Comparison of techniques to examine the diversity of fungi in adult patients with cystic fibrosis

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This study compares conventional and molecular techniques for the detection of fungi in 77 adult cystic fibrosis (CF) patients. Three different methods were investigated, i.e., (1) conventional microbiological culture (including yeasts and filamentous fungi), (2) mycological culture with CF-derived fungal specific culture media, and (3) Non-culture and direct DNA extraction from patient sputa. Fungi isolated from environmental air samples of the CF unit were compared to fungi in sputa from CF patients. Fungi \((n = 107)\) were detected in 14/77(18%) of patients by method 1, in 60/77 (78%) of patients by method 2 and with method 3, in 77/77(100%) of the patients. The majority of yeasts isolated were *Candida albicans* and *C. dubliniensis*. *Exophiala (Wangiella) dermatitidis*, *Scedosporium apiospermum*, *Penicillium* spp., *Aspergillus fumigatus* and *Aspergillus versicolor* were also identified by sequence analysis of the rDNA short internal transcribed spacer (ITS2) region. Conventional laboratory analysis failed to detect fungi in 63 patients mainly due to overgrowth by Gram-negative organisms. Mycological culture with antibiotics dramatically increased the number of fungi that could be detected. Molecular techniques detected fungi such as *Saccharomyces cerevisiae*, *Malassezia* spp., *Fuscoporia ferrea*, *Fusarium culmorum*, *Acremonium strictum*, *Thanatephorus cucumeris* and *Cladosporium* spp. which were not found with other methods. This study demonstrates that several potentially important fungi may not be detected if mycological culture methods alone are used. A polyphasic approach employing both enhanced mycological culture with molecular detection will help determine the presence of fungi in the sputa of patients with CF and their healthcare environment.

**Keywords** *Aspergillus fumigatus*, *Candida albicans*, *Scedosporium apiospermum*, *Wangiella dermatitidis*, *Exophilia dermatitidis*, ABPA, invasive disease, lung transplantation, PCR

Introduction

The lungs of cystic fibrosis (CF) patients are often colonized or infected in infancy and early childhood with such organisms as *Staphylococcus aureus* and *Haemophilus influenzae*. These may damage the epithelial surfaces, leading to increased attachment of, and eventual replacement by organisms such as *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. Yeasts and filamentous fungi are also frequently recovered from respiratory specimens derived from CF patients. Allergic bronchopulmonary aspergillosis (ABPA) is well recognized as a severe complication associated with an accelerated decline in lung function [1]. ABPA affects up to 15% of CF patients [1] and is mainly caused by *Aspergillus fumigatus* and other *Aspergillus* spp., but other fungi such as *Candida* spp. and...
Trichosporon beigeli can also cause allergic bronchopulmonary mycosis [2,3]. However, only Aspergillus spp. have been recognized as being associated with pulmonary symptoms in CF patients and there have been only a few reports of species other than members of this genus being of clinical significance in CF patients. In addition, Aspergillus spp. and other fungi may be of particular importance in the context of CF lung transplantation. The incidence of all types of fungal infections in lung transplantation recipients is around 5–10%. Pulmonary aspergillosis is the most frequently observed fungal infectious complication of heart-lung or isolated lung transplantation and invasive fungal infection is often fatal, with the mortality rate following Aspergillus infection in such patients remaining around 70% [4]. Fatal invasive fungal infections due to Candida spp., Scedosporium spp. and Paeilomyces spp. in CF patients after lung transplantation are beginning to be reported [5–7].

Although fungal infections are associated with a higher mortality rate than either bacterial or viral infections, the presence of fungi in the airways of CF patients is often not considered as a significant clinical criterion [8]. With improving methods for the detection of fungi, the prevalence and diversity of fungi in CF patients has been increasing in the last few years. According to the data from the Cystic Fibrosis Foundation, the occurrence rate of Aspergillus spp. in CF patient sputum samples has effectively doubled in the last decade (1995–2005) from 6% to 13%. Some studies have reported a correlation between antibiotic therapy and the increased occurrence of fungi [9]. However, despite interest and knowledge of bacterial lung infections, the prevalence, diversity, effects and symptoms of fungi in CF is still largely unknown.

