recipient, and 227 from 123 patients of the center of Verona. All patients with positive cultures for *A. fumigatus* were included.

Demographic data and previous antifungal treatments were collected for each case.

2.2. Identification and screening for azole resistance

A total of 423 isolates, identified as *A. fumigatus* species complex on the basis of macroscopic and microscopic morphology, were screened on Sabouraud dextrose agar (SDA, Biolife, Milano, Italy) plates added of azoles, to detect azole resistance. Briefly, conidia from a 3-day-old culture on SDA were suspended in sterile distilled water added of 0.05% Tween 20 (Sigma-Aldrich, St. Louis USA) (about 0.5 McFarland standard). The suspension was inoculated on three plates prepared in-house containing SDA supplemented with azoles and on one plate of SDA used as control. The plates were prepared by adding azoles dissolved in dimethyl sulphoxide (Sigma-Aldrich) to reach a final concentration of 4 mg/L, 1 mg/L and 0.5 mg/L of itraconazole (Sigma-Aldrich), voriconazole (Sigma-Aldrich), and posaconazole (Sigma-Aldrich), respectively [7]. An *A. fumigatus* resistant strain (IUM 11-0396) was used to validate each test. Plates were incubated at 37 °C and examined after 48 h.

2.3. Susceptibility testing

Isolates able to grow on azoles-containing agar plates were tested by broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology [8]. The minimal inhibitory concentration (MIC) of itraconazole, voriconazole, posaconazole and isavuconazole (Basilea Pharmaceutica, Switzerland) was determined visually as the lowest concentration of drug giving a complete inhibition of fungal growth. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were included as quality control in each test. According to the EUCAST breakpoints, isolates with MIC of itraconazole and voriconazole ≥ 4 mg/L, those with MIC of posaconazole ≥ 0.5 mg/L and those with MIC of isavuconazole ≥ 2 mg/L were considered resistant. Those with MIC of itraconazole, voriconazole and isavuconazole ≤ 1 mg/L, those with MIC of posaconazole ≤ 0.125 mg/L were considered susceptible [9–11].

2.4. Genotyping testing and cyp51A sequencing

Genomic DNA was extracted from the azole-resistant isolates (one single colony from each patient) using PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA).

A. fumigatus sensu stricto isolates were identified by amplification and sequencing of a portion of the β-tubulin gene, as described previously [4].

The entire *cyp51A* gene was amplified and sequenced as described previously [4]. The *cyp51A* sequence from *A. fumigatus* strain 237 (GeneBank accession number: AF338659) was used as wild-type reference. The *cyp51A* gene promoter was amplified using the primers PA5 and PA7 as described by Mellado et al. [5].

2.5. Microsatellite analysis

Resistant isolates were genotyped by microsatellite analysis using the primers STRAf3A, STRAf3B, STRAf3C, STRAf4A, STRAf4B and STRAf4C as described previously [12]. The allelic profiles were compared with those of resistant environmental isolates previously genotyped [4]. In order to compare the results of CF and environmental isolates, the isolate IUM 11-0396 was used to normalize the sizes. Microsatellite profiles were imported into GenAlEx 6.5 [13] for genetic similarity matrix calculation. MEGA software (version 4.0) [14] was used to design a dendrogram by the UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) clustering algorithm, using the distance matrix generated by GenAlEx 6.5.

3. Results

3.1. Patients and isolates

A total of 423 *A. fumigatus* isolates from respiratory samples of 220 patients affected by CF were collected. The median age of the patients with *A. fumigatus* in sputum was 25 years (range 3–63), 113 were males (51.4%). No patient referred to the CF center of Verona had received an antifungal drug, while 22 of 97 patients of the CF center of Milano had received an azole in the six months prior to the isolation of the fungus, namely 21 patients had been treated with itraconazole and one with voriconazole; 55 patients did not receive antifungal therapy and 20 were exposed to itraconazole between one and ten years before enrolment.

