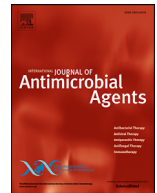




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Short Communication

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

Loïc Simon^{a,b}, Tanguy Déméautis^{a,c}, Damien Dupont^{a,d}, Rolf Kramer^{a,e}, Héloïse Garnier^a, Isabelle Durieu^f, Agathe Sénéchal^g, Philippe Reix^h, Sébastien Couraudⁱ, Gilles Devouassoux^{c,j}, Bruno Lina^{k,l}, Meja Rabodonirina^{a,l}, Martine Wallon^{a,d}, Eric Dannaoui^{m,n}, Florence Persat^{a,c}, Jean Menotti^{a,c,*}

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

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

^c Université Claude Bernard Lyon 1, EA7426 équipe Inflammation et immunité de l'épithélium respiratoire, Oullins, France

^d Université Claude Bernard Lyon 1, Centre de Recherche en Neurosciences de Lyon, Inserm U1028, CNRS UMR5292, équipe WAKING, Lyon, France

^e European Public Health Microbiology Training Programme, European Centre for Disease Prevention and Control, Stockholm, Sweden

^f 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

^g Hospices Civils de Lyon, Hôpital Louis Pradel, Département de Pneumologie et de Transplantation Pulmonaire, Hospices Civils de Lyon, Bron, France

^h 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

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

^j Hospices Civils de Lyon, Hôpital de la Croix-Rousse, Service de Pneumologie, Lyon, France

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

^l Université Claude Bernard Lyon 1, Centre International de Recherche en Infectiologie, Inserm U1111, Lyon, France

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

ⁿ Université Paris-Est Créteil, Équipe Dynamyc, EA 7380, Créteil, France

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ABSTRACT

Resistance of *Aspergillus fumigatus* to triazoles has been reported increasingly in Europe. As few data are available from Southern France, the objectives of this study were to assess the burden of *A. fumigatus* isolates with azole resistance from clinical specimens in Lyon, and explore the resistance mechanisms involved. In this retrospective cross-sectional study, 221 consecutive *A. fumigatus* isolates from respiratory samples were identified from an 8-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. All samples were tested for susceptibilities to itraconazole, voriconazole, posaconazole and isavuconazole using concentration gradient strips, and the results were confirmed using the EUCAST broth microdilution method. Resistance mechanisms were investigated by sequencing 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 isolate had F46Y, M172V and E427K polymorphisms. No mutations were identified in the *cyp51A* promoter, but significant induction of *cyp51A* and *cyp51B* gene expression was observed for all four and three isolates, respectively. Significant induction of *atrF* and *cdr1B* gene expression was 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 induction of expression of the *cyp51* genes and two genes encoding efflux transporters was evidenced, underlying the diversity of resistance mechanisms to be explored.

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1. Introduction

Aspergillus fumigatus is a ubiquitous mould, and is 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 inhibition of lanosterol 14- α -demethylase involved in the biosynthesis pathway of ergosterol, the main sterol in the fungal membrane [1]. However, resistance of *A. fumigatus* strains to triazole antifungals has been reported increasingly worldwide, and is becoming a concern for patient management [4–11]. The main mechanisms of resistance described to date are: (i) mutations in the *cyp51A* gene encoding lanosterol 14- α -demethylase or its promoter [4–7,12]; (ii) overexpression of *cyp51* [12]; and (iii) overexpression of multi-drug efflux pumps [12]. The objectives of this study were to assess the burden of *A. fumigatus* isolates with azole resistance from clinical specimens in Lyon, and to evaluate the resistance mechanisms involved.

2. Materials and methods

2.1. Study design

This study was conducted in the Laboratory of Parasitology-Mycology of 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 the laboratory routine.

2.2. Isolates

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

2.3. Antifungal susceptibility testing and fungal cultures

Determination of minimum inhibitory concentrations (MICs) was performed for all *A. fumigatus* isolates using concentration gradient strips for itraconazole, voriconazole, posaconazole (Etest, bioMérieux) and isavuconazole (Liophilchem, Roseto degli Abruzzi, Italy), in accordance with the manufacturers' recommendations. All isolates showing resistance with concentration gradient strips were tested using the EUCAST standardized broth microdilution method for confirmation. Susceptibility or resistance profiles to the different antifungals were defined according to the 2020 EUCAST clinical breakpoints after 48-h 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 cultured in liquid Sabouraud + 4 mg/L itraconazole medium at 35°C for 16 h for further analyses [8].

