Unusual *Aspergillus* species in patients with cystic fibrosis

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Poorly sporulating *Aspergillus* isolates from patients with cystic fibrosis (CF) are generally identified in routine procedures as *Aspergillus* spp. In this study, we identified and characterized 11 isolates belonging to two unusual *Aspergillus* species of the section *Fumigati* (*A. lentulus* and *Neosartorya pseudofischeri*) recovered from four different patients. *Aspergillus lentulus* was found occasionally during a 10-year follow-up study of one CF patient colonized by *A. fumigatus*. *Neosartorya pseudofischeri* was isolated from three patients followed in different European hospitals. This species was recovered from two sputum samples of one patient, and from four successive samples of the two other patients, suggesting that it may be responsible for chronic colonization. Both species were isolated together with *A. fumigatus*. Isolates from both species did not grow at 50°C, and DNA sequence analysis, together with further morphological observations permitted identification at the species level. Growth at different temperatures and antifungal susceptibility were also investigated. All the isolates of *N. pseudofischeri* exhibited a very low susceptibility to voriconazole (VRZ) whereas a very low susceptibility to VRZ and amphotericin B was seen with the *A. lentulus* isolates.

**Keywords** Neosartorya pseudofischeri, Aspergillus lentulus, cystic fibrosis, airway colonization

**Introduction**

Patients with cystic fibrosis are at high risk of colonization of the airways by various microorganisms, mainly bacteria, but also several filamentous fungal species. The latter include, in particular, some *Aspergillus* or *Scedosporium* species, *Exophiala dermatitidis* and the more recently described fungus *Geosmithia argillacea*. Among the *Aspergillus* species *A. fumigatus* is the most common agent of chronic colonization of the airways with a high prevalence rate, ranging up to 56% [1]. Other *Aspergillus* species recovered from respiratory specimens of CF patients, some of which may be responsible for chronic colonization, include *A. terreus, A. flavus, A. niger, A. nidulans* and *A. versicolor*, with a varying prevalence rate within the different European countries [2–6]. In the routine laboratory, other unusual or poorly sporulating *Aspergillus* species are generally identified as *Aspergillus* spp. However, identification of clinical *Aspergillus* isolates at the species level may be important as they may differ in their antifungal susceptibility patterns [7]. For instance, some unusual *Aspergillus* species exhibit a very low *in vitro* susceptibility to some of the antifungal drugs commonly used for the treatment of *A. fumigatus* infections, i.e., itraconazole (ITZ) and voriconazole (VRZ). The prevalence rate of these ‘uncommon’ species is unknown, possibly low, but probably underestimated as molecular methods are required to ascertain reliable identification at the species level. Moreover, their clinical significance may be important. Here, we report four cases of colonization of...
the airways in CF patients by some unusual Aspergillus species belonging to the section Fumigati. They were initially identified as Aspergillus spp. due to discrepancies in their key features with respect to A. fumigatus. Detailed analyses confirm that these isolates belonged to A. lentulus or N. pseudofischeri, two species which can be isolated from the environment and show a worldwide distribution [8].

Case reports

Patient 1

Patient 1 from Angers, France, is a male, born in December 1979, who is homozygous for the F508 Del mutation, and was diagnosed with CF at the age of 5 years. The patient was colonized by Staphylococcus aureus at the age of 8 years, by A. fumigatus and S. apiospermum at 11 years of age, and by Pseudomonas aeruginosa and Candida albicans at 12 years, respectively. Since at this time, antibody studies for A. fumigatus and S. apiospermum started to be positive, the patient received discontinuously ITZ from eight years. Sixty-one multiple and sequential isolates of A. fumigatus were collected for molecular typing during a 9-year period (1991–2000) [9]. During morphological reassessment of these isolates, one ‘atypical’ poorly sporulating isolate was observed among the five collected from one sputum sample (13 January 1999).

