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High prevalence of triazole resistance in *Aspergillus fumigatus*, especially mediated by TR/L98H, in a French cohort of patients with cystic fibrosis

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Objectives: Triazole resistance in *Aspergillus fumigatus* due to a single azole resistance mechanism (TR/L98H) is increasingly reported in European countries. Data from patients with cystic fibrosis (CF) are limited. Our study aimed to investigate the prevalence and molecular mechanisms of azole resistance in *A. fumigatus* in a cohort of patients with CF.

Methods: Eighty-five *A. fumigatus* isolates from 50 CF patients, collected between January 2010 and April 2011, were retrospectively analysed for azole resistance using agar plates containing 4 mg/L itraconazole. MICs of itraconazole, voriconazole and posaconazole were determined according to EUCAST methodology for each isolate able to grow on this medium. Species identification was performed by sequencing of the β-tubulin gene. Sequencing analysis of the *cyp51A* gene and its promoter region was conducted.

Results: Nine isolates (four patients, 8% prevalence) were able to grow on itraconazole-containing agar plates. Itraconazole resistance was confirmed by EUCAST methodology (MICs >2 mg/L). All isolates had mutations in the *cyp51A* gene at residues previously involved in azole resistance: L98H ($n=5$), M220T ($n=4$) and G54R ($n=1$). One patient had three genetically distinct azole-resistant isolates identified during the study. The isolates with L98H that were recovered from three patients (6% prevalence) also had the 34 bp tandem repeat in the promoter region of *cyp51A* (TR/L98H) and displayed multiazole resistance.

Conclusions: We report an 8% prevalence of itraconazole resistance in CF patients in our centre, mostly driven by TR/L98H (6%). Our data confirm that TR/L98H occurs in France and can be highly prevalent in CF patients.

Keywords: *cyp51A* mutations, multi-azole resistance, *A. fumigatus*, CFTR

Introduction

Aspergillus fumigatus is widespread in the environment and is the main *Aspergillus* species responsible for human diseases in both immunocompromised and immunocompetent hosts. Inherently resistant to fluconazole, *A. fumigatus* is usually susceptible to the other triazole antifungal drugs, such as itraconazole, voriconazole or posaconazole, and voriconazole is the first-line therapy for invasive aspergillosis. Since the first two published cases in 1997,[1] an ever-growing number of studies focusing on acquired azole resistance in both clinical and environmental *A. fumigatus* isolates suggest that azole resistance is increasing.[2,3] Azole resistance mainly results from substitutions in lanosterol 14α-demethylase (encoded by the *cyp51A* gene).[4] In the Netherlands, the main mechanism involves a 34 bp tandem repeat in the promoter region of the *cyp51A* gene along with a leucine to histidine substitution at residue 98 (also referred to as TR/L98H), which confers multiazole resistance.[3] The main hypothesis explaining why TR/L98H is found in >90% of *A. fumigatus* azole-resistant isolates from the Netherlands relies on the widespread use of azole compounds in agriculture.[5,6] Outside the Netherlands, isolates with

TR/L98H have been reported in most European countries, including Belgium, Denmark, the UK and Spain, and recently outside Europe, e.g. in India, suggesting a global spread of this resistance mechanism.[2,5,7–9] Although TR/L98H has also been recently reported in France, there are limited data regarding its prevalence in this country.[10,11] Here, we present the results of a 16 month retrospective study to determine the frequency and molecular mechanisms of azole resistance in a cohort of patients with cystic fibrosis (CF).

Patients and methods

One hundred and forty-two sputum and bronchial aspiration samples (mean number of samples per patient = 2.8; 1–18 samples per patient) from 50 CF patients (mean age = 21 years; 5–46 years of age) admitted to the Pneumology Department of Nantes University Hospital (France) during January 2010 to April 2011 were analysed retrospectively. Ninety-seven of the 142 (68.3%) samples were positive for *A. fumigatus*. Twenty-six patients (52%) had several positive samples (two to six samples). Eighty-five of the 97 isolates (12 unavailable for analysis) were included in this study.

