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

Loïc SIMON[1,2], Tanguy DÉMÉAUTIS[1,3], Damien DUPONT[1,4], Rolf KRAMER[1,5], Héloïse GARNIER[1], Isabelle DURIEU[6], Agathe SÉNÉCHAL[7], Philippe REIX[8], Sébastien COURAUD[9], Gilles DEVOUASSOUX[10,3], Bruno LINA[11,12], Meja RABODONIRINA[1,12], Martine WALLON[1,4], Eric DANNAOUI[13,14], Florence PERSAT[1,3], Jean MENOTTI[1,3*]

1 - Hospices Civils de Lyon, Institut des Agents Infectieux, Service de Parasitologie et Mycologie médicale, Hôpital de la Croix-Rousse, Lyon, France.

2 - Centre Hospitalier Universitaire de Nice, Service de Parasitologie-Mycologie, Université Côte d'Azur, Nice, France.

3 - Université Claude Bernard Lyon 1, EA7426 équipe Inflammation et immunité de l'épithélium respiratoire, Oullins, France.

4 - Université Claude Bernard Lyon 1, Centre de Recherche en Neurosciences de Lyon, Inserm U1028, CNRS UMR5292, équipe WAKING, Lyon, France.

5 - European Public Health Microbiology Training Programme, ECDC, Stockholm, Sweden.

6 - Hospices Civils de Lyon, Hôpital Lyon Sud, Service de Médecine Interne et Vasculaire, Centre de Ressources et de Compétences de la Mucoviscidose Adultes, Pierre-Bénite, France.

7 - Hospices Civils de Lyon, Hôpital Louis Pradel, Département de Pneumologie et de Transplantation Pulmonaire, Hospices Civils de Lyon, Bron, France.

8 - Hospices Civils de Lyon, Hôpital Femme-Mère-Enfant, Service de Pneumologie et Allergologie Pédiatriques, Centre de Ressources et de Compétences de la Mucoviscidose Enfants, Bron, France.

9 - Hospices Civils de Lyon, Hôpital Lyon Sud, Service de Pneumologie Aiguë Spécialisée et Cancérologie Thoracique, Pierre Bénite, France.

10 - Hospices Civils de Lyon, Hôpital de la Croix-Rousse, Service de Pneumologie, Lyon, France.

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11 - Hospices Civils de Lyon, Institut des Agents Infectieux, Service de Virologie, Hôpital de la Croix-Rousse, Lyon, France.

12 - Université Claude Bernard Lyon 1, Centre International de Recherche en Infectiologie, Inserm U1111, Lyon, France.

13 - Université de Paris, Assistance Publique – Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Microbiologie, Unité de Parasitologie-Mycologie, Paris, France.

14 - Université Paris-Est Créteil, Équipe Dynamyc, EA 7380, Créteil, France.

***Corresponding author:** Jean Menotti, Laboratoire de Parasitologie et Mycologie Médicale, Institut des Agents Infectieux, Hôpital de la Croix-Rousse, Hospices Civils de Lyon / Université Claude Bernard - Lyon 1, 103 grande rue de la Croix-Rousse 69317 Lyon cedex 04, France. Phone: +33472003728, e-mail: jean.menotti@univ-lyon1.fr

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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.

1. INTRODUCTION

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

2. MATERIALS AND METHODS

2.1 Study design

This study was conducted in the laboratory of Parasitology-Mycology of the Lyon University Hospitals, France. From February to September 2017, all respiratory samples from patients attending the inpatient and outpatient wards of the Pulmonary Medicine Departments were 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 $2^{-\Delta\Delta Ct}$ 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**

142 **3.1 Study population and azole resistance**

143 During an eight-month period, 221 consecutive *Aspergillus sp.* isolates from the section
144 *Fumigati* were obtained by culture from 195 patients' respiratory specimens. All isolates were
145 confirmed as *A. fumigatus* by PCR and sequence analysis of the β-tubulin gene. Susceptibility
146 testing identified four isolates to be resistant to azole antifungals **(Table 1)**. All of them were
147 resistant to itraconazole (all with MIC>8 mg/L), voriconazole (range 4 - >8 mg/L), and
148 isavuconazole (range 4 - >8 mg/L); three of them were in addition resistant to posaconazole (range
149 0.5 - >8 mg/L) whereas the fourth was in the area of technical uncertainty (MIC=0.25 mg/L)
150 **(Table 1)**. These four isolates were from four different patients. In total, the prevalence of azole
151 resistance in our study population was 2.1% (4/195). The resistant isolates were grown in culture
152 from respiratory specimens from three patients with cystic fibrosis (for isolates A, B and C) and
153 one patient with autosomal dominant hyperimmunoglobulinemia E (for isolate D). All four
154 patients previously received prolonged antifungal treatments: itraconazole for the three patients
155 with cystic fibrosis and treatments with itraconazole, voriconazole and posaconazole for patient D.
156 Among the 195 patients included, 70 had cystic fibrosis: the prevalence of azole resistance in
157 patients with cystic fibrosis in our cohort was thus 4.3% (3/70). Consequently, the prevalence of
158 azole resistance in patients from Pulmonary Medicine Departments, excluding cystic fibrosis
159 patients, was 0.8% (1/125).

160 **3.2 DNA sequence analyses of the *cyp51A* gene**

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

165 **3.3 Gene expression of the azole-resistant strains**

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

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

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

4. DISCUSSION

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

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

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

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

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

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

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

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

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

DECLARATIONS

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Competing Interests: PR has received educational grants outside the submitted work from Vertex and personal fees from Zambon and Gilead. SC has received research grants outside the submitted work from Astra-Zeneca, MSD, Pfizer and Roche, personal fees from Astra-Zeneca, MSD and Roche, and non-financial support from Astra-Zeneca and Roche. FP has received aid for attending symposia from Gilead and Pfizer. JM has received a research grant outside the submitted work from MSD and aid for attending symposia from Gilead, MSD, Pfizer and Roche Diagnostics. All other authors: none to declare.

