

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

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# ▶ To cite this version:

C. Dauchy, N. Bautin, S. Nseir, G. Reboux, R. Wintjens, et al.. Emergence of Aspergillus fumigatus azole resistance in azole-naïve patients with chronic obstructive pulmonary disease and their homes. Indoor Air, 2017, 28, pp.298-306. 10.1111/ina.12436 . hal-02399473

# HAL Id: hal-02399473 https://hal.science/hal-02399473

Submitted on 12 Dec 2019

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# Emergence of Aspergillus fumigatus azole-resistance in azole-naïve COPD patients and their homes

| Journal:                      | Indoor Air  |
|-------------------------------|---|
| Manuscript ID                 | Draft   |
| Manuscript Type:              | Original Article  |
| Date Submitted by the Author: | n/a   |
| Complete List of Authors:     | Dauchy, Camille; Centre Hospitalier Regional Universitaire de Lille<br>Bautin, Nathalie; Centre Hospitalier Regional Universitaire de Lille<br>Nseir, Saad; Centre Hospitalier Regional Universitaire de Lille<br>Reboux, Gabriel; University Hospital, Mycology;<br>Wintjens, René; Universite Libre de Bruxelles Faculte de Pharmacie<br>Le Rouzic, Olivier; Centre Hospitalier Regional Universitaire de Lille<br>Sendid, Boualem; Centre Hospitalier Regional Universitaire de Lille<br>Viscogliosi, Eric; Institut Pasteur de Lille, Centre d'Infection et d'Immunité<br>de Lille<br>Le Pape, Patrice; Universite de Nantes Pole Sante<br>Arendrup, Maiken; Statens Serum Institut, Unit of Mycology<br>Gosset, Philippe; Institut Pasteur de Lille, Centre d'Infection et d'Immunité<br>de Lille<br>Fry, Stéphanie; Centre Hospitalier Regional Universitaire de Lille<br>Fréalle, Emilie; Centre Hospitalier Regional Universitaire de Lille, ; Institut<br>Pasteur de Lille, Centre d'Infection et d'Immunité |
| Keywords:                     | Aspergillus fumigatus, COPD, Dwelling, Mold environmental exposure, Azole-resistance, Electrostatic dust collector  |
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# Emergence of *Aspergillus fumigatus* azole-resistance in azole-naïve COPD patients and their homes

Running Head: A. fumigatus azole-resistance in COPD

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Abstract Azole-resistant Aspergillus fumigatus (ARAF) has been reported in COPD patients, but has not been specifically assessed so far. Here, we evaluated ARAF prevalence in azolenaïve COPD patients and their homes, and ARAF circulation between 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 between August 2011 and February 2015. A. fumigatus was detected in respiratory samples from 11/41 patients (26.8%) and in 15/36 domiciles (41.7%). Cyp51A sequencing and selection on itraconazole-medium of clinical (n=68) and environmental (n=48) isolates yielded ARAF detection in 1/41 COPD patients (2.4%) and 2/36 patient's homes (5.6%). The clinical isolate had no CYP51A mutation. Two environmental isolates from two patients harbored TR<sub>34</sub>/L98H mutation, and one had an H285Y mutation. Coexistence of different *cvp51A* genotypes and/or azole-resistance profiles was detected in 3/8 respiratory and 2/10 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 COPD patients and their homes supports the need for further studies to assess the clinical impact of azoleresistance in our region.

**Keywords:** *Aspergillus fumigatus*, COPD, dwelling, mold environmental exposure, azoleresistance, electrostatic dust collector

### **Practical implications**

This paper reports the presence of azole-resistant *Aspergillus fumigatus* (ARAF) isolates in azole-naive COPD patients and their homes, and a new CYP51A mutation "H285Y" associated with azole resistance, found in an environmental isolate. The high prevalence of ARAF in dwellings from COPD patients indicates that domestic mold environmental

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 exposure could be a risk factor for the acquisition of ARAF in susceptible hosts. This risk should be taken into account for the management of patients prone to invasive aspergillosis.

