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1 Azole resistance in *Aspergillus fumigatus* isolates from respiratory specimens in Lyon
2 University Hospitals, France: prevalence and mechanisms involved

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ABSTRACT

Aspergillus fumigatus resistance to triazoles is increasingly reported in Europe. As few data are available in Southern France, our objectives were to assess the burden of *A. fumigatus* isolates with azole resistance from clinical specimens in Lyon and explore the involved resistance mechanisms. In this retrospective cross-sectional study, 221 consecutive *A. fumigatus* isolates from respiratory samples were identified during an eight-month period from 195 patients attending the Pulmonary Medicine Departments of Lyon University Hospitals. Morphological identification was confirmed by sequence analysis of the β -tubulin gene. Itraconazole, voriconazole, posaconazole, and isavuconazole susceptibilities were tested for all samples with concentration gradient strips and confirmed with EUCAST broth microdilution method. The resistance mechanisms were investigated by sequencing of the *cyp51A* gene and its promoter, and by expression analysis of *cyp51* and genes encoding several efflux transporters. Four isolates exhibited azole resistance. Three isolates presented with polymorphisms in an intronic region of *cyp51A* and one with simultaneously the F46Y, M172V and E427K polymorphisms. No mutation was identified in the *cyp51A* promoter, but significant inductions of *cyp51A* and *cyp51B* gene expression were observed for all four and three isolates, respectively. Significant inductions of *atrF* and *cdr1B* gene expression were observed for two and three isolates, respectively. No significant induction of *MDR1/2/3/4*, *MFS56* and *M85* gene expression was observed. To conclude, the observed prevalence of azole resistance was 2.1%. Significant inductions of the expression of the *cyp51* genes and two genes encoding efflux transporters were evidenced, underlying the diversity of resistance mechanisms to be explored.

75

76 1. INTRODUCTION

77 *Aspergillus fumigatus* is a ubiquitous mould and the main species responsible for invasive
78 aspergillosis in immunocompromised patients and other forms of aspergillosis in patients with
79 chronic lung diseases (1,2). Mould-active triazoles are the first-line antifungal treatments for
80 invasive or chronic pulmonary aspergillosis (2,3), based on the inhibition of the lanosterol 14- α -
81 demethylase involved in the biosynthesis pathway of ergosterol, the fungal membrane major sterol
82 (1). However, resistance of *A. fumigatus* strains to triazole antifungals is increasingly reported
83 worldwide, becoming a worrying issue for patient management (4–11). The main mechanisms of
84 resistance described so far are: (i) mutations in the *cyp51A* gene encoding the lanosterol 14- α -
85 demethylase or in its promoter (4–7,12); (ii) overexpression of *cyp51* (12); (iii) overexpression of
86 multidrug efflux pumps (12). Our objectives were to assess the burden of *A. fumigatus* isolates
87 with azole resistance from clinical specimens in Lyon, and to evaluate the involved resistance
88 mechanisms.

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91 2. MATERIALS AND METHODS

92 2.1 Study design

93 This study was conducted in the laboratory of Parasitology-Mycology of the Lyon
94 University Hospitals, France. From February to September 2017, all respiratory samples from
95 patients attending the inpatient and outpatient wards of the Pulmonary Medicine Departments were
96 plated on chromID™ *Candida* (CAN2) agar plates and Sabouraud chloramphenicol agar tubes

(bioMérieux, Marcy-l'Étoile, France), and incubated at 35°C for 7 days, as part of our laboratory routine.

2.2 Isolates

Every mould grown in culture was microscopically identified with Lactophenol Cotton Blue stain (Becton-Dickinson, Franklin Lakes, NJ, USA). All *Aspergillus sp.* isolates belonging to the section *Fumigati* were then submitted to DNA extraction with the MagNAPure Compact Nucleic Acid Isolation Kit I (Roche, Mannheim, Germany) and identified by PCR and sequence analysis of the highly conserved β -tubulin gene (4).

2.3 Antifungal susceptibility testing and fungal cultures

Determination of Minimum Inhibitory Concentrations (MICs) were performed for all *A. fumigatus* isolates by concentration gradient strips (CGS) for itraconazole, voriconazole, posaconazole (ETEST®, bioMérieux) and isavuconazole (Liophilchem) according to the manufacturer's recommendations. All isolates showing resistance with CGS were then tested with EUCAST standardised broth microdilution method for confirmation. Susceptibility or resistance profiles to the different antifungals were defined according to the 2020 EUCAST clinical breakpoints after 48-hour cultures at 35°C (<https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/>). Thus, isolates were considered resistant when MIC was >1 mg/L for itraconazole and voriconazole, >2 mg/L for isavuconazole, and >0.25 mg/L for posaconazole. Isolates that were resistant to azoles were then cultured in liquid Sabouraud + 4 mg/L itraconazole medium at 35°C for 16 hours for further analyses (8).