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

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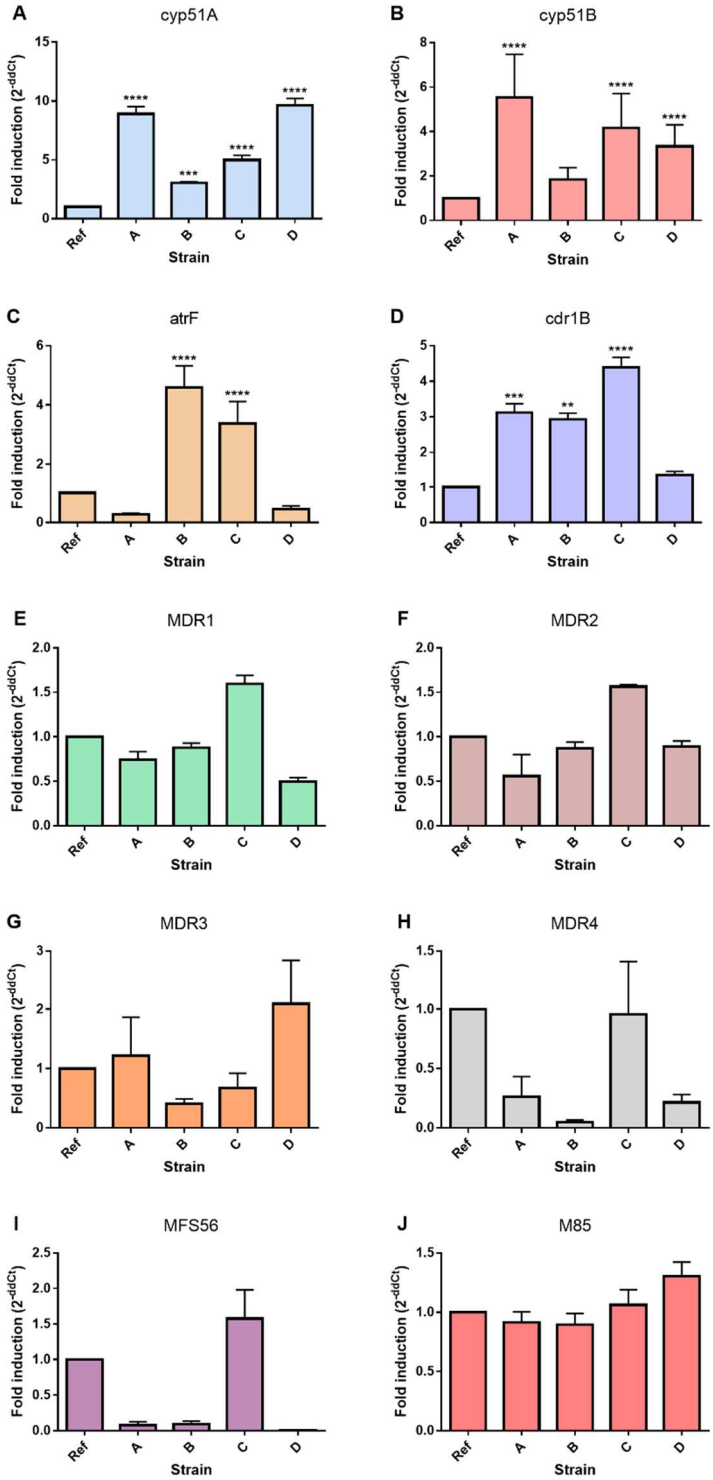
326 **Table 1:** Minimum Inhibitory Concentrations (EUCAST broth microdilution method) of azole-
327 resistant *A. fumigatus* strains, sequence analysis and gene overexpression results.

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

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

328



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 ± 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

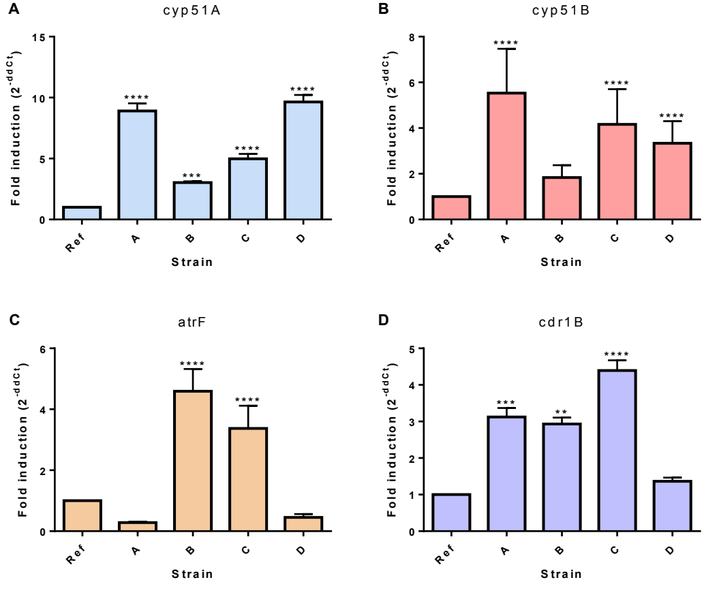
Minimum Inhibitory Concentrations (EUCAST broth microdilution method) of azole-resistant *A. fumigatus strains*, sequence analysis and gene overexpression results.

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

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)



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