Azole resistance was screened by subculturing each isolate on agar plates containing 4 mg/L itraconazole. Plates were prepared in-house and contained RPMI 1640 medium (Sigma–Aldrich, Saint-Quentin Fallavier, France) supplemented with 2% D-glucose (Sigma–Aldrich) buffered with 3-(*N*-morpholino)propanesulphonic acid (MOPS; 0.165 M final concentration, Sigma–Aldrich) at pH 7.0 and 1.5% Bacto agar (Difco, Pont de Claix, France). Briefly, fresh conidia from a 7-day-old culture on Sabouraud dextrose agar slants with chloramphenicol (bioMérieux, Marcy l'Étoile, France) were suspended in sterile water at a turbidity equivalent to that of a 0.5 McFarland standard. Plates were inoculated by dipping a sterile swab into the inoculum suspension and swabbing the entire agar surface. Plates were then incubated at 35°C for 72 h. Two *Aspergillus ustus* isolates were included as positive controls in each set of experiments. Itraconazole resistance was evaluated by Etest® (AB Biodisk, bioMérieux, France) for each isolate that was able to grow on azole-containing agar plates. MICs of itraconazole, voriconazole and posaconazole were therefore determined by the reference microdilution method according to EUCAST (CNRMA, Institut Pasteur, Paris, France).[12] Isolates with MICs >2 mg/L were considered resistant to itraconazole[13] and voriconazole,[4] and isolates with MICs >0.25 mg/L were considered resistant to posaconazole.[14]

Species identification of itraconazole-resistant isolates was performed by amplification and sequencing of the β-tubulin gene.[15] Each of these isolates was further subjected to the amplification and sequencing of the *cyp51A* gene, as described previously,[10] and of a 234 bp region of its promoter using the primers AFTR-F (5′-TAATCGCAGCACCACTTCAG-3′) and AFTR-R (5′-GCCTAGGACAAGGACGAATG-3′). Nucleotide sequences were compared with the reference sequence of the *A. fumigatus* azole-susceptible strain CM-237 (GenBank accession number AF338659). Genotyping of the itraconazole-resistant isolates was performed using four microsatellite markers with a global discriminatory power of 0.994, as described previously.[16] Briefly, amplification was carried out in a 20 μL reaction mixture containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1 mM each dNTP, 0.1 μM each primer (Sigma, Paris, France) and 1 U of Amplitaq Gold *Taq* DNA polymerase (Applied Biosystems, Meylan, France). After an initial denaturation step at 94°C for 5 min, samples were amplified by 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and elongation at 72°C for 30 s, and then a final extension at 72°C for 30 min. Next, 2 μL of the PCR product was mixed with 13 μL of HiDi Formamide (Applied Biosystems) containing 0.5 μL of 6-carboxy-X-rhodamine-labelled Geneflo 625 size standard (Eurx, Gdansk, Poland). Capillary electrophoresis was performed using the ABI Prism 3730XL sequencer and allele sizes were calculated with GeneMapper software (version 4; Applied Biosystems). Since the four microsatellite markers consist of dinucleotide repeats, the alleles were considered to be different when a 2 bp difference was observed.

Results and discussion

Nine of the 85 isolates (four patients, 8% global prevalence) were able to grow on itraconazole-containing agar (Table 1). Sequencing of the β-tubulin gene confirmed that these isolates were *A. fumigatus sensu stricto*. Each isolate also had high MICs by Etest®. Itraconazole resistance was confirmed using EUCAST methodology, with all isolates having MICs >2 mg/L. Distinct patterns of antifungal susceptibility were observed for voriconazole and posaconazole (Table 1). Importantly, five out of the nine isolates, coming from three patients (Patients 1, 2 and 3, Table 1), displayed multiazole resistance (resistance to itraconazole, voriconazole and posaconazole). Previous exposure to mould-active azoles (mostly itraconazole) was recorded for each of these patients.

To provide further insight into the mechanisms responsible for azole resistance in these isolates, we amplified the *cyp51A* coding sequence and its promoter. The nine itraconazole-resistant isolates displayed mutations in the *cyp51A* gene at residues previously linked to azole resistance: L98H ($n=5$), M220T ($n=4$) and G54R ($n=1$) (Table 1). All isolates with L98H also displayed the 34 bp tandem repeat (TR/L98H) and were recovered from three patients (6% global prevalence in CF). As expected, isolates with TR/L98H displayed multiazole resistance.[4] Patient 3 had three *A. fumigatus* isolates with distinct azole resistance mechanisms on three separate samplings. Compared with TR/L98H, M220T was associated with itraconazole resistance, but lower MICs of voriconazole. Combination of M220T with G54R was associated with resistance to posaconazole, but voriconazole retained significant *in vitro* activity (MIC = 0.25 mg/L).

Overall, whereas the acquisition of TR/L98H in Patients 1, 2 and 3 can hardly be excluded from having arisen from long-term azole therapy (as none of these patients was 'azole naive'), the main hypothesis is that these patients were contaminated by *A. fumigatus* itraconazole-resistant isolates from their environment.[5] This hypothesis is supported by microsatellite typing data, as Patients 1, 2 and 3 displayed almost identical TR/L98H genotypes (Table 1). In stark contrast, other isolates (including itraconazole-susceptible isolates from these patients) had completely different genotypes (Table 1).

