Emergence of *Aspergillus fumigatus* azole resistance in azole-naive patients with chronic obstructive pulmonary disease and their homes

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Abstract

Azole-resistant *Aspergillus fumigatus* (ARAF) has been reported in patients with chronic obstructive pulmonary disease (COPD) but has not been specifically assessed so far. Here, we evaluated ARAF prevalence in azole-naive COPD patients and their homes, and assessed whether CYP51A mutations were similar in clinical and environmental reservoirs. Sixty respiratory samples from 41 COPD patients with acute exacerbation and environmental samples from 36 of these patient's homes were prospectively collected. *A. fumigatus* was detected in respiratory samples from 11 of 41 patients (27%) and in 15 of 36 domiciles (42%). Cyp51A sequencing and selection on itraconazole medium of clinical (n = 68) and environmental (n = 48) isolates yielded ARAF detection in 1 of 11 *A. fumigatus* colonized patients with COPD (9%) and 2 of 15 *A. fumigatus*-positive patient's homes (13%). The clinical isolate had no CYP51A mutation. Two environmental isolates from two patients harbored TR34/L98H mutation, and one had an H285Y mutation. Coexistence of different cyp51A genotypes and/or azole resistance profiles was detected in 3 of 8 respiratory and 2 of 10 environmental samples with more than one isolate, confirming the need for a systematic screening of all clinically relevant isolates. The high prevalence of ARAF in patients with COPD and their homes supports the need for further studies to assess the prevalence of azole resistance in patients with *Aspergillus* diseases in Northern France.

KEYWORDS

*Aspergillus fumigatus*, azole resistance, chronic obstructive pulmonary disease, dwelling, electrostatic dust collector, mold environmental exposure
Patients with chronic obstructive pulmonary disease (COPD) are at risk for Aspergillus colonization and invasive aspergillosis (IA), which has a 1.6%-3.9% prevalence and is associated with high mortality. Azole-resistant Aspergillus fumigatus (ARAF) has been reported in the UK in four itraconazole-treated COPD patients (3 with chronic pulmonary aspergillosis and one with IA), in one patient with IA who had received voriconazole for 2 days in Denmark, and in two azole-naïve patients colonized with TR46/Y121F/T289A A. fumigatus in the Netherlands. However, although the emergence of ARAF is increasingly studied in hematology IA (1%-15.9% prevalence), cystic fibrosis (4.5%-8% prevalence), or intensive care units (ICU) patients (4.5%-26% prevalence), the epidemiology and potential clinical impact of A. fumigatus azole resistance in patients with COPD have not been specifically assessed so far.

Furthermore, the presence of ARAF isolates has also been increasingly reported in the environment in several European countries, in Asia and in South America, supporting the role of azole fungicides used in agriculture in the emergence of drug resistance. As most patients acquire A. fumigatus from the environment, the emergence and spread of azole-resistant strains in the environment will put more humans at risk. However, only a few studies have combined environmental assessment and clinical data, two of them reporting ARAF isolates (TR34/L98H or TR46/Y121F/T289A) in the soil around the home of patients with IA due to isolates carrying the same mutation. Interestingly, in one of these two studies, ARAF isolates were further found in the patient’s home. Another study compared the prevalence and CYP51A mutations of clinical and environmental ARAF in different geographic areas of Germany and found a higher prevalence of TR34/L98H and TR46/Y121F/T289A isolates in the environment than in clinical isolates (4%-15% vs 0%-3%), whereas some other mutations, such as G54W or M220L, were only present in clinical isolates. Furthermore, except a study in which TR34/Y121F/T289A isolates were found in the home of patients with IA, and another one assessing eight domiciles in the Netherlands, most environmental ARAF were isolated from soil or outdoor/indoor air samples in hospitals.

In this study, we aimed to determine the prevalence of ARAF in azole-naïve COPD patients and their homes, and assess whether CYP51A mutations of ARAF isolates were similar in clinical and environmental reservoirs, in order to evaluate if the domestic mold environmental exposure could be a risk factor for the acquisition of ARAF isolates in these patients.