The clinical significance of fungal infection is not clear and increasing information on the topic is difficult due to the insensitivity of fungal culture methods. Yeasts and filamentous fungi may be present in CF sputum, as a result of three mechanisms, i.e., (i) co-incident presence due to airborne introduction from the environment, or contamination of the oropharynx and mouth due to foodstuffs, (ii) colonization of the airways without any clinical sign, similar to the presence of a commensal organism; and (iii) pathogenic role in a disease process. Unlike the gastrointestinal tract, our understanding of their presence in a commensal-like fashion is limited as fungi should not be present in the airways. Although in the majority of the cases no clinical signs are associated with the recovery of filamentous fungi from sputum samples, one cannot disregard the fact that the development of these fungi (which cannot be considered as commensal organisms) in the airways may contribute to the inflammatory reaction which progressively leads to the deterioration of the lung function in CF patients.

Although CF clinicians are now becoming more aware of the presence of fungi in sputum specimens of their patients, and are subsequently beginning to consider the clinical significance of these organisms in patients, to date, there have been relatively few studies describing the ecological mycobiota of the CF lung, mainly due to a lack of reliable methods to undertake such studies. Although the application of molecular techniques to aid in the detection of fungi in clinical infection has been well studied and published, there has only been a single recent report to date, employing panfungal PCR techniques to detect fungi in spuata of CF patients [10]. Hence it was for these novel reasons that the current study was designed and performed. In addition, we were interested in determining the presence of other fungi recently reported in CF, including Scedosporium apiospermum and Exophiala dermatitidis. These two organisms are beginning to be reported with increasing frequency and pathogenicity [11–17].

In order to determine the occurrence of fungi in CF sputum samples, this study compared conventional and PCR-based techniques for the detection of yeasts and filamentous fungi and investigated the prevalence and diversity of fungal species in spuata from 77 adult CF patients in Northern Ireland. Three different methods were compared to detect fungi in sputum, i.e., (1) conventional non-selective culture, (2) mycological culture with CF-derived fungal specific culture media, and (3) direct DNA extraction and fungal PCR from sputum. Contemporaneous environmental air samples were also taken from the cystic fibrosis unit to examine the prevalence of fungi in the environment and to compare this data to that obtained in the analysis of spuata from CF patients.

**Materials and methods**

**Patients**

Sputum samples from 77 individual adult (>18 years old; female 52% and male 48%) CF patients attending the Northern Ireland Regional Adult CF Centre, Belfast City Hospital, were collected over the 13 month study period, from December 2004–January 2006. Patients were selected consecutively on their ability to produce a sputum specimen in either an out-patient or in-patient setting. Each patient enlisted into the study had only one sputum specimen examined. The median age of CF patients examined was 28.5 years (range 18–59 years) and the majority of spuata from patients examined (68%) were obtained from those attending as out-patients for routine review or as a result of complications not requiring their admission. The remaining 32% of patients in the study were in-patients suffering from acute pulmonary exacerbation of their pulmonary condition. Sputum was collected from all

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patients immediately after a standardized session of physiotherapy from a state-registered physiotherapist and all sputa were stored at ambient temperature, prior to microbiological processing.

**Study design**

Isolation and identification of yeasts and filamentous fungi in sputa from these patients consisted of three components, namely:

(i) **Non-selective conventional culture.** Individual sputum samples were diluted 1:1 in Sputasol (Oxoid SR089A, Oxoid Ltd., Poole, England) and inoculated on Columbia blood agar base (Oxoid CM0331) supplemented with 5% [v/v] defibrinated horse blood (Oxoid SR0050) to be incubated at 37°C for 48 h. Isolated colonies of yeasts or filamentous fungi with distinct morphologies were examined to obtain their identification at the species level. Yeasts were identified biochemically using API ID 32C test strips (bioMérieux) or other biochemical identification systems [18], and filamentous fungi were identified on the basis of their macroscopic and microscopic characters according to standard descriptions, as previously described [18].

