Aspergillus species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on Aspergillus fumigatus azole resistance.

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Aspergillus species and other moulds in respiratory samples from cystic fibrosis patients: A laboratory-based study with focus on azole-resistance in *Aspergillus fumigatus*

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Abstract

Respiratory tract colonisation by moulds in cystic fibrosis (CF) patients were analysed with particular focus on the frequency, genotypes and underlying mechanism of azole resistance among *Aspergillus fumigatus* isolates. Clinical and demographic data were also analysed.

3336 respiratory samples from 287 CF patients were collected during two six-month periods in 2007 and 2009. Clinical and demographic data were retrieved from the CF database. Azole resistance was detected using an itraconazole screening agar (4 mg/L) and the EUCAST method. *cyp*51A gene sequencing and STRAF genotyping were performed for isolates from patients harbouring azole resistant *A. fumigatus*.

Aspergillus spp. were present in 145 patients (51%) of whom 63 (22%) were persistently colonised. Twelve patients (4%) harboured other moulds. Persistently colonised patients were older, provided more samples and more often had a chronic bacterial infection. 6/133 patients (4.5%) harboured azole intermediate/resistant *A. fumigatus* isolates five of whom had isolates with Cyp51A alterations (M220K, TR/L98H, TR/L98H+S297T+F495I, M220I+V101F, and Y431C, respectively). All six patients were previously exposed to azoles. Genotyping revealed 1) microevolution for *A. fumigatus* isolates received consecutively over the 2-year period; 2) susceptible and resistant isolates (not involving TR/L98H isolates) of identical or very closely related genotypes (two patients); and 3) two related susceptible isolates and a third unrelated resistant isolate with a unique genotype and the TR/L98H resistance combination (one patient).

Aspergilli were frequently found in Danish CF patients with 4.5% *A. fumigatus* being azole resistant. Genotyping suggested selection of resistance in the patient as well as being achieved from the environment.

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Introduction

Moulds are frequently recovered from cultures of respiratory samples from children and adults with cystic fibrosis (CF). *Aspergillus fumigatus* is the predominant species with reported prevalence rates from 6% to nearly 60% (1). Among other *Aspergillus* species, *A. terreus* is the most common (2). Other fungi such as *Scedosporium* spp., *Exophiala dermatitidis*, *Acrophialophora fusispora* and recently *Geosmithia argillacea* have also been detected (3-6). However, overall reported rates are influenced by both laboratory factors, e.g. type of culture media used, incubation time, as well as patient related factors, e.g. age of patients, applied definitions of transient or persistent mould colonisation and sampling frequencies (1;7).

Aspergillus may cause several pulmonary manifestations in CF patients. Allergic bronchopulmonary aspergillosis (ABPA) is recognised as a severe complication and characterized by an accelerated decline in lung function. ABPA in CF affects mainly older children and adults with a prevalence rate of 6-25% (8;9). *Aspergillus* bronchitis is observed in CF patients not fulfilling the criteria for ABPA but with positive respiratory tract cultures and clinical improvement with appropriate antifungal treatment (10;11). Invasive aspergillosis in the absence of lung transplantation is rare among CF patients, but has been reported (12). Finally, *Aspergillus* may colonise the lungs without causing apparent clinical disease and the effects on lung function are not clear (13;14). Genotyping studies of *A. fumigatus* isolates indicate that patients may be simultaneous or sequentially colonised with several different strains, but eventually a single strain becomes dominant (15-17).

Itraconazole is often used for the treatment of chronic non-invasive forms of aspergillosis (10) and has proven effective and steroid-sparing in ABPA (18). Voriconazole is recommended as first-line therapy for invasive aspergillosis (19) and posaconazole for prophylaxis in severely immunocompromised patients (20). Resistance to itraconazole in *A. fumigatus* is well recognised and was first reported in 1997 in three clinical isolates obtained in the late 1980s (21). The main mechanism of azole resistance currently reported is alterations to the drug target. Mutations in the gene (*cyp*51A) which encodes this target may result in structural modifications of the target enzyme and subsequently decrease affinity of the azole compounds. Azole-

resistance has been reported in azole exposed patients (22-24) but also in azole-naïve Dutch patients and in the environment in the Netherlands (23;25) and Denmark (26). However, a recent study found no azole resistant isolates in their CF patients (27).