* Corresponding author. Address: 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. Tel.: +33 472003728

E-mail address: jean.menotti@univ-lyon1.fr (J. Menotti).

2.4. Sequence analysis of the *cyp51A* gene

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

2.5. RNA extraction and reverse transcriptase real-time PCR

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

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

2.6. Statistical analysis

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

3. Results

3.1. Study population and azole resistance

Over an 8-month period, 221 consecutive *Aspergillus* spp. isolates from the section *Fumigati* were obtained by culture from the respiratory specimens from 195 patients. All isolates were confirmed as *A. fumigatus* by PCR and sequence analysis of the β -tubulin gene. Susceptibility testing revealed that four isolates were resistant to azole antifungals (Table 1). All four isolates were resistant to itraconazole (all with MIC > 8 mg/L), voriconazole (MIC range 4–> 8 mg/L) and isavuconazole (MIC range 4–> 8 mg/L); three of these isolates were also 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 the study population was 2.1% (4/195). The resistant isolates were grown in culture from respiratory specimens from three patients with cystic fibrosis (Isolates A, B and C) and one patient with autosomal-dominant hyperimmunoglobulinemia E (Isolate D). All four patients had previously received prolonged antifungal treatment: itraconazole for the three patients with cystic fibrosis; and itraconazole, voriconazole and posaconazole for the patient with autosomal-dominant hyperimmunoglobulinemia E. Among the 195 patients included, 70 had cystic fibrosis; the prevalence of azole resistance in patients with cystic fibrosis in the study cohort was thus 4.3% (3/70). Consequently, the prevalence of azole resistance in patients from pulmonary medicine departments, excluding patients with cystic fibrosis, 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*

Table 1

Minimum inhibitory concentrations (MICs) (EUCAST broth microdilution method) of azole-resistant *Aspergillus 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 overexpression
	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>

ITZ, itraconazole; VRZ, voriconazole; PSZ, posaconazole; ISA, isavuconazole; WT, wild type.

gene for Strain D (F46Y, M172V, E427K) (Table 1). No mutations were 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

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

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

No significant differences in expression of the *MDR1/2/3/4* genes or the *MFS56* and *M85* genes (Fig. 1E-J) were observed for the four azole-resistant strains compared with 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 for invasive and chronic aspergilloses. This study on a large number of consecutive patients identified three findings. First, the prevalence of azole resistance in this study was low, but higher in the subpopulation of patients with cystic fibrosis. Second, this 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, this study invites the authors to continue monitoring azole sensitivity in *A. fumigatus* isolates.

The overall prevalence of azole resistance in this study (approximately 2%) can be specified according to the patients' background. Choukri et al. reported similar prevalence but in an cohort of immunocompromised, immunocompetent and cystic fibrosis patients without distinction [14]. Other studies reported in France gave different percentages for the prevalence of *A. fumigatus* resistance in different patient groups [5,6,15]. Regarding patients from pulmonology wards excluding patients with cystic fibrosis, the prevalence of azole resistance found in the present study (0.8%) was lower than that reported recently by Guegan et al. on a similar

number of patients (2.8%) [15]. Focusing on patients with cystic fibrosis in the present study, the prevalence of azole resistance was 4.3%, which is close to the prevalence rate found in Paris by Burgel et al. [6], and slightly lower than the prevalence rate of 6.8% found in Nantes by Laverne et al. [5]. Guegan et al. found a 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 prevalence rate for resistance 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) overuse of azole antifungals for patient treatment, particularly for long-term treatment; and (ii) large-scale use of azoles 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 the present 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 been observed in the past, and none of them had been correlated definitively 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. This study found significant induction in expression of the *cyp51A* gene for all four azole-resistant strains. Furthermore, despite scarce descriptions of overexpression of *cyp51B* in the literature [12], this study found significant induction of expression of the *cyp51B* gene for three strains, including two from patients with cystic fibrosis.