3.2. Screening for azole resistance and susceptibility testing

Thirteen *A. fumigatus* isolates from eight patients were able to grow on at least one azole-containing agar plate.

Broth microdilution method, according to EUCAST, confirmed itraconazole and isavuconazole resistance in 12 isolates (the thirteenth was intermediate for itraconazole). Eleven of these isolates were also resistant to posaconazole. Two isolates were resistant to voriconazole, and the others were intermediate. One isolate showed a multiple resistance to azoles (Table 1). Only one isolate was susceptible to isavuconazole (Table 1).

Table 1
MICs, *cyp51A* alterations, allelic profiles at six microsatellite loci for azole-resistant *A. fumigatus* isolates and demographics of CF patients.

Patient	Age (yr)/sex	Isolate#	Sample date (d/m/y)	Antifungal treatment	MIC (mg/L)				*cyp51A* alteration	Fragment size as bp (number of repeats)					
					ITZ	VRZ	PSZ	ISZ		STRAf3A	STRAf3B	STRAf3C	STRAf4A	STRAf4B	STRAf4C
1	22/M	14-0006/A	27/01/14	ITZ	>16	2	1	4	TR_{34}/L98H	202	164	75	185	179	225
		14-0006/B			>16	2	1	4							
		14-0006/C			>16	2	1	4							
		14-0096/A	13/06/14	ITZ	>16	2	1	4	TR_{34}/L98H	n.d.	n.d	n.d	n.d	n.d	n.d
		14-0096/B			>16	2	1	4							
		14-0096/C			>16	2	1	4							
2	35/F	14-0042	17/03/14	none	>16	1	1	4	TR_{34}/L98H	294	160	78	185	187	173
3	20/F	14-0071	14/05/14	none	>16	1	0.25	4	TR_{34}/L98H	199	164	75	185	179	225
4	32/F	14-0198	17/09/14	ITZ	>16	4	1	16	TR_{34}/L98H	199	158	85	178	184	185
5	26/F	14-0199	22/09/14	none	16	4	0.5	4	TR_{34}/L98H	199	164	75	185	179	225
6	22/M	14-0215	14/11/14	none	>16	1	1	0.25	F219I	208	164	78	250	254	161
7	36/M	14-0218	14/11/14	none	>16	2	1	4	TR_{34}/L98H	310	158	80	178	184	185
8	11/F	15-0055	22/04/15	ITZ	16	1	0.25	4	TR_{34}/L98H	142	164	80	178	187	161

ITZ: itraconazole; VRZ: voriconazole; PSZ: posaconazole; ISZ: isavuconazole; n.d.: not done.

No resistant strain was isolated in the CF center of Verona. The azole-resistant isolates were cultured from eight single patients out of the 97 followed in the CF center of Milano (prevalence rate 8.2%). In addition, strains from a same patient isolated five months later were analyzed.

Median age of patients harboring resistant strains was 24 years (range11–36); five were females. Three patients harboring resistant isolates had received a long-term itraconazole therapy, while five had not azole exposure (Table 1).

Few information concerning living habits of patients harboring a resistant isolate were available: one lived in the countryside (patient # 7) and one of the two living in the city, handled loam (patient # 2).

3.3. Genotyping testing and cyp51A sequencing

All resistant isolates were identified as *A. fumigatus* sensu stricto.

In order to identify mutations related to azole resistance, the entire *cyp51A* gene was sequenced. The t364a point mutation, resulting in the L98H substitution, combined with the 34-bp tandem repeat in the promoter region, was found in all resistant isolates except one, in which the t726a point mutation, resulting in the F219I substitution, was present (Table 1).