# **1 INTRODUCTION**

Patients with chronic obstructive pulmonary disease (COPD) are at risk for *Aspergillus* colonization<sup>1,2</sup> and invasive aspergillosis (IA), which has a 1.6-3.9% prevalence,<sup>3,4</sup> and is associated with high mortality.<sup>5</sup> Azole-resistant *Aspergillus fumigatus* (ARAF) has been reported in the UK in four itraconazole-treated COPD patients (three with chronic pulmonary aspergillosis and one with IA<sup>6</sup>), in one patient with IA who had received voriconazole for 2 days in Denmark,<sup>7</sup> and in two azole-naïve patients colonized with TR<sub>46</sub>/Y121F/T289A *A*. *fumigatus* in the Netherlands.<sup>8</sup> However, although the emergence of ARAF is increasingly studied in hematology IA (1%-15.9% prevalence<sup>9–11</sup>), cystic fibrosis (4.5%-8% prevalence<sup>11–13</sup>) or intensive care units (ICU) patients (4.5% prevalence<sup>10</sup>), the epidemiology and potential clinical impact of *A. fumigatus* azole-resistance in COPD patients has 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.<sup>14,15</sup> Since 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 (TR<sub>34</sub>/L98H or TR<sub>46</sub>/Y121F/T289A) in the soil around the home of hematology patients with IA due to isolates carrying the same mutation.<sup>16,17</sup> Interestingly, in one of these two studies, ARAF isolates were further found in the patient's home.<sup>17</sup> Another study compared the prevalence and CYP51A mutations of clinical and environmental ARAF in different geographic area of Germany and found a higher prevalence of TR<sub>34</sub>/L98H and TR<sub>46</sub>/Y121F/T289A isolates in the environment than in clinical isolates (4-15% *vs.* 0-3%),

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whereas some other mutations, such as G54E or M220L were only present in clinical isolates.<sup>18</sup> Furthermore, except a study in which  $TR_{46}/Y121F/T289A$  isolates were found in the home of patient with IA,<sup>17</sup> and another one assessing eight domiciles in the Netherlands,<sup>19</sup> most environmental ARAF were isolated from soil or outdoor/indoor air samples in hospitals.<sup>20–22</sup>

In this study, we aimed to determine the prevalence of ARAF in azole-naïve COPD patients and their homes, and assess ARAF circulation between clinical and environmental reservoirs, in order to evaluate the potential clinical impact of azole-resistance 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 obtained by a 30 sec to 1 min gargling with 10 mL of sterile NaCL 0.9%). 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  $\mu$ ® (Bertin Technologies) in some 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 min at 37°C, and 10  $\mu$ L were cultured on half-diluted Sabouraud agar medium with 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  $\mu$ L of sample were cultured similarly without pre-treatment. EDC washing, which was performed no later than 48 h post-sample retrieval, consisted of shaking for 10 min in 20 mL of sterile PBS with 0.1% Tween 80 in a Stomacher, as previously described<sup>23</sup>, and 100  $\mu$ L of the collected solution were 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 10<sup>6</sup> conidia/ml suspension and streaking it across the surface of an ITZ medium, which was incubated at 37°C for 4 days.

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# 2.3 Phenotypic and genotypic confirmation of A. fumigatus identification