At our centre, with a tradition of close monitoring of patients and aggressive treatment of infections, all three azoles are frequently used in the CF population. Thus, in the present study, we aimed to explore the prevalence and dynamics of moulds in respiratory tract samples from a cohort of approximately 300 CF patients, and particularly the frequency, genotypes and underlying mechanism of azole resistance among the detected *A. fumigatus* isolates. Furthermore, we analysed clinical and demographic data in patients with and without *Aspergillus* spp. in their respiratory samples.

Materials and methods

Patients and samples. Since 1971 all patients at the CF centre at Copenhagen University Hospital Rigshospitalet have been followed with monthly visits in the outpatient clinic for clinical status, pulmonary function and microbiological investigations of lower respiratory tract secretions. Presently, approximately 300 patients are managed and more than two-thirds are homozygous for the ΔF508 CFTR mutation. Patients had a confirmed diagnosis of CF based on abnormal sweat electrolytes, characteristic clinical features or mutations in the gene encoding the CFTR protein. Information regarding CFTR mutations, chronic bacterial infections, lung transplantation (LTX), CF related diabetes, presence of ABPA, lung function (forced expiratory volume in 1 second, percent predicted [FEV1%] patient mean per year), according to ATS guidelines (28) using reference values from Polgar (29), body mass index z scores ([zBMI], patient mean per year) using reference values from Nysom (30) and antifungal therapy was accessed using the Copenhagen CF centre database. The administration of itraconazole, voriconazole and posaconazole was recorded as weeks of therapy. ABPA was defined according to consensus criteria (8). Patients were treated with azoles according to the following guidelines: *Aspergillus* colonisation was treated if symptomatic and no response was achieved on anti-bacterial therapy. First line was itraconazole (10 mg/kg/day), followed by voriconazole (200-400 mg x 2/day, dose adjusted if weight was below 40 kg) or posaconazole (400 mg x 2/day). ABPA was treated with an azole in combination with systemic steroid therapy. Duration of therapy was based on response, but generally three to six months. Therapeutic drug monitoring (TDM) was performed using a bio-assay at Statens Serum Institut, although not systematically.

A total of 3,336 respiratory samples from 287 CF patients were included prospectively during two sample periods: Sample period I: 1 July – 31 December 2007 (1,715 samples from 266 patients) and sample period II: 1 July – 31 December 2009 (1620 samples from 274 patients). 253 patients (88.2%) out of 287 patients provided samples in both periods. Six died during or between sample periods. The respiratory sample types

were sputum, endo-laryngeal suction, tracheal suction or broncho-alveolar lavage (BAL). Samples such as oral or nasal swabs and sinus samples were excluded.

In an attempt to graduate the dynamics of mould colonisation, patients were classified as 1) "mould negative" if no moulds were detected in any of the respiratory samples (n=130); 2) "new *Aspergillus* cases" if patients were negative for *Aspergillus* or no samples were provided in 2007 and *Aspergillus* were present in 2009 (n=44); 3) "persistent *Aspergillus* colonisation" if *Aspergillus* species were detected in both sample periods (n=63); 4) "*Aspergillus* colonisation cleared" if patients were *Aspergillus* positive in 2007, but negative in 2009 (n=38); 5) "other moulds" if other moulds than *Aspergillus* spp. were detected in one or both samples periods (n=12).

Plating and identification. At the department of clinical microbiology respiratory samples were investigated using the following media (SSI Diagnostika, Hillerød, Denmark): 5% horse blood agar, chocolate agar, Sabouraud glucose (pH 4) agar, 7.5 % NaCl agar, "blue agar" (modified Conradi-Drigalski's medium), *B. cepacia* agar containing colistin and gentamycin and on a 14 cm susceptibility testing plate with disks containing anti-pseudomonas antibacterials. Plates were incubated at 37 °C and investigated daily for growth of bacteria and moulds for up to five days. If moulds were observed, the plate was referred to the mycology reference laboratory at Statens Serum Institut for identification and susceptibility testing of the isolate(s). *Aspergillus* spp. were identified to complex/section level according to morphological and phenotypic criteria after a minimum of three days incubation using Sabouraud glucose agar (pH4) (SSI Diagnostika, Hillerød, Denmark) and vegetable juice agar (SSI Diagnostika, Hillerød, Denmark) at 37 °C (31). *A. fumigatus* complex isolates with reduced susceptibility to one or several azoles were further incubated at 48°C to separate *A. fumigatus* sensu stricto from cryptic *A. fumigatus* complex species. *Aspergillus* isolates that could not be identified to the species level were sequenced as described below. Non-*Aspergillus* moulds were identified according to morphological and phenotypic criteria after a minimum of three days incubation using Sabouraud glucose agar