3.4. Microsatellite analysis

Microsatellite typing was used to analyze the relatedness of clinical resistant isolates and environmental azole-resistant strains isolated in an area surrounding Milan during a previous survey [4]. A total of 27 isolates were typed, namely 17 environmental isolates, all harboring the L98H substitution, eight resistant clinical isolates, one from each patient, one azole-resistant strain (IUM 13-0110/1) isolated one year before from patient # 3 harboring resistant isolate (IUM 14-0071), and one azole-susceptible isolate (IUM 14-0131) from patient # 7, who developed resistance after 4 months (Fig. 1).

Five isolates harboring the L98H substitution (IUM 14-0006A, IUM 14-0198, IUM14-0042, IUM14-0218, IUM15-0055), as well as the clinical isolate harboring the F219I substitution (IUM 14-0215) and the susceptible isolate (IUM 14-0131) resulted unrelated.

Two patients were found to have an identical genotype, patients # 3 (IUM 14-0071) and # 5 (IUM 14-0199); the same genotype was also observed in the strain isolated from the same patient one year before (IUM 13-0110/1). Identical genotypes were observed in one clinical resistant isolate (IUM 14-0218) and two environmental strains (IUM 11-0317D and IUM 11-0396) as well as in another clinical isolate (IUM 14-0006A) and another environmental strain (IUM 11-0317C).

A nearly identical genotype was observed between clinical isolate and an environmental isolate in four cases (IUM 14-0198 and 11-0104D; 14-0042 and 11-0087A; 14-0199 and 11-0317C; 14-0071 and 11-0317C).

4. Discussion

A number of studies had investigated azole resistance in European CF patients and the prevalence of resistance ranged from 3.4% in Cologne (Germany) up to 8% in France [1,15–18]. To our knowledge, this is the first study concerning azole resistance in *A. fumigatus* isolates from Italian CF patients. The frequency of detection of resistance varies considerably between the two centers involved in the study: no resistance was detected in the Verona center, while a prevalence of 8.2% was observed in the Milano center that is comparable to the French data [16].

All the resistant isolates in Milano had the mutations in the *cyp51A* gene and the predominant mutation was TR_{34}/L98H, displayed by isolates from seven out of eight patients.

Differences between the two Italian centers could be caused by a different use of azoles or by environmental factors. No antifungal treatment was administered in the Verona center, while 22.7% of the patients of the Milano center received azoles in the six month prior to the isolation of the fungus. No resistant isolates were found in the environmental samples collected near Verona, even if the number was limited (unpublished data). In addition, microsatellite analysis seems to confirm the environmental origin as six patients resulted colonized by a genotype identical (patients # 1 and # 7) or nearly identical (patients # 2, # 3, # 4, and # 5) to that of environmental isolates. Four of these patients (# 2, # 3, # 5, and #7) had not received azoles, and one (patient # 7) lived in the country and another (patient #2) did gardening.

The TR_{34}/L98H alteration resulted associated with poor in vitro activity of azoles. Strains were resistant or intermediate to itraconazole, posaconazole, voriconazole and isavuconazole. On the contrary, the isolate with F219I alteration, resistant to itraconazole and posaconazole, intermediate to voriconazole was susceptible to isavuconazole (MIC of 0.25 mg/L). These data suggest that also the F219I mutant, as well as the majority of the G54 and some of the M220 mutants [19], remains susceptible to this new azole.

With this study, it was possible to document the presence, also in Italy, of azole-resistant *A. fumigatus* in CF patients. TR34/L98H is confirmed to be the main resistance mechanism and the environmental origin of the resistance seems to be probable since azole resistance was also found in naïve patients and identical genotype in clinical and environmental isolates was observed. Therefore, in vitro susceptibility testing of *A. fumigatus* isolates should be performed also in azole naïve patients requiring antifungal treatment, and particularly in patients for whom a lung transplant is planned.

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Conflict of interest statement

We have no conflicts of interest to declare.