*A. fumigatus* identification was confirmed by culture at 50°C for 3 days, and, at the genotypic level, by ITS-rDNA and beta-tubulin sequencing, after DNA extraction using QIAamp® DNA Mini Kit (Qiagen, Courtaboeuf, France), as previously described.<sup>13,24</sup> ITS-rDNA and beta-tubulin sequences were deposited in GenBank database under accession numbers KX788215 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 three fragments of 725 bp, 705 bp and 826 bp, respectively: CYP51AF-1 (5'-TAATCGCAGCACCACTCCAG-3') and CYP51AR-1 (5'-GACATCCTTGWGCTTGCCGTTGAG-3'), CYP51AF-2 (5'-TCTACCTGGGCGTTCAGGG-3') CYP51AR-2 (5'and CTTCGAGGACTTTTGGCTGTGAG-3'), CYP51AF-3 (5'-(5'-AACCCTGTTGATGGCTGGTC-3') and CYP51AR-3 GCAACAACACTTCAGGGCCA-3'). Amplification reactions were performed in a final volume of 50  $\mu$ L containing 5  $\mu$ l template DNA, 10  $\mu$ l reaction buffer (5X), 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.4 µM 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 min at 95°C, followed by 45 cycles consisting of denaturation for 1 min at 95°C, annealing for 1 min at 55°C and extension for 1 min at 72°C, followed by a final

extension for 15 min 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 (IVU) 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.<sup>25</sup> EUCAST criteria were used to define susceptibility and resistance.<sup>26</sup>

# 2.6 Homology modeling of A. fumigatus CYP51A

To assess the azole-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<sup>27</sup>) was used as the 3D template, as the two proteins share 63.6% sequence identity. 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.<sup>28</sup> Among the models, the one with the lowest energy was selected as the final model.

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

The mean age of patients was  $63.8 \pm 9.7$  years old, and M/F sex-ratio was 3.2. *A. fumigatus* colonization was detected in respiratory samples from 11/41 patients (26.8%), 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 out of 36 EDCs (1 to 19 colonies), yielding a 41.7% frequency of *A. fumigatus* domestic exposure. These 15 EDCs corresponded to five *A. fumigatus* colonized patients and 10 non-colonized patients, and yielded 41 isolates. Seven supplementary isolates were obtained from air or swab samples.

# 3.2 Phenotypic and genotypic confirmation 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 two patients and 13 environmental strains from six patient's homes, whereas only two environmental strains presented beta-tubulin gene mutations, one synonymous and one non-synonymous, all other sequences having 100% identity (Table 1).

# 3.3 Cyp51A mutations in clinical and environmental isolates

*Cyp51A* sequencing yielded five mutation profiles in five clinical and five environmental isolates from six different patients (Table 1).  $TR_{34}/L98H$  mutation was detected in two

environmental isolates from two patients (n°7 and n°22). Mutations F46Y-M172V-N248T-D255E-E427K were detected in two clinical isolates from the same patient (n°24) and in one environmental isolate (n°7). Another environmental isolate from patient n°9's EDC presented mutations F46Y-M172V-E427K. Mutation A284T was detected in three clinical isolates from one patient (n°41). Lastly, mutation H285Y was detected in one environmental isolate from patient n°22.

*Cyp51A* synonymous mutations were detected in 13 isolates which presented G1696A mutation, including three environmental isolates from three patients ( $n^{\circ}2$ , 7 and 30) and 10 clinical isolates from two patients ( $n^{\circ}21$  and 34). Nine clinical isolates from the same patient ( $n^{\circ}42$ ) presented the non-synonymous mutation C1562T.

# 3.4 Selection on ITZ medium and MIC determination

Culture was positive on ITZ medium for 7 out of 116 isolates, including the two  $TR_{34}/L98H$ , the H285Y, one clinical isolate from patient n°34 with no CYP51A mutation, and the three A284T isolates which had a very weak growth. MIC determination yielded pan-azole resistance for the two  $TR_{34}/L98H$ , the CYP51A WT and the H285Y isolates (for which MICs were confirmed by the EUCAST Development Laboratory for fungi, Copenhagen, Denmark), but the remaining isolates (three A284T, three F46Y-M172V-N248T-D255E-E427K and one F46Y-M172V-E427K) were all ITZ and VOR susceptible, and had a discreet increase in POS and IVU MICs (Table 2).