(pH4) (SSI Diagnostika, Hillerød, Denmark) and vegetable juice agar (SSI Diagnostika, Hillerød, Denmark) at 37 °C or room temperature. *Scedosporium* isolates were subcultured on Sabouraud agar containing cycloheximide (0.5 mg/mL) and chloramphenicol (0.05 mg/mL) (SSI Diagnostika, Hillerød, Denmark) to distinguish between *S. apiospermum* complex and *S. prolificans*.

Susceptibility testing and screening for itraconazole resistance in *Aspergillus*. Unique *Aspergillus* isolates (> 6 months apart) underwent EUCAST susceptibility testing (32) as part of the clinical routine in 2007. Subsequent isolates were stored at -80 °C, and screened for azole-resistance in parallel with isolates from 2009 using an itraconazole containing agar (4 mg/L) (SSI Diagnostika, Hillerød, Denmark) (25). Isolates which grew on the itraconazole agar were further examined according to EUCAST method (32). Manufacturers and stock solutions (5000 mg/L in DMSO) were as follows: dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Brøndby, Denmark); itraconazole (Sigma-Aldrich, Brøndby, Denmark); voriconazole (Pfizer, Ballerup, Denmark), posaconazole (Schering-Plough, Glostrup, Denmark). Final drug concentration range of itraconazole, voriconazole and posaconazole were 4 to 0.03 mg/L. Minimal inhibitory concentrations (MICs) were determined visually as a no-growth end point at 48 h incubation time. Four *A. fumigatus* isolates with prominent but not absolute growth inhibition in the highest itraconazole concentration were further analyses using an extended range up to 32 mg/L. Quality control strains *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were included but read after 24 h of incubation (33). In addition, resistant isolates were tested for susceptibility to amphotericin B and caspofungin using E-test strips (AB Biodisk, Solna, Sweden) on RPMI-1640-2% glucose agar (SSI Diagnostika, Hillerød, Denmark). For caspofungin, aberrant growth in the inhibition zone was ignored.

Clinical breakpoints for azoles and *Aspergillus* spp. have not yet been established by CLSI or EUCAST. However, epidemiological and clinical cut-offs have been proposed for *A. fumigatus* and azoles (34-36). In this study we

used the following cut-off values defining susceptibility: itraconazole and voriconazole ≤ 1 mg/L and posaconazole ≤0.5 mg/L.

Molecular identification and detection of resistance mechanisms. DNA was extracted from fungal cultures as previously described (37). Three isolates of *A. thermomutatus* were identified by sequencing of the β-tubulin gene using primers βtub1 (5'-AATTGGTGCCGCTTTCTGG-3') and βtub2 (5'-AGTTGTCGGGACGGAATAG-3') as both PCR and sequencing primers (38). An isolate of *A. ustus*, two *A. nidulans* and two *Penicillium* sp. were identified using universal primers ITS1 (5'-CGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGAT-ATGC-3') (39;40). The promoter and full coding region of the *cyp*51A gene was amplified by PCR using the primers Cyp51_0F (5'-TCATATGTTGCTCAGCGG -3') and Cyp51_4R (5'-CCTATTCCGATCACACCAAA-3') and, in addition, the primers Cyp51_1R (5'-CATTGAGCAAGATTGCCG-3'), Cyp51_2F (5'-CGGCAATCTTGCT-CAATG-3'), Cyp51_2R (5'-GGTGAATCGCGCAGATAGT-3'), Cyp51_3F (5'-ACTATCT-GCGCGATT-CACC-3'), Cyp51_3R (5'-GTCAAGATCCTTGTACTGGAGC-3') and Cyp51_4F (5'-CTCCA-GTACAAGGATCTTGAC-3') were used for the sequencing of the full PCR product on both DNA strands (41). The sequences were compared to the sequence of an azole-susceptible wild-type *A. fumigatus* isolate (GenBank accession no. AF338659).