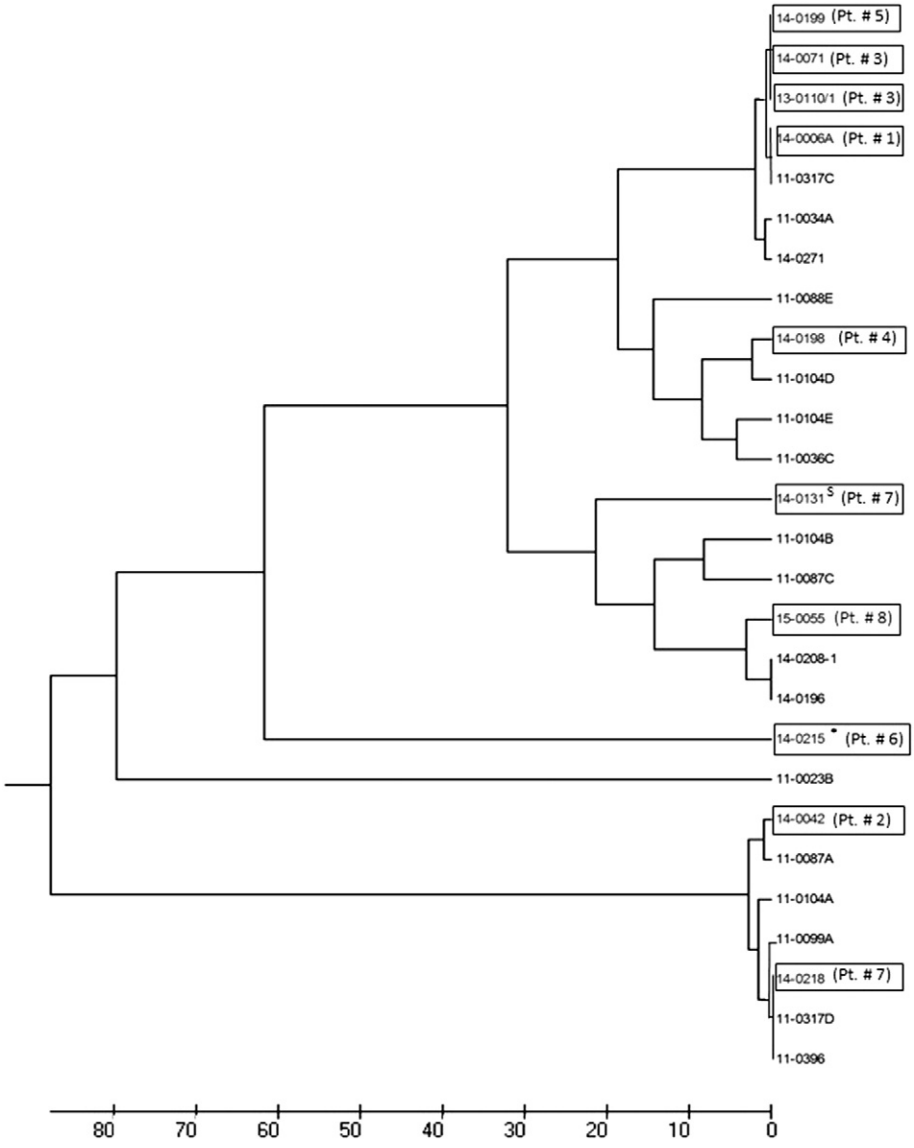

Fig. 1. Dendrogram of microsatellite genotyping analysis of environmental and clinical (bordered) *A. fumigatus* isolates. The percentage of dissimilarity among the genetic profiles is indicated along the horizontal ax (distance matrix generated by GenAIEx 6.5). * isolate with the F219I substitution; ^{S} susceptible isolate.

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References

[1] Mortensen KL, Jensen RH, Johansen HK, Skov M, Pressler T, Howard SJ, et al. *Aspergillus* species and other moulds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. J Clin Microbiol 2011;49:2243–51.

[2] Touati K, Nguyen Do Ngoc L, Delhaes L. The airway colonization by opportunistic filamentous fungi in patients with cystic fibrosis: recent updates. Curr Fungal Infect Rep 2014;8:302–11.

