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SHORT COMMUNICATION

Laboratory Diagnosis and Characterization of Fungal Disease in Patients with Cystic Fibrosis (CF): A Survey of Current UK Practice in a Cohort of Clinical Microbiology Laboratories

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Abstract There is much uncertainty as to how fungal disease is diagnosed and characterized in patients with cystic fibrosis (CF). A 19-question anonymous electronic questionnaire was developed and distributed to ascertain current practice in clinical microbiology laboratories providing a fungal laboratory service to CF centres in the UK. Analyses of responses identified the following: (1) current UK laboratory practice, in general, follows the current guidelines, but the scope and diversity of what is currently being delivered by laboratories far exceeds what is detailed in the guidelines; (2) there is a lack of standardization of fungal tests amongst laboratories, outside of the current guidelines; (3) both the UK CF Trust Laboratory Standards for Processing Microbiological Samples from People with Cystic Fibrosis and the US Cumulative Techniques and Procedures in Clinical Microbiology (Cumitech) Guidelines 43 Cystic Fibrosis Microbiology need to be updated to reflect both new methodological innovations, as well as better knowledge of fungal disease pathophysiology in CF; (4) there is a need for clinical medicine to decide upon a stratification strategy for the provision of new fungal assays that will add value to the physician in the optimal management of CF patients; (5) there is also a need to rationale what assays should be performed at local laboratory level and those which are best served at National Mycology Reference Laboratory level; and (6) further research is required in developing laboratory assays, which will help ascertain the clinical importance of 'old' fungal pathogens, as well as 'emerging' fungal pathogens.

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Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which is located on the long arm of human chromosome 7 [1]. All disease-causing mutations in the *CFTR* gene prevent the chloride ion channel from functioning properly, leading to a blockage of the movement of salt and water into and out of cells. As a result of this blockage, cells that line the passageways of the lungs, pancreas, and other organs produce abnormally thick, sticky mucus which obstructs the airways and glands, causing the characteristic signs and symptoms of CF. The most life-threatening complications, in CF patients, are pulmonary inflammation and infection, from bacteria and fungi becoming trapped in the thick, tenacious secretions in the airways, resulting in a vicious cycle of infection and inflammation. Chronic lung infection is responsible for more than 90% of deaths in adults with CF [2].

Although the predominant infections associated with the CF lung are associated with bacteria, yeasts and filamentous fungi are frequently recovered from respiratory specimens from these patients, especially with the increased use of *B. cenocepacia* selective agars, which support fungal detection. These bacterial selective media contain high concentrations of several antibiotics, enhancing growth of highly antibiotic-resistant bacterial species, including *B. cenocepacia*, which will inadvertently support the growth of fungi. However, it is important that laboratories do not rely solely on these for the isolation of fungi, but instead employ fungal selective agar to attempt the isolation of these eukaryotes.

There is a growing awareness amongst CF physicians of the importance of fungal infections in patients with CF, largely due to: (1) the increasing incidence of fungal disease in CF patients; (2) emerging novel mechanisms of fungal pathophysiological disease, for example *Aspergillus* bronchitis; (3) the limited number of effective therapies that are available or their association with dose-limiting toxicities; (4) the fact that fewer symptoms bring the infection to the attention of the patient and physician early on; and (5) the difficulties to make an early diagnosis because of the lack of sensitive tests for the detection of invasive fungal infections [3]. Culturing *Aspergillus fumigatus,* per se, is not an indication for treatment, but this fungus has a wide range of clinical presentations and, when combined with the presence of co-habiting bacterial pathogens, such as *Pseudomonas aeruginosa*, makes it more difficult to attribute clinical significance to the presence of the fungus. Reliable laboratory detection of fungi is thus the cornerstone of subsequent clinical considerations.

Currently, it is difficult to estimate the prevalence of fungal infections in patients with CF. This is due mainly to a lack of monitoring of the presence of fungi in microbiological cultures of sputum, in most CF registries/databases in the UK and Europe. The US Cystic Fibrosis Foundation (CFF) Registry data, however, indicate an approximate doubling of prevalence of fungi being detected in the sputum of patients with CF, from 1995 to 2005, rising from approximately 6% in 1995 to approximately 13% in 2005. Such a rise as this could be the cumulative effect of the ad hoc introduction of novel and improved laboratory methods, as well as greater awareness of fungi disease in CF. In the absence of such data, the clinical significance of fungi is indicated mainly through reports in the scientific/medical literature. To date, the majority of reports have included clinically significant fungi such as *Aspergillus* spp., *Scedosporium* species, and *Exophilia dermatitidis* [3].