(ii) **Mycological culture with CF-derived fungal specific culture media.** Undiluted sputum samples were inoculated on two different fungal selective media, namely, Sabouraud dextrose agar (SDA) and Medium B+. SDA was obtained commercially from Oxoid Ltd, Basingstoke, UK (CM0041) and was prepared in accordance with the manufacturer’s instructions. Medium B+ was a novel fungal selective medium, which was developed specifically for the detection of fungi in sputum from CF patients [19]. The medium consisted of dextrose (16.7 g/l), yeast extract (30 g/l), peptone (6.8 g/l) and agar (20 g/l) and was supplemented with the following combination of antibiotics to suppress the growth of bacterial co-habitants of CF sputa, most notably the Gram-negative organisms, *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, as well as *Staphylococcus aureus*, including ceftazidine (32 mg/l), chloramphenicol (50 mg/l), colistin (24 mg/l) and cotrimoxazole (128 mg/l). All inoculated plates of both media were incubated aerobically at 22°C for three weeks. Following incubation, the identification of fungal colonies with distinct morphologies was made by using rDNA PCR and sequencing techniques, as described below.

(iii) **Direct DNA extraction from sputum and colonies and fungal rDNA PCR & sequencing.** All DNA isolation procedures were carried out in accordance with the DNA contamination management guidelines of Millar et al. [20] and in a Class II Biological Safety Cabinet (MicroFlow, England). This was in a room physically separated from that used to set up nucleic acid amplification reaction mixes, and also from the ‘post-PCR’ room, in order to minimize contamination and the possibility of false positive results. Total genomic DNA (including fungal genomic DNA), was extracted directly from individual CF sputum samples in accordance with the following procedures. CF sputa were mixed with sterile Sputolysin (Calbiochem, La Jolla, CA, USA) in a ratio of 1:1 [v/v] and incubated for 15 min at 37°C. Following this, the sputum/sputolysin mixture was centrifuged at 13,000 g for 10 min. The supernatant was discarded and the pellet was resuspended in 200 ul Tris-HCl (10 mM, pH 8.0). Pelleted fungi were suspended in 1.5 ml Tris HCL (10 mM, pH 8.0) and were subjected to 6 cycles of freeze-thawing in liquid nitrogen/boiling. Genomic fungal DNA was further extracted from isolates employing the Roche High Purity PCR Template Preparation Kit (Roche Ltd., England), in accordance with the manufacturer’s instructions. Extracted DNA was transferred to a clean tube and stored at −80°C prior to PCR studies. For each batch of extractions, negative extraction controls containing all reagents minus isolates, were performed. All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and the amplification and post-PCR room in order to minimize contamination.

For fungal identification, DNA was amplified with ITS1 and ITS4 PCR primer set targeting the 18S-ITS1-5.8S-ITS2-28S rRNA and internal transcribed spacer region [21]. For those samples that were negative by PCR with ITS1 and ITS4 (<10%), a nested PCR was performed with ITS3 and ITS4 primer set targeting the 5.8S-ITS1-28S rRNA internal transcribed spacer region to improve sensitivity of fungal detection. PCR reaction mixes (50 μl) contained: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 200 μM (each) dATP, dCTP, dGTP and dTTP; 1.25U of Taq DNA polymerase (Amplitaq; Perkin Elmer), 0.2 μM (each) of the broad-range/universal interspace (ITS) rDNA primers, ITS3/ITS4 [21] and 4 μl of DNA template, containing circa. 50ng DNA/ml extract. Following a ‘hot start’, the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler; 96°C for 3min followed by 40 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 10 min, followed by a final extension at 72°C for 10 min. During each run molecular grade water (Biowhittaker Inc. Maryland, USA) instead of DNA was included randomly as a negative control and *Candida albicans* DNA, from a well-characterized wildtype strain, was included as a positive control.