2 | METHODS

2.1 | Patients recruiting and clinical and environmental sampling

Forty-one patients with acute exacerbation of COPD, who had not received antifungal systemic treatment within the 6 previous months, were prospectively included in Lille University Hospital (France) from August 2011 to February 2015, resulting in a collection of 60 respiratory samples (47 sputa, and 13 oropharyngeal washes (OPW) obtained by a 30 seconds to 1 minute gargling with 10 mL of sterile NaCl 0.9%). The location of the patient’s homes was noted, and environmental samples were collected within the month following the patient’s recruitment. Samples included 36 electrostatic dust fall collectors (EDCs) that had been exposed for 10 weeks in the patient’s bedroom (from 36 patient’s homes) and sent to the laboratory by mail, together with swabs and air samples collected with Coriolis ™ (Bertin Technologies) in 10 patient’s homes.

The study was approved by the local ethics committee (North-West Ethics Committee, University of Rouen, France, referral number 2010-031), and it was registered at ClinicalTrials.gov (identifier NCT02318524).

2.2 | Mycological analysis of clinical and environmental samples, and screening for A. fumigatus azole-resistant isolates

Sputum samples were half-diluted with a solution of acetylcysteine (10% in water; Digest-EUR, Eurobio), incubated for 30 minutes at 37°C, and 10 µL was cultured on Sabouraud agar medium diluted half and containing 0.5 g/L amikacin (SAB) at 30°C, chromogenic agar medium at 37°C, and erythritol agar medium at 24°C for 7 days. For OPW, 10 µL of sample was cultured similarly without pretreatment. EDC washing, which was performed no later than 48-hour postsample retrieval, consisted of shaking for 10 minutes in 20 mL of sterile PBS with 0.1% Tween 80 in a Stomacher, as previously described, and 100 µL of the collected solution was cultured on SAB at 30°C, benomyl or DG18 agar media at 24°C for 7 days. A. fumigatus and other molds were identified by macroscopic and microscopic characteristics. When more than 10 A. fumigatus isolates were detected, clinical and environmental samples were further cultured on Sabouraud agar medium containing 4 mg/L of itraconazole (ITZ) at 37°C for 4 days, for selection of azole-resistant isolates. Otherwise, each A. fumigatus clinical and environmental isolate was screened for detection of azole resistance by dipping a sterile swab into a 10⁵ conidia/mL suspension
and streaking it across the surface of an ITZ medium, which was incubated at 37°C for 4 days.

### 2.3 | Phenotypic and genotypic confirmations of A. fumigatus identification

Aspergillus fumigatus identification was confirmed by culture at 50°C for 3 days, and, at the genotypic level, by internal transcribed spacer (ITS)-rDNA and beta-tubulin sequencing, after DNA extraction using QIAamp® DNA Mini Kit (Qiagen, Courtaboeuf, France), as previously described. ITS-rDNA and beta-tubulin sequences were deposited in GenBank database under accession numbers KX788447 to KX788446.

### 2.4 | Cyp51A sequencing

Detection of cyp51A alterations was performed for all A. fumigatus clinical and environmental isolates by sequencing of the cyp51A gene and its promoter. The three following primer pairs were used for amplification of 3 fragments of 725, 705, and 826 bp, respectively: CYP51AF-1 (5′-TAATCGCAGCACCTCCAG-3′) and CYP51AR-1 (5′-GACATCCTTGWGCTTGCCGTTGAG-3′), CYP51AF-2 (5′-TCTACCTGGGCGTTCAGGG-3′) and CYP51AR-2 (5′-CTTCGAGACATCCTTGCGCGGTTGAG-3′), CYP51AF-3 (5′-CTTCGA GGACTTTTGGCTGTGAG-3′) and CYP51AR-3 (5′-CTTCGA GGACTTTTGGCTGTGAG-3′). Amplification reactions were performed in a final volume of 50 μL containing 5 μL template DNA, 10 μL reaction buffer (5X), 1.5 mmol L−1 MgCl₂, 200 μmol L−1 of each dNTP, 0.4 μmol L−1 of each primer and 2 units Taq polymerase (GoTaq® DNA polymerase, Promega, France). The PCR cycling protocol was performed in a GenAmp 2700 Engine (Applied Biosystems, Life Technologies, Saint Aubin, France) as follows: one initial denaturation step of 10 minutes at 95°C, followed by 45 cycles consisting of denaturation for 1 minute at 95°C, annealing for 1 minute at 55°C and extension for 1 minute at 72°C, followed by a final extension for 15 minutes at 72°C. Cyp51A sequences were deposited in GenBank database under accession numbers KX788447 to KX788562.