2.4 Sequence analysis of the *cyp51A* gene

120 Molecular resistance mechanisms were assessed by sequence analysis of the *cyp51A* gene
121 and its promoter region (7) using the CBS144.89/A1163 strain of *A. fumigatus* as reference wild-
122 type strain.

123 2.5 RNA extraction and reverse transcriptase real-time PCR

124 Total RNA extractions from grown cultures in liquid Sabouraud with itraconazole were
125 performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

126 Gene expression of *cyp51A*, *cyp51B* and several efflux transporters (namely *atrF*, *cdr1B*,
127 *MDR1/2/3/4*, *MFS56* and *M85*) (8) were quantified by reverse transcriptase real-time PCR using
128 the High Capacity cDNA Reverse Transcription Kit and the PowerUp Sybr® Green Master Mix
129 (Thermo-Fisher, Waltham, Massachusetts, USA) on the Applied Biosystems 7500 Real-Time PCR
130 System (Thermo-Fisher). Expression levels were normalized using the $2^{-\Delta\Delta C_t}$ method with the
131 expression of the CBS144.89/A1163 strain as reference and the β -tubulin housekeeping gene as
132 endogenous control (8).

133 2.6 Statistical analysis

134 Two-way analysis of variance (ANOVA) with Tukey's multiple comparisons post-hoc test
135 was performed with Prism v6.04 (GraphPad Software) to compare the gene expression of the
136 different strains, after itraconazole exposure, with the basal gene expression of the
137 CBS144.89/A1163 azole susceptible *A. fumigatus* reference strain. A $P < 0.05$ was considered as
138 statistically significant.

139

140

141 3. RESULTS

3.1 Study population and azole resistance

During an eight-month period, 221 consecutive *Aspergillus sp.* isolates from the section *Fumigati* were obtained by culture from 195 patients' respiratory specimens. All isolates were confirmed as *A. fumigatus* by PCR and sequence analysis of the β -tubulin gene. Susceptibility testing identified four isolates to be resistant to azole antifungals (**Table 1**). All of them were resistant to itraconazole (all with MIC>8 mg/L), voriconazole (range 4 - >8 mg/L), and isavuconazole (range 4 - >8 mg/L); three of them were in addition resistant to posaconazole (range 0.5 - >8 mg/L) whereas the fourth was in the area of technical uncertainty (MIC=0.25 mg/L) (**Table 1**). These four isolates were from four different patients. In total, the prevalence of azole resistance in our study population was 2.1% (4/195). The resistant isolates were grown in culture from respiratory specimens from three patients with cystic fibrosis (for isolates A, B and C) and one patient with autosomal dominant hyperimmunoglobulinemia E (for isolate D). All four patients previously received prolonged antifungal treatments: itraconazole for the three patients with cystic fibrosis and treatments with itraconazole, voriconazole and posaconazole for patient D. Among the 195 patients included, 70 had cystic fibrosis: the prevalence of azole resistance in patients with cystic fibrosis in our cohort was thus 4.3% (3/70). Consequently, the prevalence of azole resistance in patients from Pulmonary Medicine Departments, excluding cystic fibrosis patients, was 0.8% (1/125).

3.2 DNA sequence analyses of the *cyp51A* gene

Two polymorphisms were found in an intron of the *cyp51A* gene for *A. fumigatus* azole-resistant strains A, B and C. Three polymorphisms were found within the open reading frame of the *cyp51A* gene for strain D (F46Y, M172V, E427K) (**Table 1**). No mutation was found in the promoter of the *cyp51A* gene for any of the four azole-resistant *A. fumigatus* strains.

3.3 Gene expression of the azole-resistant strains

166 Compared to the CBS144.89/A1163 azole susceptible reference strain, significant
167 induction of the expression of the *cyp51A* gene was found for all four strains, with mean mRNA
168 inductions ranging from 3.0 to 9.6 folds (**Fig. 1A**). There was also a significant induction of the
169 expression of the *cyp51B* gene for strains A, C and D with mean mRNA inductions ranging from
170 3.3 to 5.5 folds (**Fig. 1B**), whereas mean fold induction for strain B was 1.8 with no statistical
171 significance compared to the CBS144.89/A1163 strain.