Patient 2

Patient 2 from Giens, France, is again a male, born in 1982, who was homozygous for the F508 Del mutation and was diagnosed with CF at the age of 5 months. He was colonized by S. aureus since the initial diagnosis and later by A. fumigatus and Achromobacter xylosoxidans subsp. xylosoxidans at the ages of 17 and 22 years, respectively. Serology for A. fumigatus always remained negative. Nevertheless, one year after his initial colonization by A. fumigatus, the patient received discontinuously and successively ITZ and VRZ. In November 2008, an ‘atypical’ poorly sporulating isolate was recovered and the same observation was made 5 months later (April 2009).

Patient 3

This male patient from Angers, France, also homozygous for the F508 Del mutation was diagnosed prenatally in 1998. The patient was colonized by methicillin-resistant S. aureus and developed Pseudomonas aeruginosa infection at the age of 2 and 5 years, respectively. The patient was then colonized by several hyphomycetes including Aspergillus fumigatus since he was 7 years old, Scedosporium apiospermum and Geosmithia argillacea at 8 years of age, and S. prolificans at 9 years, respectively. Two years after colonization by A. fumigatus, the patient developed Aspergillus-derived asthma and he was treated by ITZ and corticoids. Two years later (18 June 2009), A. fumigatus was isolated together with an ‘atypical’ poorly sporulating isolate. Isolates with the identical morphology were recovered 2, 4 and 6 months later (25 August 2009, 5 October 2009 and 14 December 2009).

Patient 4

For this male patient from Herscheid, Germany, born in 1984 and colonized by S. aureus since early childhood, CF was diagnosed at the age of 3 years with homozygosity for the F508 Del mutation and presence of IVS8-T tract 7T variant. At his initial medical examination in November 2003 in Aachen, Germany, he was diagnosed with S. aureus, Achromobacter xylosoxidans subsp. xylosoxidans and an ‘atypical’ poorly sporulating Aspergillus isolate. The patient was followed until November 2006 and then lost for further observation, but he was still alive in November 2009. Isolates with the same atypical morphology were collected on 27 July 2004, 4 October 2004 and 19 July 2005, whereas two typical isolates of A. fumigatus were recovered from a sputum sample collected on 4 September 2006. During the follow-up, there were no signs for APBA, especially no specific anti-A. fumigatus antibodies detectable, as well as no pancreatic insufficiency or signs of diabetes mellitus. Despite intermittent antibiotic treatment with ceftazidime and tobramycine, A. xylosoxidans could not be eradicated. Due to the relative good condition of the patient, no attempt was made to eradicate this atypical Aspergillus isolate during his medical attendance in Aachen.

Materials and methods

Fungal isolates

All the isolates presented in this study (Table 1) were recovered from sputum samples. They were preserved and referenced in the BCCM/IHEM Collection (Scientific Institute of Public Health, Brussels, Belgium (http://bccm.belspo.be/db/ihem_search_form.php)). The reference strains of A. lentulus and N. pseudofischeri (Table 1) were obtained from the CBS (Centraalbureau voor Schimmelcultures) Culture Collection (Utrecht, The Netherlands) and the reference strain of A. fumigatus (AF293) from the National Collection from Pathogenic Fungi (NCPF, Bristol, UK), respectively.

Molecular identification

Molecular identification was performed by sequencing of β-tubulin gene. Genomic DNA was extracted according to
Table 1 Isolates of Aspergillus lentulus and Neosartorya pseudofischeri: sources, isolation dates, conidiation rate and antifungal susceptibility.