**Detection of amplicons**

Following amplification, aliquots (8 μl) were removed from each reaction mixture and examined by electrophoresis...
(100V, 45 min) in gels composed of 2% (w/v) agarose (Gibco Ltd., Paisley, Scotland, UK) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) and stained with ethidium bromide (5 μg/100ml). Gels were visualized under UV illumination with a gel image analysis system (UVP Products Ltd., England) and all images were archived as digital graphic bitmap files (*.bmp).

**Sequencing of rDNA PCR amplicons**

PCR amplicons were identified by both cloning and direct sequencing techniques. All amplicons were purified using the QIA quick PCR purification kit (QIAGEN Ltd., UK) eluted in molecular grade water, prior to sequencing, in order to remove dNTPs, polymerases, salts and primers. For samples in which fungal DNA was extracted from spuma and more than one band was detected by electrophoresis after PCR amplification, the QIAGEN PCR clonings® kit (QIAGEN Ltd., UK) was used in accordance with the manufacturer’s instructions in order to separate all of the different amplified fungal DNA. Following this, the plasmids including the target fungal DNA were extracted with QIAprep® Spin Miniprep Kit (QIAGEN Ltd., UK) in accordance with the manufacturer’s instructions.

Both PCR amplicons and cloned plasmids, including the target DNA, were sequenced by the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit and ABI PRISM 3100 automated sequencer, according to the manufacturer’s instructions. Appropriate primers ITS1, ITS3 (for the PCR amplicons) and -20 M13 forward (for the plasmids: 5‘-GTAAACGACGGCCAGTG-3’) were employed as sequencing primers. Resulting sequences were confirmed from chromatogram analysis and confirmed sequences were compared with those stored in the GenBank using the BLAST alignment software (http://www.ncbi.nlm.nih.gov/BLAST). Sequence similarity was determined in accordance with criteria described previously [22]. Where a sequence from the short ITS region was not able to yield an identity to the species level, then the longer ITS region was recruited, as a means of improving the speciation. All short ITS sequences were subsequently submitted to GenBank and a corresponding accession number was issued.

**Examination of indoor air from CF inpatient and outpatient environmental sites**

Fifteen sites within the Regional Adult Cystic Fibrosis Centre, Belfast City Hospital were targeted for examination of the indoor air environment. These included the out-patient clinic and two different in-patient wards (Pseudomonas aeruginosa and Burkholderia cenocepacia wards), as detailed in Table 1. The mycological components of indoor air at these locations, under normal hospital conditions, were sampled both qualitatively and quantitatively, employing an electrical slit air sampler (CF Casella Ltd., London, England), in accordance with the manufacturer’s instructions. After thorough decontamination of the air slit sampler with absolute ethanol, the sampler was placed at each location and approximately 1.400 l of air for the specific location was drawn in for a 2 min period, over the microbiological agar media employed. For mycological examination, triplicate agar plates [diameter = 8 cm] (Sterilin Ltd., UK) of SDA (Oxoid CM0041) were employed. After incubation of the plates at 22°C for 2 weeks, all resulting yeasts and filamentous fungi were identified employing rDNA PCR and direct sequencing techniques, as detailed above.

**Conventional identification of environmental fungal isolates**

All fungal isolates were grown on SDA at both 22°C and 37°C, for the purposes of identification. Resulting filamentous colonies were examined both macroscopically and microscopically, in terms of their colonial features, ability to grow at 22°C and 37°C, and microscopic characteristics, and identified in accordance with previously published algorithms/criteria for the identification of medically important fungi [18].