There are two superfamilies of efflux pumps known to allow azole efflux outside the fungal cell: ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters [19]. The present study evaluated the gene expression of six ABC transporters (*atrF*, *cdr1B* and *MDR1/2/3/4*). Significant induction of expression of the *atrF* and *cdr1B* genes was found in two and three of the azole-resistant *A. fumigatus* strains, respectively. Even if these transporters have been linked to azole resistance [8,12], descriptions of this mechanism are much scarcer than that implying *cyp51A*, and only induction of *cdr1B* gene expression was evidenced by Fraczek et al. [8]. The present study did not find any induction of expression of the *MDR1/2/3/4* genes, whereas others have reported that

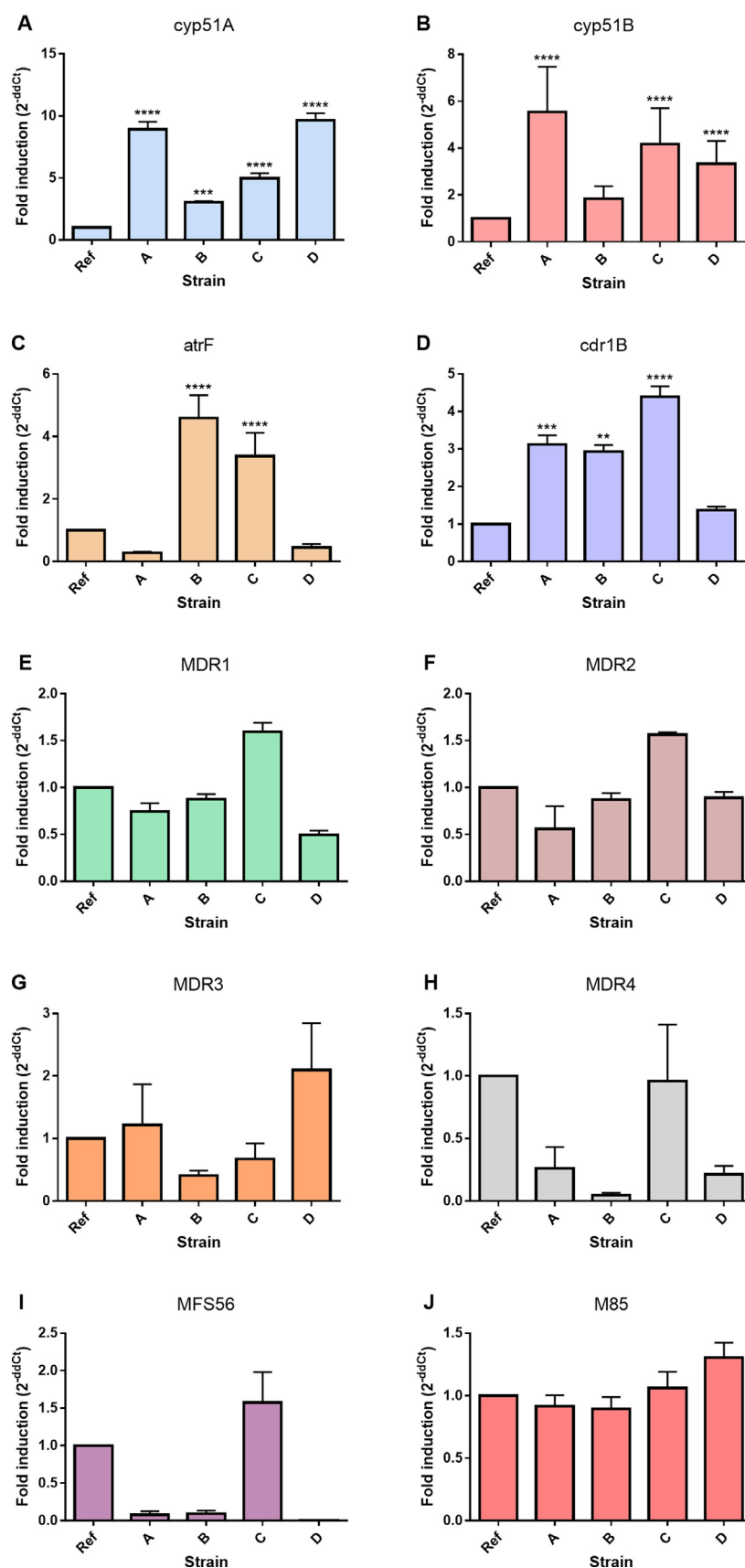


Fig. 1. Gene expression of (A) *cyp51A*, (B) *cyp51B*, (C) *atrF*, (D) *cdr1B*, (E-H) *MDR 1/2/3/4*, (I) *MFS56* and (J) *M85* compared with CBS144.89/A1163 reference strain. The data are represented as the mean \pm standard deviation of duplicate samples and are representative of the data obtained from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

these genes are upregulated by azole exposure or overexpressed in some resistant strains [12]. Considering *MF556* and *M85* genes, this study did not find any induction of expression.

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

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Competing interests

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Ethical approval

Not required.

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