Genotyping. The following *A. fumigatus* isolates were selected for typing: 1) 23 azole resistant and 20 azole-susceptible isolates from six patients with azole-resistant isolates, 2) 10 azole-susceptible isolates from two patients with azole-susceptible isolates only and 3) Four azole-resistant (TR/L98H) environmental isolates (26). Genotyping was performed with a panel of nine short tandem repeats as described previously (42).Microevolution was defined as one repeat evolution, indicating high degree of relatedness between isolates. Construction of phylogenetic tree was performed using four microsatellite markers (3A, 3C, 4A and 4B)…

Statistical analysis. Fisher's exact test or Chi-square test was used to compare two groups of categorical data. Groups of continuous data that were not normally distributed were compared using Kruskal-Wallis test. Mann-Whitney test was used for comparison of two groups of continuous data without normal distribution. *P* values less than 0.05 were considered statistically significant.

Results

Cohort characteristics. A total of 3,336 respiratory samples from 287 patients with CF were included in this study. *Aspergillus* spp. were present in 145 patients (51%) of which 63 patients (22%) were persistently colonised, whereas 12 patients (4%) harboured other moulds (Table 1). Patients persistently colonised with *Aspergillus* and those who cleared their *Aspergillus* colonisation were significantly older (median years, 23.0 and 25.0 vs. 17.5), provided more respiratory samples (median number of samples, 12 and 12 vs. 10) and were more often chronically colonised with bacteria (65% and 74% vs. 48%) than those who were negative for moulds. A higher proportion of patients with persistent *Aspergillus* colonisation had ABPA than those with no moulds (14% vs. 5%). Fewer persistently colonised patients had diabetes than mould negative patients (14% vs. 28%). Patients who cleared their *Aspergillus* colonisation were more often recipients of lung transplantation than mould negative patients (29% vs. 8%). Finally, we observed that more females than males were persistently colonised with *Aspergillus* than patients with no moulds (60% vs. 51%), however this was not significant (*P*=0.22).

Neither baseline lung function nor BMI z-scores varied between the groups. Patients with other moulds present had the highest proportion of ABPA, provided most respiratory samples and had the poorest lung function.

Detection of *Aspergillus* spp. and other moulds. A total of 1,715 and 1,621 samples from 266 and 274 patients were analysed in 2007 and 2009, respectively. *Aspergillus* spp. was the most frequently recovered genus with a constant detection rate among patients in the two periods (38.0% in 2007 vs. 38.3% in 2009). In both periods, *A. fumigatus* was the most common species (37.2% vs. 33.2% of the cases), followed by *A. flavus* (4.1% vs. 4.4% of the cases) and *A. terreus* (1.9% vs. 2.6% of the cases). More patients harboured *A. niger* in 2009 than in 2007 (4.7% vs. 0.8 %). Finally, one patient harboured an other *Aspergillus* species (*A. nidulans*) in 2007 and four patients in 2009 (*A. nidulans*, *A. versicolor*, *A. thermomutatus, A. ustus*). Among other moulds,

Penicillium spp. and *Paecilomyces* spp. were isolated more frequently from patients in 2009 (5.5% and 2.6% vs. 0.8% and 0.8% in 2007, respectively). Other moulds were detected in 1 – 3 patients per sampling period and included *Scedosporium apiospermum, Fusarium* spp*., Acremonium* spp*., Trichoderma* spp*., Scopulariopsis* spp*.,* other *hyaline hyphomycetes, Cladosporium* spp*.,* other *dematiaceous* fungi, *Rhizopus microsporus* and -*Mycocladus corymbifer* . Thus, overall 41% and 44.5% of the patients harboured at least one mould in the two study periods respectively.