One interesting finding is the description of Patient 1, who was colonized for 11 months by a unique itraconazole-resistant *A. fumigatus* isolate with TR/L98H upon genotyping. Such chronic colonization by a multiazole-resistant isolate in the course of CF, also reported in another study,[8] must be considered in antifungal management strategies after lung transplantation. However, our study also illustrates that colonization of the respiratory tract by *A. fumigatus* in the course of CF is a complex and dynamic process, as illustrated here by: (i) the recovery of multiple azole-resistant isolates from a single patient (Patient 3); and (ii) the recovery of itraconazole-susceptible isolates before (Patient 4) or after the recovery of itraconazole-resistant isolates (Patient 3).

In previous studies, the prevalence of itraconazole resistance was <1%, 4.5% and 4.6% in Portugal,[17] Denmark[8] and France,

Table 1. MICs and Cyp51A amino acid alterations for azole-resistant *A. fumigatus* isolates

Patient	Sex/age (years)	Isolate identification number	Date of isolation (day/month/year)	Growth on ITC-containing agar plates	MICs by EUCAST methodology (mg/L)			Cyp51A alteration	Length of microsatellite markers (bp)[15]			
					ITC	VRC	POS		AspA	AspB	AspC	AspD
1[a]	M/30	70041901	28/01/2010	+	≥8	2	1	TR/L98H	106	118	175	92
		70067922	30/11/2010	+	≥8	4	0.5	TR/L98H	106	118	175	92
		70070375	28/12/2010	+	≥8	4	1	TR/L98H	106	118	175	92
2[b]	M/41	70054925	01/07/2010	+	≥8	4	1	TR/L98H	106	118	175	98
3[c]	M/25	70048399	15/04/2010	+	≥8	4	0.5	TR/L98H	106	118	175	92
		70056323	15/07/2010	+	≥8	0.25	2	G54R, M220T	118	106	165	94
		70061397	06/10/2010	+[d]	≥8	0.5	0.5	M220T	104	105	175	112
		70075298	22/02/2011	−	0.25	0.5	0.125	WT	139	129	169	ND
4[e]	F/18	70053499	15/06/2010	−	0.125	0.125	0.06	WT	124	142	165	96
		70071183	07/01/2011	+	≥8	0.5	0.25	M220T	124	104	173	111
		70073662	03/02/2011	+	≥8	0.5	0.25	M220T	124	104	173	111

M, male; F, female; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; +, positive growth on ITC-containing agar plates; −, no growth on ITC-containing agar plates; WT, wild-type sequence; ND, not detected.
[a]The patient was given ITC from 2007 for chronic *Aspergillus* colonization while awaiting lung transplantation.
[b]The patient had received ITC for 6 months in 2000 for allergic bronchopulmonary aspergillosis (ABPA) and several courses of VRC before 2010.
[c]The patient was given ITC for chronic *Aspergillus* colonization from 2009 to April 2011.
[d]Growth on ITC-containing agar plate after prolonged incubation.
[e]The patient had received several courses of ITC since June 2010 for ABPA.

respectively.[11] Here, we report a high prevalence of itraconazole resistance in *A. fumigatus* isolated from CF patients at our centre (4/50 patients, 8%). Importantly, TR/L98H was the main mechanism responsible for azole resistance in our study (3/50 patients, 6%), confirming that TR/L98H is prevalent in France.[5,11] Of note, TR/L98H was not reported in the single study focusing on patients with haematological malignancies in France,[10] providing evidence that the prevalence of TR/L98H and more generally azole resistance varies from centre to centre and probably also according to the underlying diseases.[18] Indeed, azole resistance has been especially described in CF and in patients with chronic pulmonary aspergillosis or allergic bronchopulmonary aspergillosis, with a prevalence rate as high as 75%.[19] On the other hand, azole resistance has been shown to be lower but highly variable in patients with haematological malignancies, with prevalence rates varying from 0.01% to 9.4%.[10,18]

As already shown, itraconazole-containing plates are easy to use in a routine mycology laboratory, offering the possibility of screening large collections of clinical strains at low cost.[8] However, the use of a concentration of 4 mg/L itraconazole, which has also been reported by others[8,9,18] but is above the susceptibility breakpoint for *A. fumigatus* (≤1 mg/L),[13] might be a limitation of our study as some non-susceptible isolates could have been missed.

In summary, the present study highlights a high prevalence of itraconazole resistance in *A. fumigatus*, mostly driven by TR/L98H, in CF patients at our centre. From a more global perspective, nationwide and multicentre surveys involving medical centres responsible for the management of patients at high risk of invasive aspergillosis, such as those with CF, are urgently needed to evaluate the burden of azole resistance in *A. fumigatus*.

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Transparency declarations

None to declare.

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