172 Concerning the *atrF* efflux transporter gene, mRNA expression was significantly induced
173 for both strains B and C, with mean folds of 4.6 and 3.4, respectively (**Fig. 1C**), whereas strains A
174 and D showed no significant variations compared to the CBS144.89/A1163 reference strain. For
175 *cdr1B* gene expression, strains A, B and C showed significant induction with mean folds ranging
176 from 2.9 to 4.4 (**Fig. 1D**), whereas strain D showed no significant variation.

177 No significant results were observed in the expression of the *MDR1/2/3/4* genes or the
178 *MFS56* and *M85* genes (**Fig. 1E-J**) with the four azole-resistant strains, compared to the
179 CBS144.89/A1163 reference strain.

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181

182 4. DISCUSSION

183 The emergence of *A. fumigatus* azole-resistant strains could become a major issue on a
184 global scale (13) as mould-active triazoles are the first-line treatment of invasive and chronic
185 aspergilloses. Our study on a large number of consecutive patients identified three findings. First,
186 the prevalence of azole resistance in our study is globally low but higher in the subpopulation of
187 patients with cystic fibrosis. Second, our study provides insights regarding the different
188 mechanisms of resistance that can be observed in patients with cystic fibrosis or primary

189 immunodeficiency, who are submitted to a selective pressure with frequent long-term azole
190 antifungal treatments. Third, it invites to continue monitoring azole sensitivity in *A. fumigatus*
191 isolates.

192 The overall prevalence of azole resistance in our study, around 2%, can be specified
193 according to the patients' background. Choukri *et al.* reported a similar prevalence but in an cohort
194 of immunocompromised, immunocompetent and cystic fibrosis patients without distinction (14).
195 Other studies reported in France gave different percentages of *A. fumigatus* resistance prevalence
196 in different patient groups (5,6,15). Regarding patients from pulmonology wards excluding cystic
197 fibrosis patients, our prevalence of azole resistance was 0.8%, lower than the 2.8% recently
198 reported by Guegan *et al.* on a similar number of patients (15). Focusing on patients with cystic
199 fibrosis in our study, we found a prevalence of azole resistance of 4.3%, close to the prevalence
200 found in Paris by Burgel *et al.* (6) and a bit lower than the 6.8% prevalence found in Nantes by
201 Lavergne *et al.* (5). Guegan *et al.* found a very much higher prevalence of azole resistance
202 (34/123, 27.6%) in a cohort including the same type of patients (15). However, they considered
203 isolates with E-test results in the area of technical uncertainty as resistant isolates and the majority
204 of the resistant isolates in their study were resistant to itraconazole but not to voriconazole;
205 considering isolates resistant to voriconazole, only 5% (27/539) were resistant, corresponding to a
206 resistance prevalence of 10.9% (13/119 patients).

207 An increasing number of countries in Europe, America, Asia and Oceania have reported
208 the emergence of azole-resistant strains of *A. fumigatus* in the past decade (13). This recent
209 concern about azole resistance could be due to a strong and durable selection pressure by azole
210 antifungals. Two major hypotheses to explain this phenomenon seem to stand out: (i) an overuse
211 of azole antifungals for patients' treatments, for long-term therapies in particular; (ii) a large-scale
212 use of azole as pesticides for farming activities (13,16). A previous study on nine patients with

213 azole-resistant invasive aspergillosis showed that four of them had no previous history of azole
214 therapy, supporting the hypothesis of an environmental route of emergence (17).

215 Several mutations in the *cyp51A* gene of some *A. fumigatus* strains were described as
216 responsible for azole resistance (9,17,18). In our study, three non-synonymous polymorphisms in
217 the *cyp51A* gene were found in the same azole-resistant strain, resulting in F46Y, M172V and
218 E427K substitutions. All three polymorphisms had already been observed in the past without any
219 of them being able to be definitively correlated with a resistance phenotype (9).

220 As azole antifungals bind to Cyp51, the overexpression of *cyp51* genes induces an
221 increased quantity of corresponding enzyme synthesis allowing the fungi to prevent the effect of
222 antifungals. We found significant inductions of the expression of the *cyp51A* gene for all four
223 azole-resistant strains. Furthermore, despite scarce descriptions of overexpression of *cyp51B* in the
224 literature (12), we found significant inductions of the expression of the *cyp51B* gene for three
225 strains, including two cystic fibrosis patient.