Azole susceptibility for *Aspergillus* isolates. Overall, itraconazole MICs ≥2 mg/L was found in *Aspergillus* isolates from eight out of 145 (5.5%) patients. From a total of 133 patients with *A. fumigatus*, six (4.5%) had intermediate/resistant isolates (range, 1 to 9 resistant isolates per patient). Five patients harboured isolates for which the MICs for itraconazole, voriconazole and posaconazole were >4 mg/L, 0.5-4 mg/L and 0.5->4 mg/L, respectively. One patient harboured four isolates where growth was notably reduced in wells with a concentration of itraconazole of 2 mg/L or more. However, using a stereo microscope and a stringent no growth endpoint the MICs were >32 mg/L and thus indicated as 2/>32 mg/L (Table 3). In addition, one patient out of nine with *A. terreus* had two isolates (both detected in 2007) for which the itraconazole and voriconazole MICs were higher than expected (4 mg/L and 2-4 mg/L, respectively). Finally, one patient had three *A. thermomutatus* isolates (detected in 2009) for which the itraconazole, voriconazole and posaconazole MICs were >4 mg/L, 4->4 mg/L and 0.125-0.25 mg/L, respectively.

Mutations in the *cyp*51A gene of *A. fumigatus*. Seven different amino acid substitutions of the Cyp51A target enzyme were detected in 18 azole-resistant *A. fumigatus* isolates from five patients (Table 5 and 6). In three patients (R1-3) only intermediate/resistant isolates were found, while in three patients (R4-6) susceptible as well as resistant isolates were recovered. A substitution of leucine with histidine at codon 98 in combination with a 34-bp tandem repeat (TR/L98H) in the promoter region of the *cyp*51A gene was detected in 1/1 and 7/9

isolates from patients R2 and R5, respectively. Furthermore, in seven of these eight isolates this was combined with two additional substitutions (S297T and F495I). Noticeably, in one sample from patient R5 two distinct azole-resistant isolates were recovered, one with the TR/L98H+S297T+F495I substitutions, the second with the TR/L98H substitution only. In two patients substitutions were detected at the amino acid hot spot position 220 (patient R1: M220K and patient R4: M220I) and for patient R4 an additional substitution (V101F) was also found. From patient R3 we found four itraconazole intermediate resistant isolates with an Y431C substitution. No Cyp51A substitutions were found in three azole-resistant isolates from patients R4 and R6. One of the Cyp51A substitutions was associated with multi azole-resistance (M220K), while the M220I and TR/L98H substitutions were consistently resistant to itraconazole and posaconazole but variably susceptible to voriconazole.

Exposure to anti-*Aspergillus* azoles. All six patients with azole-resistant *A. fumigatus* isolates had been exposed to mould active azoles for a total of 46 to 278 weeks prior to the detection of the resistant isolate and overall for more weeks than patients with no mould or susceptible *Aspergillus* isolates (Table 5). Patients R1 and R4 with the hot-spot mutation M220 received azole-therapy for a prolonged period of 260 and 278 weeks, respectively. The remaining patients with azole-resistant isolates were treated with azoles for a shorter period of 30-175 weeks, and not at all in the years 2001-2003. TDM (?) was performed on a total of 20 serum samples from four of the six patients since 2007, showing drug concentrations below the recommended level from each of the patients.

Genotyping. Microsatellite typing was used to analyse the relatedness of isolates obtained from patients R1-6, S1-2 and four previously reported (26) environmental azole resistant isolates harbouring the TR/L98H substitution (T11, T18, T22 and R13) (Table 6). In three patients (R1, R3 and R4) identical or nearly identical genotypes were found within each individual despite that both azole susceptible as well as azole resistant

isolates were included for patients R4. Each of the three patients (R5, R6 and S1) harboured multiple isolates which had two or more unrelated genotypes. The azole resistant isolates from patient R5 were unrelated to the susceptible isolates, whereas the genotypes for resistant and susceptible isolates from patient R6 were identical or nearly identical. A unique azole resistant isolate from patient R2 had an identical genotype as the seven isolates from patient R5. Figure 1 shows a phylogenetic tree based on four microsatellite markers of the genetic relatedness of the isolates in comparison with isolates from Manchester for reference. The resistant clinical isolates (both with and without Cyp51A substitutions) and environmental isolates are distributed among susceptible isolates, and both Danish and Manchester isolates are distributed among each other.