Evolving laboratory technology and methodologies aids in the isolation, identification, and characterization of aetiological agents of fungal disease in CF patients. Coupled with this, various laboratory guidelines exist to guide Clinical Microbiology Laboratories, in the employment of suitable techniques to employ, to support laboratory workup of respiratory specimens, and to guide clinicians in patients' management. It was therefore the aim of this study to examine how a cohort of NHS Clinical Microbiology Service Laboratories, supporting CF centres in the UK, were performing CF fungal diagnosis.

A 19-question anonymous electronic questionnaire was developed and posted on the SurveyGizmo platform for completion. This questionnaire is also available at the following link: www.surveygizmo.eu/s3/90008906/Fungal-Laboratory-Questionnaire-for-Cystic-Fibrosis.

The questionnaire was designed in four sections, exploring NHS service laboratory aspects of: (1)

fungal isolation; (2) fungal identification; (3) fungal characterization; and (4) promoting best practice. The questionnaire was distributed amongst NHS Consultant Respiratory Physicians and NHS Consultant Paediatricians involved in the clinical care of patients with CF at recognized UK CF centres. A request was made to 94 paediatric and adult CF consultants in the UK to forward the questionnaire onto their Consultant Microbiologist, who supports the CF centre, in terms of NHS Microbiology Laboratory Service provision. Questionnaires were duly completed and returned via the SurveyGizmo platform for analyses.

Responses to the questionnaire were received from 11 publically funded NHS Clinical Microbiology laboratories in the UK, supporting either a CF Adult Service or a CF Paediatric Service. Collated responses to the specific questions asked are shown in Table 1.

The goal of any clinical microbiology laboratory supporting the routine processing of sputum and other respiratory specimens from CF patients is to provide a robust and effective service, in a timely and cost-effective manner. Any assays that are performed need to add clinical value and aid the physician in the clinical decision-making process. Driving forces, namely the development of novel techniques of isolation and characterization of fungi and new insights into the pathophysiology of fungal disease in CF patients, make the methodological techniques to be in a constant state of evolution, thus requiring periodic rationalization to ensure NHS routine service fungal assays are keeping pace with methodological innovation, as well as emerging knowledge on disease driving what assays to optimally employ.

Currently, there are at least two laboratory standards in the UK and USA, respectively, namely the UK CF Trust Laboratory Standards for Processing Microbiological Samples from People with Cystic Fibrosis [4] and the US Cumulative Techniques and Procedures in Clinical Microbiology (Cumitech) Guidelines 43 Cystic Fibrosis Microbiology [5]. Many countries may have their own national standards in place. A comparison of these standards is shown in Table 2. When we compare the findings of this questionnaire, against these current guidelines for the laboratory processing of sputum for fungi from CF patients, responses to this questionnaire were generally within compliance of these guidelines. This study indicated that many laboratories are currently performing several more assays that are presently listed in the laboratory guidelines, with a high degree of non-standardization in assays not defined in the guidelines. Our questionnaire showed that most laboratories are employing basic fungal detection media, mainly SDA with or without antibiotics. Most laboratories are cautious about employing enhanced specific fungal culture media, such as DRBC-benomyl, Sce-Sel+ or Scedo-Select III, to aid with the isolation of fungi from CF sputum; none were using these recently described media [6].

Whilst this study received responses from 11 NHS Clinical Microbiology laboratories, this should not be interpreted as being fully reflective of practice in all NHS laboratories. However, we believe that the responses received from these 11 laboratories are a microcosm of UK laboratory practice and a reasonable reflection of what UK laboratories are currently doing.

The biggest challenge resulting from analysis of this questionnaire is the lack of standardization of methods across these laboratories. Previously, Borman et al. [7] investigated the consequences of the lack of standardization of fungal methodologies across eight laboratories and concluded that without more sophisticated molecular methods, the aetiological role of 'rarer' filamentous fungi in pulmonary exacerbations will remain hidden.