<table>
<thead>
<tr>
<th>Source</th>
<th>HEM or CBS number</th>
<th>Molecular identification</th>
<th>Isolation date</th>
<th>Conidiation rate</th>
<th>MIC 95 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>CBS 175.97</td>
<td>A. lentulus</td>
<td></td>
<td>++</td>
<td>8 8 1</td>
</tr>
<tr>
<td>Reference</td>
<td>CBS 116883</td>
<td>A. lentulus</td>
<td></td>
<td>+</td>
<td>2 4 1</td>
</tr>
<tr>
<td>Type strain</td>
<td>CBS 208.92</td>
<td>N. pseudofischeri</td>
<td></td>
<td>++</td>
<td>0.5 4 2</td>
</tr>
<tr>
<td>Reference</td>
<td>NCPF 7367</td>
<td>A. fumigatus</td>
<td></td>
<td>+++</td>
<td>2 1 0.5</td>
</tr>
<tr>
<td>Patient 1</td>
<td>IHEM 22112</td>
<td>A. lentulus</td>
<td>13 Jan 1999</td>
<td>++</td>
<td>4 8 2</td>
</tr>
<tr>
<td></td>
<td>IHEM 22113</td>
<td>A. fumigatus</td>
<td>13 Jan 1999</td>
<td>+++</td>
<td>1 2 1</td>
</tr>
<tr>
<td></td>
<td>IHEM 22114</td>
<td>A. fumigatus</td>
<td>13 Jan 1999</td>
<td>+++</td>
<td>1 1 0.5</td>
</tr>
<tr>
<td></td>
<td>IHEM 22115</td>
<td>A. fumigatus</td>
<td>13 Jan 1999</td>
<td>+++</td>
<td>2 0.5 1</td>
</tr>
<tr>
<td></td>
<td>IHEM 22116</td>
<td>A. fumigatus</td>
<td>13 Jan 1999</td>
<td>+++</td>
<td>2 1 0.5</td>
</tr>
<tr>
<td>Patient 2</td>
<td>IHEM 22905</td>
<td>N. pseudofischeri</td>
<td>Nov 2008</td>
<td>++</td>
<td>0.5 4 1</td>
</tr>
<tr>
<td></td>
<td>IHEM 23044</td>
<td>N. pseudofischeri</td>
<td>Apr 2009</td>
<td>++</td>
<td>0.5 4 1</td>
</tr>
<tr>
<td></td>
<td>IHEM 23045</td>
<td>A. fumigatus</td>
<td>Apr 2009</td>
<td>+++</td>
<td>2 1 &gt;16</td>
</tr>
<tr>
<td>Patient 3</td>
<td>IHEM 23161</td>
<td>N. pseudofischeri</td>
<td>18 Jun 2009</td>
<td>+++</td>
<td>0.25 8 1</td>
</tr>
<tr>
<td></td>
<td>IHEM 23434</td>
<td>N. pseudofischeri</td>
<td>25 Aug 2009</td>
<td>+++</td>
<td>0.5 8 0.5</td>
</tr>
<tr>
<td></td>
<td>IHEM 23435</td>
<td>N. pseudofischeri</td>
<td>05 Oct 2009</td>
<td>+++</td>
<td>1 8 0.5</td>
</tr>
<tr>
<td></td>
<td>IHEM 23463</td>
<td>N. pseudofischeri</td>
<td>14 Dec 2009</td>
<td>+++</td>
<td>1 8 1</td>
</tr>
<tr>
<td>Patient 4</td>
<td>IHEM 23146</td>
<td>N. pseudofischeri</td>
<td>Nov 2003</td>
<td>++</td>
<td>0.25 8 1</td>
</tr>
<tr>
<td></td>
<td>IHEM 23147</td>
<td>N. pseudofischeri</td>
<td>27 Jul 2004</td>
<td>++</td>
<td>0.25 4 1</td>
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<td></td>
<td>IHEM 23148</td>
<td>N. pseudofischeri</td>
<td>04 Oct 2004</td>
<td>++</td>
<td>0.5 8 2</td>
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<td></td>
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<td>N. pseudofischeri</td>
<td>19 Jul 2005</td>
<td>++</td>
<td>0.5 8 1</td>
</tr>
<tr>
<td></td>
<td>IHEM 23150</td>
<td>A. fumigatus</td>
<td>04 Sep 2006</td>
<td>+++</td>
<td>2 0.5 0.5</td>
</tr>
<tr>
<td></td>
<td>IHEM 23151</td>
<td>A. fumigatus</td>
<td>04 Sep 2006</td>
<td>+++</td>
<td>2 0.5 0.25</td>
</tr>
</tbody>
</table>

Growth at different temperatures of incubation was evaluated for the 22 isolates listed in Table 1 (A. fumigatus, n = 8; A. lentulus, n = 3; N. pseudofischeri, n = 11). Aliquots (10 μl) of a conidial suspension containing about 10^5 conidia/ml were spotted at the centre of MEA agar plates which were incubated at 25°C, 37°C, 45°C, 48°C and 50°C [12], respectively, and the diameter of the colonies were recorded every day for up to 5 days.