# 3.5 Prevalence of TR<sub>34</sub>/L98H mutation and azole-resistance

TR<sub>34</sub>/L98H mutation was detected in 2/48 (4.2%) environmental isolates in two different patient's homes, yielding a 5.6% (2/36) prevalence of TR<sub>34</sub>/L98H mutation in the COPD 10

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patient's domestic environment. When taking into account only *A. fumigatus* positive EDCs,  $TR_{34}/L98H$  mutation was detected in 13.3% (2/15) of patient's homes. Regarding azoleresistance, four out of 116 clinical or environmental isolates (including the two  $TR_{34}/L98H$ isolates) were pan-azole resistant, yielding a 3.4% overall prevalence of azole resistance. Since one of these isolates was clinical and three were environmental, the prevalence of ITZ resistance was 1.5% (1/68) and 6.3% (3/48) in clinical and environmental isolates, respectively. Azole resistance was detected in the environment of two patients (corresponding to two  $TR_{34}/L98H$  and one H285Y isolates), yielding a 5.6% (2/36) prevalence in patient's homes, and in clinical samples from one other patient (corresponding to one WT isolate), yielding a 2.4% (1/41) prevalence in patients. When taking into account only *A. fumigatus* positive samples, pan-azole resistant isolates were detected in 9.1% (1/11) of patients and 13.3% (2/15) of patient's homes.

# 3.6 Circulation of isolates within and between clinical and environmental reservoirs

Coexistence of F46Y-M172V-N248T-D255E-E427K or A284T and WT azole-sensitive isolates in the same sample was found in sputa from patients n°24 and 41, respectively (Table 1). Mutated and WT isolates also coexisted in EDCs from patients n°7 and 22, where one TR<sub>34</sub>/L98H isolate was associated to one F46Y-M172V-N248T-D255E-E427K isolate, and one TR<sub>34</sub>/L98H was associated to one H285Y and eight WT isolates, respectively. When *cyp51A* genotypes were compared for the five patients who had both clinical and environmental isolates, four patients only presented WT isolates in both reservoirs, and one patient (n°9) harbored one environmental isolate with F46Y-M172V-N248T-D255E-E427K mutations whereas no mutation was detected in the clinical isolate.

# 3.7 Geographical distribution of clinical and environmental isolates

Clinical and environmental isolates came from patients living in different parts of the North of France Region, but most were close to Lille (Fig. 1). The two TR<sub>34</sub>/L98H environmental isolates were found in two different cities: Faches-Thumesnil and Marcq-en-Baroeul, which are located in urban areas a few kilometers at the South and North of Lille, respectively. The H285Y isolate was also detected in Faches-Thumesnil, whereas the patient who was colonized with three A284T clinical isolates was living in Marquillies, in a rural area. Isolates with F46Y-M172V-N248T-D255E-E427K and F46Y-M172V-E427K were localized in Lille and Faches-Thumesnil.

# 3.8 Modeling of A. fumigatus CYP51A: structural localization of H285

In the 3D model of CYP51A, the H285 residue was a buried residue located at the N-terminal part of the  $\alpha$ -helix I, which is an important part of channel 1, and that pass through the protein structure and interacts in its middle with the heme cofactor (Fig. 2). H285 was then situated quite far from the heme moiety, but along to the entrance of the putative ligand access channel 1.<sup>29</sup> ITZ molecule was, contrariwise, found in the putative channel 2.

#### **4 DISCUSSION**

The 23.1% 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 UK.<sup>1,2</sup> Since species identification is rarely done when assessing indoor environments, limited data are available on *A. fumigatus* frequency in dwellings. However, the 41.7%

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frequency found in patient's homes in the present study seemed high when compared to some other studies that used culture  $(20\%^{30})$ . Since only five out of the 15 *A. fumigatus* positive EDCs originated from *A. fumigatus* colonized patient's homes, no relationship between *A. fumigatus* exposition and colonization could be evidenced. 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*.<sup>31</sup> 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 (e.g. 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.<sup>32</sup>