**Results**

Sputum samples from 77 patients with CF were analysed and a total of 107 yeasts and filamentous fungi were identified by using three different methods (Table 2). *Candida* spp. were detected in all but two patients (75/77, 97%). Of these, the most common was *Candida albicans* (45/77, 58%), followed by *Candida dubliniensis* (30/77, 39%), *Candida parapsilosis* (7/77, 9.1%) and *Candida glabrata* (4/77, 5.2%). Other yeasts such as *Saccharomyces cerevisiae* (5/77, 6.5%), *Malassezia* spp. (2/77, 2.6%), *Trichosporon* spp., (2/77, 2.6%), *Rhodotorula* sp. (1/77, 1.3%) were also detected. The most common filamentous fungi found in sputum samples were *Aspergillus* spp. (7/77, 9.1%) including *Aspergillus fumigatus* (4/77, 5.2%) and *Penicillium* spp. (7/77, 9.1%), followed by *Scedosporium apiospermum* (3/77, 3.9%), *Exophiala dermatitidis* (3/77, 3.9%), *Aureobasidium pullulans* (1/77, 1.3%), *Fuscospora fereae* (1/77, 1.3%), *Fusarium culmorum* (1/77, 1.3%), *Acremonium strictum* (1/77, 1.3%), *Thanatephorus cucumeris* (1/77, 1.3%) and *Cladosporium* spp. (1/77, 1.3%).

Using conventional microbiological culture techniques, fungi were detected in only 14/77 (18%) patient samples, of which eight were *Candida* spp. (7.8%), including 2 *Candida albicans* (2.6%), 5 *Aspergillus* spp. (6.5%), including an
Table 1  Mycological profile of both in-patient and out-patient CF environments within the Adult CF centre at Belfast City Hospital. CFU/m³, colony forming units of fungi/cubic metre of air.