[3] Stensvold CR, Jørgensen LN, Arendrup MC. Azole-resistant invasive aspergillosis: relationship to agriculture. Curr Fungal Infect Rep 2012;6: 178–91.

[4] Prigitano A, Venier V, Cogliati M, De Lorenzis G, Esposto MC, Tortorano AM. Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012. Euro Surveill 2014;19:20747.

[5] Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. Identification of two different 14-α sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol 2001;39:431–8.

[6] Vermeulen E, Maertens J, Schoemans H, Lagrou K. Azole-resistant *Aspergillus fumigatus* due to TR_{46}/Y121F/T289A mutation emerging in Belgium, July 2012. Euro Surveill 2012;17(48) [pii = 20326].

[7] Lazzarini C, Esposto MC, Prigitano A, Cogliati M, De Lorenzis G, Tortorano AM. Azole resistance in *Aspergillus fumigatus* clinical isolates from an Italian culture collection. Antimicrob Agents Chemother 2015;60:682–5.

[8] Rodriguez-Tudela JL, Arendrup MC, Arikan S, Barchiesi F, Bille J, Chryssanthou E, et al. EUCAST definitive document E.DEF 9.1: method for determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. European Committee on Antimicrobial Susceptibility Testing; 2008.

[9] Arendrup MC, Cuenca-Estrella M, Lass-Florl C, Hope WW, European Committee on Antimicrobial Susceptibility Testing Subcommittee on

Antifungal Susceptibility Testing (EUCASTAFST). EUCAST technical note on *Aspergillus* and amphotericin B, itraconazole, and posaconazole. Clin Microbiol Infect 2012;18:E248–50.

[10] Hope WW, Cuenca-Estrella M, Lass-Florl C, Arendrup MC, European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing (EUCASTAFST). EUCAST technical note on voriconazole and *Aspergillus* spp. Clin Microbiol Infect 2013;19:E278–80.

[11] European Committee on Antimicrobial Susceptibility Testing. Antifungal agents breakpoint tables for interpretation of MICs- version 8.0. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/Antifungal_breakpoints_v_8.0_November_2015.pdf; 2016. [Accessed May 5, 2016].

[12] de Valk HA, Meis JF, Curfs IM, Muehlethaler K, Mouton JW, Klaassen CH. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 2005;43:4112–20.

[13] Peakall R, Smouse PE. Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 2006;6:288–95.

[14] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9.

[15] Burgel PR, Baixench MT, Amsellem M, Audureau E, Chapron J, Kanaan R, et al. High prevalence of azole-resistant *Aspergillus fumigatus* in adults with cystic fibrosis exposed to itraconazole. Antimicrob Agents Chemother 2012;56:869–74.

[16] Morio F, Aubin GG, Danner-Boucher I, Haloun A, Sacchetto E, Garcia-Hermoso D, et al. High prevalence of triazole resistance in *Aspergillus fumigatus*, especially mediated by TR/L98H, in a French cohort of patients with cystic fibrosis. J Antimicrob Chemother 2012;67:1870–3.

[17] Bader O, Weig M, Reichard U, Lugert R, Kuhns M, Christner M. *Cyp51A*-based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. Antimicrob Agents Chemother 2013;57:3513–7.

[18] Fischer J, van Koningsbruggen-Rietschel S, Rietschel E, Vehreschild MJGT, Wisplinghoff H, Krönke M, et al. Prevalence and molecular characterization of azole resistance in *Aspergillus* spp. isolates from German cystic fibrosis patients. J Antimicrob Chemother 2014;69:1533–6.

[19] Howard SJ, Lass-Flörl C, Cuenca-Estrella M, Gomez-Lopez A, Arendrup MC. Determination of isavuconazole susceptibility of *Aspergillus* and *Candida* species by the EUCAST method. Antimicrob Agents Chemother 2013;57:5426–31.