Discussion

This study of has shown that moulds can be recovered from more than 40% of our CF patients with more than 20% being persistently colonised with *Aspergillus* spp. *A. fumigatus* was the predominant species and involved in the majority of cases of persistent colonisation. We observed that among the patients with *A. fumigatus* 4.5% harboured azole-resistant isolates and all of these patients were previously exposed to azoles. This frequency is in the high end compared to what have been reported by others (43). Furthermore, we showed that the isolates with TR/L98H resistance mechanism which we recently detected in environmental samples in Denmark already existed in the CF patients (26). Finally, we observed that patients persistently colonised with *Aspergillus* spp. were older, provided more samples, experienced more ABPA, and among them a higher proportion had chronic bacterial infections than mould negative patients.

The detection frequency of moulds varies considerably between centres. Most studies have focused on *A. fumigatus* and reported detection rates ranging from 5.9 to 57% (44-49). We included both children and adults and showed an *A. fumigatus* detection rate of more than 33% which falls in the middle of this wide range. We found that the detection rate of most mould species was stable over the two periods, but species such as *A. niger, Penicillium* spp. and *Paecilomyces* spp. were isolated from more patients in the second period. Seasonal variation was not the cause of this as both study periods were the second six months of the year. We found that *A. flavus* and *A. terreus* were second and third consistently on both sample periods. One patient was persistently colonised by *A. terreus* as the only mould species.

Other centres have reported that *S. apiospermum* and *A. terreus* are the second and third most frequently observed filamentous fungi (2). However, the routine media and incubation time used in our study are not optimal for species like *S. apiospermum* and *E. dermatitidis* and thus the prevalence rates of these are probably underestimated. If diagnostic molecular tools are applied for samples from CF patients, several fungal species may be detected even in culture negative samples (50;51).

A number of studies have investigated the clinical significance of moulds in the airways of CF patients not fulfilling the criteria for ABPA and most have been limited to *A. fumigatus* (52;53). Two recent longitudinal studies point in opposite directions. Amin et al. (13) showed that CF patients (<19 years) with *A. fumigatus* persistently present (defined as 2 or more positive cultures per year) in their lungs had more pulmonary exacerbations requiring hospitalisation compared to CF patients negative for *A. fumigatus*. Though, they used another definition for colonisation (>50% positive of respiratory cultures) and included more adult patients (21% ≥ 25 years) de Vrankrijker et al. (14) did not find a negative effect of *A. fumigatus* colonisation on the course of lung function. However, in both studies denominator data on the total number of respiratory samples per patient is not presented which complicates direct comparison and whether differences in classification of persistent colonisation could explain the opposite results. In our study, patients persistently colonised with *Aspergillus* had more pronounced obstructive lung disease than mould negative patients at baseline, but the association was not statistically significant. This may be due to the cross-sectional nature of our study and differences in definition of persistent colonisation/infection. Moreover, we found that, patients persistently colonised were older and the proportion of females were higher supporting similar findings reported by others (54). Traditionally, diabetes has been considered a predisposing condition for fungal infections, but surprisingly a recent French study showed that diabetes was less common in patients with *Aspergillus* than in those without (49). We confirm this observation among the persistently colonised patients, and thus the linkage between *Aspergillus* and less diabetes should be further explored.

Azole-resistance in *A. fumigatus* from CF patients has only been sporadically detected (22-24). To our knowledge the most comprehensive published report studied 159 isolates from 11 patients with defined as chronically colonised with *A. fumigatus* but found no azole-resistant isolates (27). However, five patients harboured 18 (11%) isolates which were classified as intermediate susceptible to itraconazole (MICs of 2 mg/L) and/or to posaconazole (MIC of 0.5 mg/L) using CLSI method (55). The underlying molecular mechanisms were not explored. We analysed 413 *A. fumigatus* isolates from 133 patients, and found azole resistant isolates in

the samples from six patients (4.5%) and seven different substitutions in Cyp51A. Six of the substitutions have been reported previously (22;25), but to our knowledge the V101F substitution is novel. This was found in combination with a confirmed resistance mechanism (M220) and it is unclear whether it is associated with susceptibility. Furthermore, we found the Y431C amino acid substitution in four isolates with no other target gene mutations. The isolates showed variable growth on the itraconazole agar and prominent but not absolute growth inhibition visible using a stereo microscope in the itraconazole wells of 2-32 mg/L of the EUCAST plate. The Y431C substitution has previously been found in a multi azole resistant isolate in a cohort from Manchester (22). Though, the present findings suggest a potential role of this alteration in resistance development, other factors such as presence of efflux pumps or over-expression of target gene may be required to result in a multi azole resistant phenotype observed in Manchester.