226 There are two superfamilies of efflux pumps known to allow azole efflux outside the fungal
227 cell: ABC (ATP-binding cassette) and MFS (major facilitator superfamily) transporters (19). In
228 this study, we evaluated the gene expression of six ABC transporters (*atrF*, *cdr1B* and
229 *MDR1/2/3/4*). We found significant inductions of the expression of *atrF* and *cdr1B* genes in two
230 and three of the azole-resistant *A. fumigatus* strains, respectively. Even if these transporters have
231 already been linked to azole resistance (8,12), descriptions of this mechanism are much scarcer
232 than that implying *cyp51A* and only an induction of *cdr1B* gene expression was evidenced by
233 Fraczek *et al.* (8). We did not find any induction of expression of the *MDR1/2/3/4* genes, whereas
234 they were reported by others to be upregulated by azole exposure or overexpressed in some
235 resistant strains (12). Considering *MFS56* and *M85* genes, we did not find any induction of
236 expression either.

237 Our results highlight the underestimated role of efflux pumps in the resistance of *A.*
238 *fumigatus* to azoles. Other mechanisms of resistance have been recently described, such as
239 modifications in a transcriptional regulator called Negative Cofactor (NCT) complex, which may
240 lead to a transcriptional dysregulation of the ergosterol biosynthetic pathway, an increase in
241 cellular ergosterol levels, and an increase in levels of the *cdr1B* azole transporter; or such as
242 mutations in the *hmg1* gene, responsible for residue alterations in the sterol sensing domain of
243 Hmg1, involved in ergosterol biosynthesis (20,21). Taken together, all these results underline the
244 need to screen *A. fumigatus* isolates for azole resistance and the diversity of resistance mechanisms
245 to be explored.

246

247 **DECLARATIONS**

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249 **Competing Interests:** PR has received educational grants outside the submitted work from Vertex
250 and personal fees from Zambon and Gilead. SC has received research grants outside the submitted
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253 symposia from Gilead and Pfizer. JM has received a research grant outside the submitted work
254 from MSD and aid for attending symposia from Gilead, MSD, Pfizer and Roche Diagnostics. All
255 other authors: none to declare.

256 **Ethical Approval:** Not required. However, patients were informed that their clinical and
257 biological data could be used for research purposes; no patient opposed.

258

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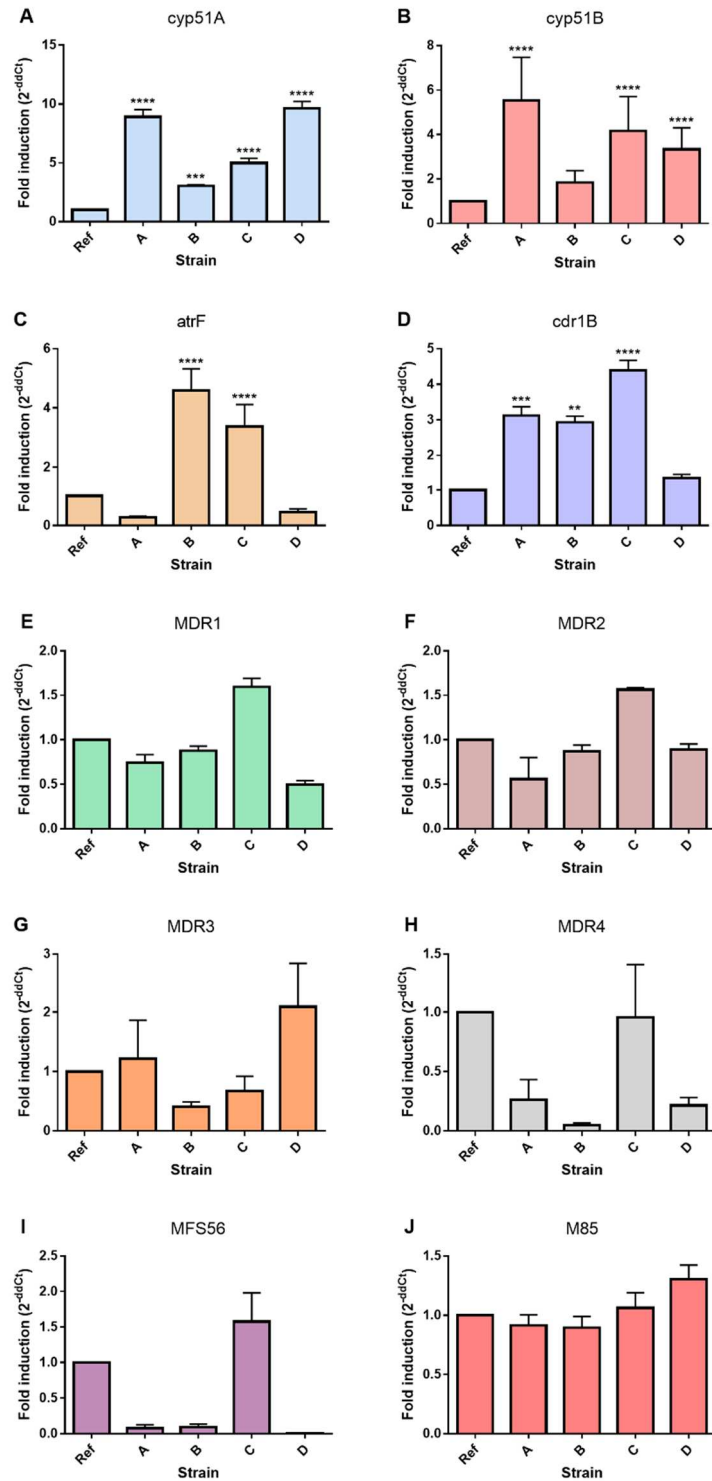
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Table 1: Minimum Inhibitory Concentrations (EUCAST broth microdilution method) of azole-resistant *A. fumigatus* strains, sequence analysis and gene overexpression results.