### 2.5 | Antifungal susceptibility testing

ITZ, voriconazole (VOR), posaconazole (POS), and isavuconazole (ISA) MICs were determined for CYP51A-mutated isolates and for isolates with positive growth on ITZ medium, using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) microbroth dilution (E.Def 9.3.1) reference method for susceptibility testing. EUCAST criteria were used to define susceptibility and resistance.

### 2.6 | Homology modeling of A. fumigatus CYP51A

To assess theazole-CYP51A interactions in mutated isolates and the potential role of CYP51A mutations in azole resistance, we modeled A. fumigatus CYP51A structure in complex with ITZ. The crystal structure of A. fumigatus CYP51B in complex with VOR (PDB id 4UYM) was used as the 3D template, as A. fumigatus CYP51A shares higher sequence identity with this protein (63.6%) than with CYP51A from other organisms with available crystal structures. For that purpose, the 3D template was first modified by docking ITZ instead of VOR, applying a 3D-structural alignment with the crystal structure of yeast CYP51 bound to ITZ (PDB id 4ZDY), and then 20 models were generated using MODELLER-9v17 program. Among the models, the one with the lowest energy was selected as the final model.

### 3 | RESULTS

#### 3.1 | Characteristics of patients with COPD; prevalence of A. fumigatus colonization and domestic exposure

The mean age of patients was 63.8 ± 9.7 years old, and M/F sex ratio was 3.2. A. fumigatus colonization was detected in respiratory samples from 11 of 41 patients (27%), with 1 to 20 colonies per sample, which yielded 68 clinical isolates. Culture was positive for all EDCs, where 1 to 159 mold colonies were detected. A. fumigatus was present in 15 of 36 EDCs (1 to 19 colonies), yielding a 42% frequency of A. fumigatus domestic exposure. These 15 EDCs corresponded to five A. fumigatus colonized patients and 10 noncolonized patients, and yielded 41 isolates. Seven supplementary isolates were obtained from air or swab samples.

#### 3.2 | Phenotypic and genotypic confirmations of A. fumigatus identification

Thermotolerance at 50°C, ITS-rDNA and beta-tubulin sequencing confirmed A. fumigatus identification for all isolates. ITS-rDNA mutations were detected in 12 clinical strains from 2 patients and 13 environmental strains from 6 patient’s homes, whereas only 2 environmental strains presented beta-tubulin gene mutations, one synonymous and one nonsynonymous, all other sequences having 100% identity (Table 1).

#### 3.3 | Cyp51A mutations in clinical and environmental isolates

Cyp51A sequencing yielded 5 mutation profiles in 5 clinical and 5 environmental isolates from 6 different patients (Table 1). TR34/L98H mutation was detected in 2 environmental isolates from 2 patients (no. 7 and no. 22). Mutations F46Y-M172V-N248T-D255E-E427K were detected in 2 clinical isolates from the same patient (no. 24) and in 1 environmental isolate (no. 7). Another environmental isolate from patient no. 9’s EDC presented mutations F46Y-M172V-E427K. Mutation A284T was detected in 3 clinical isolates from 1 patient (no. 41). Lastly, mutation H285Y was detected in one environmental isolate from patient no. 22.

Cyp51A synonymous mutations were detected in 13 isolates which presented G1696A mutation, including 3 environmental isolates from three patients (no. 2, 7 and 30) and 10 clinical isolates from two patients (no. 21 and 34). Nine clinical isolates from the same patient (no. 42) presented the nonsynonymous mutation C1562T.
**TABLE 1** Characteristics of *Aspergillus fumigatus* clinical and environmental isolates: origin, cyp51A, beta-tubulin, ITS-rDNA sequencing, and growth on itraconazole medium

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Origin of isolates</th>
<th>Number of isolates</th>
<th>cyp51A sequencing</th>
<th>Mutated β-tubulin isolates</th>
<th>Mutated ITS-rDNA isolates</th>
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<td>24</td>
<td>Sputum</td>
<td>6</td>
<td>2 F46Y, M172V, N248T, D255E, E427K</td>
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<td>41</td>
<td>Sputum</td>
<td>8</td>
<td>3 A284T</td>
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<td>Total</td>
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<td>116</td>
<td>97</td>
<td>10</td>
<td>9</td>
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</table>

Pan-azole-resistant isolates are highlighted in gray. Synonymous mutations are in italics. OPW, oropharyngeal wash; EDC, electrostatic dust collector. *Isolates obtained after direct culture on ITZ medium of respiratory samples if more than 10 *A. fumigatus* colonies had been detected on media without ITZ.
resistant (with 1-2 mg/L MICs) (Table 2).

diate or resistant (with 0.25-0.5 mg/L MICs), and ISA susceptible or
F46Y- M172V- E427K) were ITZ and VOR susceptible, POS interme-

Azole resistance was detected in the environment of two patients

Table 2, with 1-2 mg/L MICs) (Table 2).