Antifungal susceptibility testing

Broth microdilution antifungal susceptibility testing of reference strains and patients’ isolates of A. lentulus, N. pseudofischeri and A. fumigatus was performed according to CLSI 38-A2 protocol with slight modifications [13] using amphotericin B (Bristol Myers Squib, Braine l’Alleud, Belgium), itraconazole (Janssen Research Foundation, Beerse, Belgium) and voriconazole (Pfizer, Brussels, Belgium). Two-fold serial dilutions (100 μl per well) of the antifungal drugs were inoculated with 100 μl of the inoculum suspension. Inoculum suspensions were prepared by scraping the surfaces of 7-day-old potato dextrose agar slants with a solution of 0.02% Tween 80. The fungal suspensions were then adjusted to 1.0 McFarland unit, and diluted 1:50 in 2 × RPMI (2% glucose, 0.165 M morpholino propane sulfonic acid). Microtiter plates were incubated at 35°C for 48 h and read both visually and spectrophotometrically at 405 nm. The minimum inhibitory concentrations (MICs) were recorded.

Morphological characteristics and growth rate

Morphological criteria to differentiate A. fumigatus, A. lentulus and N. pseudofischeri are those in accord with the data accumulated by Samson et al. [8]. All isolates were cultured on malt extract agar (MEA) with incubation at 37°C. Texture and colour of the colonies, conidiation and its importance, vesicle form and size, and conidia form and size were recorded. The isolates of N. pseudofischeri were also grown on straw agar (containing for one liter: 50 g of grinded straw, 20 g Bacto-agar, 1 g MgSO₄, 7H₂O and 1 g KH₂PO₄) in order to stimulate the sexual reproduction.
were defined as the lowest concentration of the drugs that completely inhibited fungal growth after 48 h. All experiments were repeated twice on different days.

**Results**

**Molecular identification**

Molecular protocols were required for species identification of the poorly sporulating isolates. Sequences analyses of the ß-tubulin gene allowed us to identify isolate IHEM 22112 from patient 1 as *A. lentulus* (99% identity with the corresponding sequence EF669825.1 from strain CBS 117885, ex type strain of *A. lentulus*). Sequences obtained for the poorly sporulating isolates from patients 2, 3 and 4 exhibited 99%, 100% and 99% identity, respectively, with the ß-tubulin sequence AY870742 from the *N. pseudofischeri* strain CBS 208.92 (ex type strain of *N. pseudofischeri*).

**Morphological characteristics and growth rate**

Further morphological investigations were performed for all the isolates in order to substantiate the results from ß-tubulin sequence analysis. The colony of isolate IHEM 22112 from patient 1 on MEA plates was white at the beginning, then becoming pale grey from the centre and reverse sand with a velutinous texture. The characteristic of interspersed green/grey conidia was observed. The vesicles were subglobose (mean diameter 18.5 μm), mostly twisted, and conidia were subglobose (mean 2.8 × 2.6 μm), smooth to very finely roughened. The sporulation rate of the 11 isolates from patients 2, 3 and 4, identified as *N. pseudofischeri*, were variable from one patient to another (Table 1). However, all isolates recovered from a given patient exhibited the same sporulation rate. After several subcultures, particularly on a medium containing straw, conidiation was enhanced, except for the reference strain (type strain) which showed a very low rate of conidial formation. All patients’ isolates produced white colonies, with a cream-yellow reverse. The vesicles were subglobose with a mean diameter size of 18.6 μm (minimum size 13.8 μm and maximum size 25.8 μm). The conidia were subglobose for all the isolates, with a median size of 2.7 × 2.9 μm (minimal size 2.2 μm and maximum size 3.5 μm). All the isolates identified as *N. pseudofischeri* exhibited ascogonia (hyphal coils which are the precursor state to formation of ascomata) after 7 days of growth on MEA at 37°C (Figure 1). All attempts to obtain the full sexual state, including several subcultures on straw-containing medium, were unsuccessful for all the isolates studied including the reference strain.