<table>
<thead>
<tr>
<th>Origin of isolates [Mean quantitative count; CFU fungi/m³ room air]</th>
<th>Identification</th>
<th>GenBank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Corridor</td>
<td>Rhodotorula mucilaginosa Penicillium sp. Cladosporium cladosporioides Aspergillus sydowii Heterobasidion annosum Stereum annosum Engyodontium album Cryptococcus magnus Sclerotinia sclerotiorum Paecilomyces sp. Rhodotorula slooffiae Penicillium sp.</td>
<td>DQ679489 DQ679490 DQ679491 DQ679492 DQ679487 DQ679488</td>
</tr>
<tr>
<td>Out-patient Clinic</td>
<td>Yarrowia lipolytica</td>
<td>DQ679489</td>
</tr>
<tr>
<td>2) Physiotherapy room</td>
<td>[0.7 CFU/m³ room air]</td>
<td>Paecilomyces sp. DQ679495</td>
</tr>
<tr>
<td>3) Consulting room</td>
<td>[0.7 CFU/m³ room air]</td>
<td>Rhoxercosporidium sp. DQ679495</td>
</tr>
<tr>
<td>In patient: North ward (B. cenocepacia)</td>
<td>4) Common room</td>
<td>Trametes sp. DQ679497</td>
</tr>
<tr>
<td>5) Physiotherapy room</td>
<td>[2.5 CFU/m³ room air]</td>
<td>Penicillium sp. DQ679503</td>
</tr>
<tr>
<td>6) Treatment room</td>
<td>Penicillium sp. DQ679505</td>
<td></td>
</tr>
<tr>
<td>7) Patient room</td>
<td>Aspergillus fumigatus Yarrowia lipolytica</td>
<td></td>
</tr>
<tr>
<td>8) Shower &amp; toilet</td>
<td>Penicillium sp. Coniosporium sp. Cryptococcus sp.</td>
<td></td>
</tr>
<tr>
<td>9) Corridor</td>
<td>Aspergillus fumigatus Sporidiobolus salmonicolor</td>
<td></td>
</tr>
<tr>
<td>10) Common room</td>
<td>Penicillium corylophilum Phoma herbarum Cladosporium cladosporioides Cryptococcus sp.</td>
<td>DQ679508 DQ679509 DQ679515 DQ679516</td>
</tr>
<tr>
<td>11) Physiotherapy room</td>
<td>[5.4 CFU/m³ room air]</td>
<td>Penicillium sp. Penicillium corylophilum Cladosporium cladosporioides</td>
</tr>
<tr>
<td>12) Treatment room</td>
<td>[1.4 CFU/m³ room air]</td>
<td>Cladosporium cladosporioides</td>
</tr>
<tr>
<td>13) Patient room</td>
<td>Penicillium pinophilum Emericella sp. Penicillium digitatum</td>
<td></td>
</tr>
<tr>
<td>In patient: South ward (P. aeruginosa)</td>
<td>14) Interview room</td>
<td>Penicillium sp. Penicillium chrysogenum Blumeria sp.</td>
</tr>
<tr>
<td>15) Corridor</td>
<td>[6.8 CFU/m³ room air]</td>
<td>Penicillium sp. Aspergillus fumigatus</td>
</tr>
<tr>
<td>16) Corridor</td>
<td>[6.8 CFU/m³ room air]</td>
<td>Trametes sp. Penicillium chrysogenum Rhodotorula mucilaginosa Basidiomycete yeast</td>
</tr>
</tbody>
</table>
Aspergillus fumigatus (1.3%), and Trichosporon cutaneum in sputum from one patient (1.3%). With culture on CF specific media, fungi were found in 60/77 (78%) patient specimens. The most common fungal isolates were Candida albicans (34/77, 44%), followed by Candida dubliniensis (21/77, 27%), Aspergillus versicolor (5/77, 6.5%), Aspergillus fumigatus (4/77, 5.2%), Penicillium spp. (4/77, 5.2%), Candida parapsilosis (3/77, 3.9%), Exophiala dermatitidis (3/77, 3.9%), Scedosporium apiospermum (2/77, 2.6%), Candida glabrata (1/77, 1.3%), Rhodotorula spp. (1/77, 1.3%), Trichosporon spp. (1/77, 1.3%), Aureobasidium pullulans (1/77, 1.3%). Through the use of direct DNA extraction from sputum and fungal rDNA PCR, fungi were detected in all samples 77/77 (100%). Of these, the most common fungal isolates were Candida albicans (40/77, 52%), followed by Candida dubliniensis (27/77, 35%), Candida parapsilosis (5/77, 6.5%), Saccharomyces cerevisiae (4/77, 5.2%), Penicillium spp. (4/77, 5.2%), Candida glabrata (3/77, 3.9%), Scedosporium apiospermum (3/77, 3.9%), Trichosporon spp. (2/77, 2.6%), Malassezia spp. (2/77, 2.6%), Exophiala dermatitidis (1/77, 1.3%), Fuscospora ferrea (1/77, 1.3%), Fusarium culmorum (1/77, 1.3%), Acromonium strictum (1/77, 1.3%), Thanatephorus cucumeris (1/77, 1.3%), Cladosporium spp. (1/77, 1.3%).

Air screening in CF clinic and wards

A total of 62 fungal strains were isolated from 15 different sites within CF clinic and wards (Table 1). The most commonly isolated fungi were Penicillium spp. (16), including P. corylophilum (3), P. digitatum (2), P. pinophilum (1) and P. brevicompactum (1), followed by Aspergillus spp. (6), including Aspergillus fumigatus (3), Aspergillus versicolor (2) and Aspergillus sydowii (1), Cladosporium spp. (5), including Cladosporium cladosporioides (4), Sclerotinia sclerotiorum (3), Rhodotorula mucilaginosa (3), Yarrowia lipolytica (3), Cryptococcus spp. (3), including Cryptococcus magnus (1), Engyodontium album (2), Paecilomyces spp. (2), Trametes spp. (2), Rhodotorula slooffiae (1), Heterobasidion annosum (1), Stereum annosum (1), Rhocercosporidium spp. (1), Kondoa aeria (1), Coniothyrium spp. (1), Phoma herbarum (1), Emericella spp. (1), Phaeococcomyces chersonesos (1), Aureobasidium pullulans (1) and Blumeria spp. (1).