The 13.3% prevalence of TR<sub>34</sub>/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 TR<sub>34</sub>/L98H isolates, respectively.<sup>18,22,33,34</sup> However, contrarily to most other studies, which focused on indoor or outdoor samples around hospitals, we report the presence of TR<sub>34</sub>/L98H isolates in the domestic environment of patients who are at risk for IA. Furthermore, the two homes with TR<sub>34</sub>/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,<sup>17</sup> and further indicate that exposure to environmental ARAF isolates is not limited to rural areas where fungicides are used. Since the two patients with TR<sub>34</sub>/L98H isolates in their homes were not colonized by *A. fumigatus*, no transmission of TR<sub>34</sub>/L98H environmental isolates could be evidenced in our study. However, our data confirms that,

although most IA with TR<sub>34</sub>/L98H isolates were reported in hematology, transplant or cancer patients,<sup>8,16,35</sup> COPD patients 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 3/8 (37%) respiratory and 2/10 (20%) environmental samples where more than one *A. fumigatus* isolate had been detected. The presence of *Aspergillus* sensitive and resistant isolates has been reported previously in patients with IA.<sup>7,36</sup> The high frequencies we found 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.<sup>36</sup> The usefulness of this method was confirmed in our study where two ITZ-resistant isolates were obtained after direct sowing of one EDC and one sputum, which contained more than 10 colonies on ITZ medium.

Other CYP51A mutations included A284T, which was reported to be associated with decreased sensitivity to ITZ, VOR and POS.<sup>37</sup> However, only POS and IVU MICs were discreetly increased in our study. Interestingly, a new mutation H285Y was also identified in one environmental isolate, and was associated with pan-azole resistance, IVU MIC being correlated with VOR MIC, as previously described for ARAF isolates with other CYP51A mutations.<sup>38</sup> 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, since 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

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functional effect of this mutation, which, interestingly, is localized near the A284T and T289A mutations. However, contrarily to H285Y, T289A is not associated with decreased azole sensitivity when not combined with  $TR_{46}/Y121F$ .<sup>39</sup> Surprisingly, although  $TR_{46}/Y121F/T289A$  isolates have been reported in Belgium,<sup>40</sup> which is only at a few kilometers from Lille, or in other areas in France,<sup>41</sup> none was found in our study. However, 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  $TR_{46}/Y121F/T289A$  genotype induces high level VOR and IVU 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.<sup>42</sup> Hence, although *cyp51A* was sequenced for all the *A. fumigatus* isolates in our study, we cannot rule out that  $TR_{46}/Y121F/T289A$  isolates may have been missed by our screening procedure in samples containing more than 10 isolates.

Altogether, one out of 11 of our "azole-naïve" *A. fumigatus* colonized patients harbored ARAF, yielding a 9.1% prevalence. Regarding the low number of *A. fumigatus* colonized patients in our study, this frequency was quite similar to the ones that were reported in some studies in Belgium or in France in different situations or populations (such as hematology or ICU patients with IA, or cystic fibrosis patients).<sup>9–13</sup>

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 resistant isolates in clinical and environmental samples support 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 COPD patients

and their homes strongly supports the need for further studies to assess the clinical impact of ARAF in patients with *Aspergillus* diseases, and for potential re-evaluation concerning the current choices of antifungal therapy in our region.

# ACKNOWLEDGEMENTS

This work is dedicated to the memory of Eduardo Dei-Cas, who actively contributed to the initial design and practical achievement of the FungiCOPD project. We also would like to thank Stéphanie Delvart, Sandrine Bonaventure, Dorothée Monvillers, Michèle Wauquier, Filoména Naji, and Nausicaa Gantois for technical assistance, Dr Isabelle Durand-Joly, Pr El Moukhtar Aliouat, and Dr Annie Standaert-Vitse for their steady interest in the work and for fruitful discussions and, and all the physicians who participated to the recruitment of patients. This work was partially presented as an oral communication at the "European Congress of Clinical Microbiology and Infectious Diseases" in 2014, at the congress of the "French Medical Mycology Society" in 2016, and as a poster at the TIMM-7 in 2015. RW is a Research Associate at the National Fund for Scientific Research FNRS-FRS (Belgium).