Prevalence of TR

<table>
<thead>
<tr>
<th>CYP51A mutations</th>
<th>MIC (mg/L)</th>
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<tbody>
<tr>
<td>ITZ VOR POS ISA</td>
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<tr>
<td>TR34/L98H (n=2)</td>
<td>&gt;8 4 1.2 8-16</td>
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<tr>
<td>H285Y (n=1)</td>
<td>&gt;16 4 0.5 4</td>
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<tr>
<td>WT (n=1)</td>
<td>&gt;16 4 1 &gt;16</td>
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<tr>
<td>A284T (n=3)</td>
<td>0.25-0.5 0.25-1 0.5 2</td>
</tr>
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<td>F46Y, M172V, N248T, D255E, E427K (n=3)</td>
<td>1 1 0.5 1-2</td>
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<tr>
<td>F46Y, M172V, E427K (n=1)</td>
<td>0.5 1 0.25 1</td>
</tr>
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</table>

The following EUCAST criteria were used to define susceptibility and resis-
tance: an ITZ or VOR MIC of ≤1 mg/L was considered susceptible, a MIC of
2 mg/L intermediate and of >2 mg/L resistant; a POS MIC of ≤0.12 mg/L
was considered susceptible, a MIC of 0.25 mg/L was considered interme-
tate and of >0.25 mg/L resistant; an ISA MIC of ≤1 mg/L was considered
susceptible, a MIC of >1 mg/L was considered resistant.29 Pan-
azole-resistant isolates are highlighted in gray.

The 23% prevalence of A. fumigatus colonization we found during COPD acute exacerbation is similar to the 16.6%-28% ones that have been reported previously in Spain or in the UK.3,32 However, the 42% frequency of A. fumigatus in patient’s homes we found in the present study seems high when compared to some other studies.
As only five of the 15 A. fumigatus-positive EDCs originated from A. fumigatus colonized patient’s homes, no relationship between A. fumigatus exposure and colonization could be established. These data were consistent with those found in homes from patients with asthma, showing the absence of association between A. fumigatus isolation from sputum and dustborne levels of A. fumigatus. This absence of relationship probably results from host individual risk factors for colonization (such as corticoid or long course antibiotic treatments), and exposure to other mold sources such as food (eg, pepper, tea) or occupational or leisure activities, or inhalation of airborne mold spores when the patient is not at home. In addition, the absence of standard methods for measuring indoor mold levels renders difficult the definition of a human dose-response data and the interpretation of these data in terms of human health.

The 13% prevalence of TR34/L98H isolates in COPD patient’s homes (when taking into account only A. fumigatus-positive EDCs) was in agreement with environmental origin of this mutation and is consistent with previous data in the Netherlands, Denmark, Germany, or India where 12% (6/49), 11% (4/38), 10% (45/455), or 7% (44/630) of soil samples were found to contain TR34/L98H isolates, respectively. However, unlike most other studies, which focused on indoor or outdoor samples around hospitals, we report the presence of TR34/L98H isolates in the domestic environment of patients who are at risk for IA. Furthermore, the two homes with TR34/L98H isolates were located in urban areas. Altogether, these data confirm the presence of ARAF in the homes of patients at risk for IA that was recently reported, and further indicate that exposure to environmental ARAF isolates is not limited to rural areas where fungicides...
are used. As the two patients with TR46/L98H isolates in their homes were not colonized by *A. fumigatus*, the acquisition of ARAF isolates via domestic environmental exposure could not be confirmed in our study. However, our data confirm that, although most IA with TR46/L98H isolates were reported in hematology, transplant, or patients with cancer, patients with COPD are also exposed and at risk to be colonized by azole-resistant isolates and to further develop IA.