Five fungal isolates were not identified to species level by rDNA ITS PCR and direct sequencing, as detailed in Supplementary Table 1 (online version only). Of these, three novel species belonging to the class, Ascomycetes, the genus, Acrodontium and the order, Pleosporales, were
identified, as well as a novel Helotiales-like genus. All these fungal isolates have been archived within the culture collection of the Northern Ireland Public Health Laboratory, Belfast City Hospital. None of these fungi were detected from patients’ sputum and their closest phylolgetic neighbours have been shown to be from the plant rhizosphere and leaf litter. None have been previously shown to cause infection in CF patients or other patient populations.

Mean quantitative counts of fungi in those hospital environmental sites most associated with adult CF patients did not exceed 7 CFU/m³ room air, with the patients’ room on the *Pseudomonas aeruginosa* in-patient ward being the most heavily contaminated, with a mean airborne fungal count of 6.8 CFU/m³ room air (Table 1).

Six fungal species were detected in both patients’ sputa, as well as in the air from either out-patient or in-patient environments and included *Aspergillus fumigatus, Aspergillus versicolor*, *Aureobasidium pullulans, Cladosporium spp.*, *Penicillium spp.* and *Rhodotorula spp.* (Fig. 1).

Most CF patients (46 patients; 60%) had only one fungal species detected in their sputa, 18 patients (23%) had two fungal taxa in their specimens, 12 (16%) had three fungal taxa and only one had five fungal species present. Presence of multiple fungal species in CF sputa was detected by identifying one or more bands, following electrophoresis of the ITS3/TS4 PCR products, as shown (Fig. 2).

**Discussion**

Current US CFF Registry data indicates an approximate doubling of prevalence of fungi being detected in CF sputum from 1995 through 2005, rising from approximately 6% in 1995 to approximately 13% in 2005 (personal communication; Bruce Montgomery, Cystic Fibrosis Foundation). This increase is probably due to our better understanding of the disease and the manner in which we manage it, in particular, with new and improved antibiotic interventions. The introduction of improved antibiotic agents and the way in which they are effectively used in double or triple therapy, as well as in antibiotic cycling, has helped to address the requirement to help control chronic Gram-infections in CF patients, but has inadvertently created a niche where a diverse range of fungi may be found. Furthermore, we do not believe that this marked rise in fungal recovery is due to any significant improvements in the methodologies and equipment used at primary diagnostic clinical microbiology laboratories, who support both adult and pediatric CF centers, as these have gone largely unchanged for the past decade.

Conventional laboratory analysis employing Columbia Blood agar failed to detect fungi in 63 patients (82%). This is probably due to inappropriate culturing time (24–48 h), non-selective medium conditions and overgrowth by Gram-negative organisms, such as *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, as described previously [33, 34]. Selective mycological culture with antibiotics dramatically increased the number of fungi recovered by 60% (46