### **FUNDING SOURCES**

Acting for a healthier population, the Hauts-de-France funded this work (Environment Health program, grant N°2010-12266 & 2010-12268), which was also supported by GILEAD Sciences, MSD, and Pfizer grants.

# **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.

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#### Indoor Air - PROOF

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TABLE 1. Characteristics of A. fumigatus clinical and environmental isolates: origin, cyp51A,

*beta-tubulin, ITS-rDNA* sequencing, and growth on ITZ medium.

Pan-azole resistant isolates are highlighted in grey. Synonymous mutations are in italics. OPW=Oropharyngeal wash, EDC=Electrostatic Dust Collector

|                     |                     |              |         | Number<br>of<br>isolates |         | <i>cyp51A</i> sequencin                                     | Mutated <sup>β</sup> | Mutated <i>I</i>                 |  |
|---------------------|---------------------|--------------|---------|--------------------------|---------|---|----------------------|----------------------------------|--|
|                     | Patient<br>number O |              | solates |                          | WT      | Non synonymous mutation                                     | Synonymous mutation  | -<br><i>tubulin</i> iso<br>lates | <i>TS-</i><br><i>rDNA</i> isol<br>ates |
| A fumigatus         | 10                  |              | Sputum  | 10                       | 10      |   |                      |                                  |  |
| colonized           | 21                  | Clinical     | Sputum  | 5                        | 5       |   |                      |                                  |  |
| patients,           | 24                  |              | Sputum  | 6                        | 4       | 2 F46Y, M172V, N248T,<br>D255E, E427K                       |                      |                                  | 2 C11G                                 |
| no detection        | 34                  |              | Sputum  | 12                       | 11 + 1* |   |                      |                                  |  |
| in the<br>patient's | 41                  |              | Sputum  | 8                        | 5       | 3 A284T   |                      |                                  |  |
|                     | 42                  |              | Sputum  | 9                        |         |   | 9 C1562T             |                                  | 9 T91C                                 |
| nome                |                     |              | OPW     | 1                        | 1       |   |                      |                                  | 1 T91C                                 |
|                     |                     | Clinical     | Sputum  | 3                        | 3       |   |                      |                                  |  |
|                     | 2                   | Environment  | Air     | 1                        | 1       |   |                      |                                  | 1 T91C                                 |
|                     |                     |              | Swab    | 1                        | 1       |   |                      |                                  | 1 T91C                                 |
| A. fumigatus        |                     |              | EDC     | 4                        | 4       |   |                      | 1 V48I                           | 1 T91C                                 |
| colonized           | 0                   | Clinical     | Sputum  | 1                        | 1       |   |                      |                                  |  |
| patients &          | 9                   | Environment  | EDC     | 1                        |         | 1 F46Y, M172V, E427K  |                      | 1 G60A                           |  |
| detection in        | 18                  | Clinical     | Sputum  | 1                        | 1       |   |                      |                                  |  |
| the patient's       |                     | Environment  | EDC     | 4                        | 4       |   |                      |                                  |  |
| home                | 19                  | Clinical     | OPW     | 1                        | 1       |   |                      |                                  |  |
|                     |                     | Environment  | EDC     | 4                        | 4       |   |                      |                                  |  |
|                     | 39                  | Clinical     | OPW     | 11                       | 11      |   |                      |                                  |  |
|                     |                     | Environment  | EDC     | 3                        | 3       |   |                      |                                  | 2 T91C                                 |
| A. fumigatus        | 1                   |              | Air     | 3                        | 3       |   |                      |                                  |  |
|                     |                     |              | EDC     | 2                        | 2       |   |                      |                                  | 2 T91C                                 |
|                     | 7                   |              | Air     | 1                        | 1       |   |                      |                                  |  |
|                     |                     |              | EDC     | 2                        |         | <u>1 TR34/L98H</u><br>1 F46Y, M172V, N248T,<br>D255E, E427K |                      |                                  |  |
| the natient's       | 8                   |              | Swab    | 1                        | 1       |   |                      |                                  | 1 T91C                                 |
| home, no            | 12                  | Environment  | EDC     | 1                        | 1       |   |                      |                                  |  |
| detection in        | 22                  | Littioninoit | EDC     | 12                       | 10      | 1 TR34/L98H*<br>1 H285Y                                     |                      |                                  | 4 T91C                                 |
| samples             | 27                  |              | EDC     | 1                        | 1       |   |                      |                                  |  |
| sampies             | 29                  |              | EDC     | 1                        | 1       |   |                      |                                  | 1 T91C                                 |
|                     | 30                  |              | EDC     | 2                        | 2       |   |                      |                                  |  |
|                     | 31                  |              | EDC     | 1                        | 1       |   |                      |                                  |  |
|                     | 40                  |              | EDC     | 3                        | 3       |   |                      |                                  |  |
|                     | TOTAL 116 97 10 9   |              |         |                          |         |   | 2                    | 25                               |  |