Interestingly, coexistence of different cyp51A genotypes and/or azole resistance profiles was found in 3 of 8 (37%) respiratory and 2 of 10 (20%) environmental samples where more than one *A. fumigatus* isolate had been detected. The coexistence of *Aspergillus* sensitive and resistant isolates confirms previous reports in patients with IA. But, the frequencies we found are higher than the 12% and 2% of isolates with mixed profiles that were reported by Ahmad et al. in clinical and environmental *A. fumigatus* isolates obtained from single colonies, respectively. Altogether, these data indicate that picking a single colony for MIC determination, which is usually done in clinical practice, is not sufficient to exclude the presence of azole-resistant isolates, and confirm that MIC determination from all colonies or screening using azole-supplemented medium should be performed when patients are to be treated. The usefulness of this method was confirmed in our study where two ITZ-resistant isolates were obtained after direct culture on ITZ medium of one EDC and one sputum, which contained more than 10 colonies.

Other CYP51A mutations included A284T, which was reported to be associated with decreased sensitivity to ITZ, VOR, and POS. However, only POS and ISA MICs were discreetly increased in our study. We also identified 4 isolates harboring mutations F46Y-M172V-N248T-D255E-E427K in similar combinations, giving slightly increased POS and ISA MICs. Similar combinations, mainly associated with azole sensitivity, have been previously described in Europe, but isolates with increased azole MICs have also been reported in Europe and in the USA. Interestingly, a new mutation H285Y was also identified in one environmental isolate and was associated with pan-azole resistance, ISA MIC being correlated with VOR MIC, as previously described for ARAF isolates with other CYP51A mutations. Modeling the CYP51A indicated that the buried residue in position 285 was situated at the entrance of the ligand access channel 1. Although the mutation of a buried residue generally induces largest structural rearrangements, no large structural alteration of the channel 1 was found to be caused by the H285Y mutation. Furthermore, as ITZ was proposed to enter via another channel, the impact of H285Y modification on the ITZ susceptibility appears difficult to predict. Further investigations are required to precise the functional effect of this mutation, which, interestingly, is localized near the A284T and T289A mutations. However, unlike H285Y, T289A is not associated with decreased azole sensitivity when not combined with TR46/Y121F. Although TR46/Y121F/T289A isolates have been reported in Belgium, which is only at a few kilometers from Lille, or in other areas in France, none was found in our study. Although cyp51A sequencing was performed for all isolates, irrespectively of itraconazole agar screening result, a potential caveat is that, when more than *A. fumigatus* 10 isolates had been detected, we performed a direct screening from samples using ITZ agar only. The TR46/Y121F/T289A genotype induces high level VOR and ISA resistance but variable if any ITZ MIC elevation, and a recent study showed that for reliable detection of this genotype screening using VOR 1 mg/L agar was necessary. Hence, although cyp51A was sequenced for all the *A. fumigatus* isolates in our study, we cannot rule out that TR46/Y121F/T289A isolates may have been missed by our screening procedure in samples containing more than 10 isolates.

Altogether, one of 11 of our “azole-naïve” *A. fumigatus* colonized patients harbored ARAF, yielding a 9% prevalence. This frequency, which is among the highest ones that were reported in France, in Belgium, in the Netherlands or in Denmark in different situations or populations (such as hematology or ICU patients with IA, or cystic fibrosis patients), and is close to the 10% resistance frequency that was suggested as a threshold above which the standard treatment choice of IA with voriconazole should be reconsidered, highlights the need for a surveillance of resistance rates according to *Aspergillus* disease and risk groups in our region.

In conclusion, the presence of ARAF in indoor environments of patients at risk for aspergillosis indicates that home environment should be considered as a potential source of exposure to azole-resistant isolates in susceptible hosts, such as patients with chronic respiratory diseases, or immunocompromised patients. Furthermore, coexistence of *A. fumigatus* azole-sensitive and azole-resistant isolates in clinical and environmental samples supports the need for a detection of azole-resistant isolates on all *A. fumigatus* colonies in routine practice. Our data confirm that using azole-containing agar medium can be used either from colonies, or directly from samples. Lastly, the high prevalence of ARAF in patients with COPD and their homes strongly supports the need for further studies to assess the prevalence of ARAF in patients with *Aspergillus* diseases (including immunocompromised or cystic fibrosis patients), and for potential re-evaluation concerning the current choices of antifungal therapy in our region.

**ACKNOWLEDGEMENTS**

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**CONFLICT OF INTEREST**

S.N. has received speakers’ fees from MSD. B.S. has received grant support from Astellas, Merck, and research grant from bioMérieux. All other authors report no potential conflicts.
REFERENCES