**Fig. 1** Comparison between fungi isolated from the air in the CF Unit and fungi recovered from sputum samples. *Fungi isolated from in-patient wards; †fungi isolated only from out-patient clinics; §fungi detected from both in-patient wards and out-patient clinic environments.
The diversity of yeasts and filamentous fungi identified in adult CF patients in Northern Ireland were compared with results in previous reports (Table 2). Candida albicans was detected with the highest prevalence rate (58%), which is in accordance with the data noted in the literature [35–37]. Candida dubliniensis was detected in 39% of patients as the second most frequent fungus associated with CF sputum samples. This prevalence rate was significantly higher than the reported highest prevalence rate which is 11.1% [38]. This could be due to the misidentification of this yeast as Candida albicans, since both species share many morphological and physiological characteristics. Consequently, our investigation demonstrates the great efficacy of identification by sequence analysis of the rRNA gene ITS regions. Candida glabrata and Trichosporon spp. were also detected with a slightly higher prevalence rate than the reported rate [15,35]. The prevalence of Exophiala dermatitidis, Penicillium spp., Scedosporium apiospermum and Cladosporium spp. were in accord with results in previous reports [6,15,26,35,39]. Interestingly, Aspergillus spp. including Aspergillus fumigatus were detected at a lower rate (9.1%) as compared with the data in the literature [15]. To the best of our knowledge, this is the first report describing the isolation of Rhodotorula spp., Saccharomyces cerevisiae, Acremonium strictum, Thanatephorus cucumeris and Cladosporium spp. were identified with only this method. With regard to method 2, we incubated specimens at 22°C for a prolonged period of 2 weeks. This was based on our desire to capture any environmental fungi that might be present in the sputa which could not grow at body temperature, but may be responsible for an allergic ABPA-type immunological response. Equally, we believe that this lower incubation temperature for the extended period of incubation would allow fungi that normally proliferate optimally at body temperature to grow and be detected/identified.

Overall, the results of the comparison of three different methods for the detection of fungi in this study suggest the importance of including appropriate fungal culture media with a selective antibiotic combination in the routine microbiological analysis of respiratory secretions from CF patients. Non-culture and direct DNA extraction from sputa approach was valuable as it gave a high degree of sensitivity and speed for the detection of fungi and thus should be considered as an appropriate alternative to conventional mycological culture methods.

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mycological culture methods are not refined in order to select out genera that require additional workup. A polyphasic approach employing both enhanced mycological culture combined with molecular (rDNA) detection and identification will serve to help determine the presence of these in the sputum of patients with CF and their healthcare environment.

In conclusion, this study identified many different fungal species, which have not been described in the context of CF until this study, thus highlighting the diversity of the fungal biota associated with the CF lung. Further molecular and cellular work is now required in order to estimate the significance of these organisms in this patient population.

Acknowledgements

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References


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**Supplementary Table 1**  Description of unidentified fungi isolated from the air in healthcare in-patient and out-patient environments associated with the care of patients with cystic fibrosis

<table>
<thead>
<tr>
<th>Unidentified fungal ref</th>
<th>Geographical location</th>
<th>Colonial characteristics</th>
<th>ITS3/ITS4 bases</th>
<th>Nearest phylogenetic neighbour: GenBank Accession no. (% similarity/no. bases called)</th>
<th>Submitted GenBank Accession No.</th>
<th>Taxonomical description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Out-patient clinic corridor</td>
<td>Small white mycelium</td>
<td>285</td>
<td>Helotiales sp.: EF093148 (92%/265 bases called)</td>
<td>DQ679493</td>
<td>Helotiales-like filamentous fungus</td>
</tr>
<tr>
<td>2</td>
<td>Out-patient consulting room</td>
<td>Large white mycelium with cream centre</td>
<td>297</td>
<td>Ascomycetes AM084468 (99%; 241 bases called)</td>
<td>DQ679496</td>
<td>Ascomycetes filamentous fungus</td>
</tr>
<tr>
<td>3</td>
<td>In-patients' common room</td>
<td>Small white mycelium</td>
<td>285</td>
<td>Helotiales: EF093148 (92%/265 bases called)</td>
<td>DQ679504</td>
<td>Helotiales-like filamentous fungus</td>
</tr>
<tr>
<td>4</td>
<td>In-patient physiotherapy room</td>
<td>White translucent</td>
<td>276</td>
<td>Acrodontium crateriforme: AY843112 (94%/223 bases called)</td>
<td>DQ679507</td>
<td>Acrodontium sp.</td>
</tr>
<tr>
<td>5</td>
<td>Single-bedded in-patient's room</td>
<td>Cream colony with brown centre</td>
<td>283</td>
<td>Pleosporales DQ182.451 (96%/277 bases called)</td>
<td>DQ679486</td>
<td>Pleosporales-like filamentous fungus</td>
</tr>
</tbody>
</table>