Figure 2 shows the mean diameter of the colonies for the three species as measured at 72 h. At 25°C, *N. pseudofischeri* grew faster (39–44 mm, mean 41 mm) than *A. fumigatus* (20–25 mm, mean 21 mm) and *A. lentulus* (22–27 mm, mean 23 mm), the latter two exhibiting quite similar growth rates at this temperature. Growth was enhanced for all isolates and reference strains at 37°C. *N. pseudofischeri* grew a little slower (61–68 mm, mean 66 mm) than *A. fumigatus* (65–75 mm, mean 71 mm), and the smallest colonies were observed with *A. lentulus* (45–55 mm, mean 50 mm). Increasing the incubation temperature markedly affected the growth for *A. lentulus* and *N. pseudofischeri*. While colonies of *A. fumigatus* covered more than half of the plates after 72 h of incubation at 45°C (40–55 mm, mean 44 mm), small colonies were obtained in the case of *A. lentulus* (13–15 mm, mean 14 mm) and *N. pseudofischeri* (14–20 mm, mean 15 mm). A very restricted growth was observed at 48°C for *A. lentulus* and *N. pseudofischeri* isolates respectively, and none of them grew at 50°C.

**Antifungal susceptibility testing**

Antifungal susceptibility patterns of patients’ isolates, and of reference and type strains are shown in Table 1. As the...
reference strains, the isolate of *A. lentulus* from patient 1 exhibited a very low susceptibility to AmB and VRZ (MIC values 4 and 8 μg/ml, respectively), but it could be considered as intermediate to ITZ (MIC value of 2 μg/ml) according to Verweij et al. [14]. The 11 isolates of *N. pseudofischeri* from patients 2, 3 and 4 exhibited quite similar *in vitro* antifungal susceptibility patterns in that all had a very low susceptibility to VRZ (MIC values ranging from 4–8 μg/ml, which were very close to the MIC obtained for the type strain). According to the interpretative breakpoints proposed by Verweij et al. [14] for *A. fumigatus*, all clinical isolates were susceptible to AmB (MIC values of 0.25 or 0.5 μg/ml) and all but one were susceptible to ITZ (MIC from 0.5 to 1 μg/ml), while the last one was intermediate (MIC value of 2 μg/ml). Additionally, all but one out of the seven clinical isolates of *A. fumigatus* (IHEM 23045) recovered from these patients were susceptible to ITZ.

**Discussion**

After the re-evaluation of the taxonomy of the section *Fumigati* following a polyphasic approach, 33 species have been accepted within this section [8,15,16], comprising five distinct clades. Some of these species had sexual stages and therefore have been assigned to the teleomorphic genus *Neosartorya*. Besides *A. fumigatus*, which is the predominant member of the genus in medical mycology, only ten species have been involved in clinically proven infections [17], including *A. lentulus* and *N. pseudofischeri*. For these two species, the number of cases reported remains limited, probably because of misidentifications.

Balajee et al. [12] pointed out in 2004 after screening a collection of clinical isolates of *A. fumigatus*, the existence of poorly sporulating isolates with decreased susceptibility to antifungal drugs. Due to this particular phenotype, these isolates were reclassified one year later as a new species, *A. lentulus* [18]. Since then this species has been recovered from clinical specimens in Japan [19], Australia and the USA [20]. *Aspergillus lentulus* was also recovered, together with *A. fumigatus*, in a case of chronic obstructive pulmonary disease [21] and in a probable invasive pulmonary aspergillosis [22].

Until now, only five clinical cases involving *N. pseudofischeri* have been reported, i.e., an osteomyelitis [23], two peritonitis [24,25], an invasive pulmonary aspergillosis [26], and an invasive otitis in a hematopoietic stem cell transplant patient [11]. Likewise, this fungus was also recovered from two CF patients [11].

This study underlines the difficulties in identifying poorly sporulating *Aspergillus* species from the section *Fumigati* strictly on the basis of morphological criteria. *Aspergillus fumigatus, A. lentulus* and *N. pseudofischeri* have overlapping morphological characteristics [8] and therefore, use of sequence-based methods is required for correct identification at the species level. Moreover, it is not uncommon for clinical isolates of *A. fumigatus* to exhibit atypical phenotypes, with white or grey colonies instead of the typical dark green colour and poor sporulation. A very useful and important physiological property of *A. fumigatus* for its differentiation from the other species of the section *Fumigati* is its ability to grow at 50°C [17].