An interesting aspect of this study is the variety of different *cyp*51A mutations found which parallels what has been described in Manchester (UK) from a centre which manages patients with chronic pulmonary aspergillosis whom often receives long term azole therapy (22). In contrast, in the Netherlands more than 90% of azole resistant isolates shared the same TR/L98H resistance mechanism (23). The emergence of this resistance mechanism and a newly reported one consisting of two substitutions of Cyp51A (Y121F and T289A) plus a 46-bp tandem repeat in the gene promoter region has been linked to the use of azoles in the environment (56;57). We detected the TR/L98H resistance mechanism in nine isolates from two patients. In addition, eight of these isolates also had two extra substitutions in the Cyp51A protein (S297T and F495I). Though, any potential role in resistance of these substitutions have yet to be confirmed, they have been found in clinical and environmental samples from the Netherlands as well (25). It has previously been suggested that patients can acquire azole resistant isolates by two means: either by selection in the individual patient during treatment or via de novo inhalation of resistant isolates present in the environment. Our data supports both theories. First, there was evidence of evolution within the lung; by identification of susceptible and resistant isolates of the identical or closely related genotypes in two patients suggesting selection of resistance in vivo. Second, two

patients had the same isolates both with TR/L98+S297+F495 alterations, suggesting acquisition of a single strain which was resistant prior to colonisation. Third, none of the susceptible isolates in patients shared genotype with the TR/L98H isolates, while patients with isolates with other resistance mechanisms simultaneously harboured susceptible isolates with identical genotype. Finally, there was a trend towards less azole exposure in patients with the TR/L98H phenotype than the patients with other hot spot mutations.

The detection of azole resistance among *Aspergillus* spp. was based on the use of an itraconazole-containing medium in the majority of cases. Thus, isolates with resistance to voriconazole or posaconazole but not to itraconazole may have been mis-classified as susceptible. This has been reported in *Aspergillus lentulus* (58) and recently in environmental and clinical *A. fumigatus* isolates from the Netherlands (57) and in the UK (59). In 2007 only unique isolates (defined as >6 months apart) were susceptibility tested as part of the routine procedures and two patients with resistant isolates were identified. However, by subsequent screening of "in between" isolates, additional two patients with resistant isolates were identified. Thus, unless all isolates and possibly several isolates per patient are tested, the prevalence of azole resistance may be underdiagnosed.

Previously, it was shown that CF patients may harbour several genotypes of *A. fumigatus* in their lungs both at the same time and in serial samples (16;27). Our analysis of both azole susceptible and resistant isolates from eight patients also showed that several genotypes could be detected in a single patient as well as in a single sample. Isolates with identical or nearly identical genotypes were detected in patients R1, R3, R4 (both azole susceptible and resistant isolates. From patient R2 we had a single TR/L98H+S297T+F495I isolate with identical genotype as all the isolates with the same resistance mechanism from patient R5. We were not able to identify the source of this coincidence, and cannot exclude the possibility for mislabelling of a respiratory sample. However, transmission from patient R5 to R2 is also, at least in theory, a possibility. Alternatively, both patients could have independently acquired this resistant isolate from the environment.

In conclusion, the emergence of azole-resistance in the *A. fumigatus* isolates of CF patients is of concern. Firstly, patients may later develop *Aspergillus* bronchitis or severe complications such as ABPA. Secondly, in

patients undergoing lung transplantation an azole-resistant invasive infection may develop. Due to the fact that azoles are the only available oral group of drugs for the treatment of these conditions, clinical management of azole resistant infections is challenging. More longitudinal and prospective studies on the clinical significance of moulds in the CF respiratory tract are needed, especially in order to define which patients will benefit from the treatment of fungal colonisation outside the ABPA setting.

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