 **TABLE 2.** MIC determination for *A. fumigatus* isolates with positive growth on ITZ medium and/or CYP51A mutation.

The following EUCAST criteria were used to define susceptibility and resistance: an ITZ or VOR MIC of  $\leq 1$  mg/L was considered susceptible, a MIC of 2 mg/L intermediate and of > 2 mg/L resistant; a POS MIC of  $\leq 0.12$  mg/L was considered susceptible, a MIC of 0.25 mg/L was considered intermediate and of > 0.25 mg/L resistant; an IVU MIC of  $\leq 1$  mg/L was considered susceptible, a MIC of > 1 mg/L was considered resistant.<sup>26</sup> Pan-azole resistant isolates are highlighted in grey.

| CVP51A mutations                       | MIC (mg/L) |        |      |      |  |  |  |  |  |
|--|------------|--------|------|------|--|--|--|--|--|
| CTT STA mutations                      | ITZ        | VOR    | POS  | IVU  |  |  |  |  |  |
| TR34/L98H (n=2)                        | >8         | 4      | 1-2  | 8-16 |  |  |  |  |  |
| H285Y (n=1)                            | >16        | 4      | 0.5  | 4    |  |  |  |  |  |
| WT (n=1)                               | >16        | 4      | 1    | >16  |  |  |  |  |  |
| A284T (n=3)                            | 0.25-0.5   | 0.25-1 | 0.5  | 2    |  |  |  |  |  |
| F46Y, M172V, N248T, D255E, E427K (n=3) | 1          | 1      | 0.5  | 1-2  |  |  |  |  |  |
| F46Y, M172V, E427K (n=1)               | 0.5        | 1      | 0.25 | 1    |  |  |  |  |  |
|  |            |        |      |      |  |  |  |  |  |

**FIGURE 1.** Distribution of *A. fumigatus* clinical and environmental isolates in the North of France, and associated azole-resistance and/or CYP51A mutations.

Each patient and/or patient's home is represented by a round. The presence of clinical isolates is mentioned as a « C » in the round. Azole-resistant isolates are round in white.



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**FIGURE 2.** Ribbon illustrations of the *A. fumigatus* CYP51A 3D model. Helices/loop and strands are shown in corral and cyan, respectively. The helix I containing the H285 residue, is colored in orange. The structure is showed in two different views related by a 90-degree rotation. The protein surface is displayed in transparency. The heme cofactor (yellow carbons), ITZ (green carbons) and residue H285 (magenta carbons) are depicted as sticks and labeled in the right view, as well as the N- and C-terminal regions. The two putative ligand access channels <sup>29</sup> are labeled and indicated by arrow. Oxygen, nitrogen and iron are colored in red, blue and orange, respectively. The images were produced using the PyMOL Molecular Graphics System (Version 1.7, Schrödinger, LLC).