Resistant strain	MIC (mg/L)				Sequence analysis of <i>cyp51A</i> promoter	Sequence analysis of <i>cyp51A</i> gene	Significant gene overexpressions
	ITZ	VRZ	PSZ	ISA			
Strain A	> 8	4	0.25	4	WT	2 polymorphisms in intronic part	<i>cyp51A</i> , <i>cyp51B</i> , <i>cdr1B</i>
Strain B	> 8	4	0.5	8	WT	2 polymorphisms in intronic part	<i>cyp51A</i> , <i>atrF</i> , <i>cdr1B</i>
Strain C	> 8	4	1	> 8	WT	2 polymorphisms in intronic part	<i>cyp51A</i> , <i>cyp51B</i> , <i>atrF</i> , <i>cdr1B</i>
Strain D	> 8	> 8	> 8	> 8	WT	F46Y, M172V, E427K	<i>cyp51A</i> , <i>cyp51B</i>

MIC: Minimum Inhibitory Concentrations
ITZ: Itraconazole; VRZ: Voriconazole; PSZ: Posaconazole; ISA: Isavuconazole
WT: Wild Type



329

330 **Figure 1:** Gene expression of (A) *cyp51A*, (B) *cyp51B*, (C) *atrF*, (D) *cdr1B*, (E-H) *MDR 1/2/3/4*,
331 (I) *MFS56*, and (J) *M85* compared to CBS144.89/A1163 reference strain. The data are represented
332 as the mean \pm SD of duplicate samples and are representative of the data obtained from two
333 independent experiments (**** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$).

Azole resistance in *Aspergillus fumigatus* isolates from respiratory specimens in Lyon University Hospitals, France: prevalence and mechanisms involved

195 patients included over an eight-month period, representing 221 respiratory samples with positive culture for *Aspergillus fumigatus*

4 patients (2%) had *A. fumigatus* resistant strains (A, B, C, D)

This study underlines the need to screen isolates for azole resistance and the diversity of resistance mechanisms to be explored

Minimum Inhibitory Concentrations (EUCAST broth microdilution method) of azole-resistant *A. fumigatus* strains, sequence analysis and gene overexpression results.

Resistant strain	MIC (mg/L)				Sequence analysis of <i>cyp51A</i> promoter	Sequence analysis of <i>cyp51A</i> gene	Significant gene overexpressions
	ITZ	VRZ	PSZ	ISA			
Strain A	> 8	4	0.25	4	WT	2 polymorphisms in intronic part	<i>cyp51A</i> , <i>cyp51B</i> , <i>cdr1B</i>
Strain B	> 8	4	0.5	8	WT	2 polymorphisms in intronic part	<i>cyp51A</i> , <i>atrF</i> , <i>cdr1B</i>
Strain C	> 8	4	1	> 8	WT	2 polymorphisms in intronic part	<i>cyp51A</i> , <i>cyp51B</i> , <i>atrF</i> , <i>cdr1B</i>
Strain D	> 8	> 8	> 8	> 8	WT	F46Y, M172V, E427K	<i>cyp51A</i> , <i>cyp51B</i>

MIC: Minimum Inhibitory Concentrations ITZ: Itraconazole; VRZ: Voriconazole; PSZ: Posaconazole; ISA: Isavuconazole; WT: Wild Type

(A) *cyp51A*, (B) *cyp51B*, (C) *atrF*, and (D) *cdr1B* gene expression

(**** P<0.0001; *** P<0.001; ** P<0.01)