The full sexual state i.e., presence of cleistothecia containing asci and ascospores could not be observed for all the *N. pseudofischeri* isolates in this study, and even if they had been seen, *N. pseudofischeri* can only be differentiated from *Neosartorya fischeri* on a morphological basis by use of scanning electron microscopy with visualization of the surface ornamentation of ascospores due to the presence of nonanastomosing, elongate equatorial ridges [25]. As ascogonia have been observed for all the isolates studied, we could refer all of them to the teleomorph *N. pseudofischeri*, instead of the corresponding anamorph *Aspergillus thermomutatus*.

The study of the optimum growth temperature of the three species *A. fumigatus, A. lentulus* and *N. pseudofischeri* is highly informative. It was shown that *A. fumigatus* and *N. pseudofischeri* have a quite similar growth rate at 37°C. Coexistence and long-term persistence of both species in the airways of patients with CF was therefore possible and this reinforces the hypothesis that *N. pseudofischeri* can be considered as an agent of chronic colonization in CF.

As suggested in a conference of general agreement from the French Society of Pneumology [28] for *Pseudomonas aeruginosa* in CF patients, a chronic colonization of the airways could be defined as the isolation of a microorganism from three successive samples over a 6-month period, which was the case for at least two of the patients colonized by *N. pseudofischeri* (patients 3 and 4). Nevertheless, genotyping of *A. fumigatus* isolates from CF patients have shown co-colonization by multiple genotypes, whereas some genotypes were isolated recurrently and capable of prolonged colonization [9,27]. Such studies should be performed for *N. pseudofischeri* in order to demonstrate the long-term persistence of some genotypes and thereby confirming its ability to cause a chronic colonization of the airways in CF patients.

The slower growth of *A. lentulus* at 37°C hinders its competition and coexistence with *A. fumigatus*, suggesting that this species can only transiently colonize the respiratory tract of patients. The lack of fitness of *A. lentulus* in case of co-colonization with *A. fumigatus* may be one of the reasons why this species was isolated only once from patient 1 during a 10-year follow-up period. Likewise, in the case of invasive aspergillosis with a mixed culture of *A. fumigatus* and *A. lentulus*, it is also highly probable that the presence of *A. lentulus* was only occasional and that the causative agent of the invasive disease was *A. fumigatus* [21,22].
Both \textit{A. lentulus} and \textit{N. pseudofischeri} recovered in this study from patients with CF are poorly susceptible to current antifungal drugs. \textit{A. lentulus} showed high in vitro MIC values for AmB and VRZ, whereas \textit{N. pseudofischeri} isolates had high VRZ MICs. These MIC values were in agreement with reports in the literature \cite{7,11,12,17,19,29}. Verweij \textit{et al.} \cite{14} proposed interpretative breakpoints for isolates of \textit{Aspergillus} spp., and isolates with MIC values $> 2$ $\mu$g/ml for VRZ and ITZ could be considered as resistant. We also showed that \textit{N. pseudofischeri} may coexist with isolates of \textit{A. fumigatus} exhibiting a very low susceptibility to ITZ. The fact that \textit{A. fumigatus} was reported to colonize the bronchial mucus of CF patients in the form of biofilms may explain higher MICs \cite{30}. Additionally, one may speculate that repeated therapies with antifungals in CF patients colonized by \textit{A. fumigatus} may account for their very low susceptibility to ITZ. Antifungal treatments may also lead to elimination of susceptible \textit{A. fumigatus} isolates and favour persistence of less susceptible species such as \textit{A. lentulus} and \textit{N. pseudofischeri}.

In conclusion, this study underlines the importance of identification at the species level of unusual or poorly sporulating \textit{Aspergillus} isolates since some species within the \textit{Fumigati} section may be poorly susceptible to some of the current antifungal drugs.

\textbf{Declaration of interest:} The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

\textbf{References}