Most recently, the February 2018 issue of *Mycopathologia* (Volume 183; Issue 1) contains 25 articles which are highly relevant to this area. In particular, the paper by Chen et al. [8] discusses the challenges in laboratory detection of fungal pathogens in the airways of CF patients. In this article, the authors highlight and discuss in detail the repertoire of available mycological laboratory techniques (cultural and molecular methods) to support accurate isolation, identification, and characterization of fungal organisms from CF respiratory specimens and conclude that guidelines for standardized processing of respiratory specimens are urgently needed. Following on from this call for urgent standardization of methods, the paper by Coron et al. [9] takes on this challenge to standardize cultural/isolation methods with the 'MucoFong' programme, whereby sputa from 243 CF patients from seven CF centres in France were studied over a 15-month period. Six fungal culture media were compared, and the study concluded that four of these media, namely CHROMAgar *Candida* medium incubated at 37 °C, Sabouraud dextrose agar with chloramphenicol and gentamicin at 25 °C, Sabouraud

Table 1 Responses to CF laboratory fungal questionnaire from 11 UK NHS microbiology service laboratories

Survey question	Survey response
Fungal isolation	
1. Under which of the following circumstances does your laboratory attempt to isolate fungi from CF patients:	Eleven (91.7%) respondents answered on all submitted specimens and one respondent added 'any specimen except cough swabs'
On all submitted specimens	
Only on request from the submitting clinician	
Other (e.g. high-risk patients/post-transplant)	
2. Do you routinely use a selective medium for isolation of fungi from CF specimens?	Ten respondents (91%) routinely use the fungal selective medium, Sabouraud (SAB), for isolation of fungi from CF specimens. There was wide variation in the incubation time and temperature employed, including:
	SAB for 5 days
	SAB at 30 and 37 °C
	SAB for 2 days at 37 °C + 5 days at 30 °C
	SAB + chloramphenicol at 30 °C + 37 °C for 48 h + 5 days
	SAB in CO_2 at 37 °C for 5 days
	SAB + gentamicin + chloramphenicol at 37 °C for 5 days
	SAB at 35–37 °C for 48 h
	No laboratory reported using other CF selective media
3. Does your laboratory employ molecular methods for the detection of fungi from CF specimens? (either in-house or referral outside)	Two (18.2%) laboratories employ molecular methods
4. Does your laboratory employ other methods, (e.g. precipitin testing, galactomannan), different to Q2 and Q3 above?	Eight (72.7%) laboratories employ other methods (e.g. precipitin testing, galactomannan). Four of these would refer to another laboratory for testing, whilst one can do tests as special request, although their *Aspergillus* PCR and susceptibility testing are referred to the Mycology reference Laboratory
Fungal identification	
5. On isolation of a fungus from a CF specimen, which of the following do you attempt to identify? in-house or via a Mycology Reference Laboratory	All of the 11 respondents attempt to identify all filamentous fungi that are recovered, and 3 (27.3%) attempt for yeasts
6. What methods of fungal identification do you employ to achieve this?	Six (54.5%) use in-house and 3 (27.3%) refer to the Mycology Reference Laboratory. The remaining two respondents commented, 'a combination of conventional mycology in-house or reference laboratory for non-*Candida'* and 'some ID in-house via conventional phenotypic methods, others sent to reference laboratory'
	Of those who had answered in-house, three use conventional mycology, one uses conventional and MALDI-TOF mass spectrometry, one uses microscopy, 18S rDNA sequencing and the final comment was, 'phenotypic, then 18S rDNA sequencing if no identification or uncertain identification (then reference laboratory if confirmation required)'
7. What is your laboratory's practice for isolating, identifying and testing antifungal susceptibility on *Candida* spp. and/or other yeasts?	Identification of black yeasts: 7/11 laboratories
	Identification of other yeasts: 2/11 (by MALDI-TOF mass spectrometry)
	Antifungal susceptibility testing:
	Routinely performed on all yeast species: 0/11
	Performed only on non-*albicans* yeast species: 5/11
	Susceptibility testing performed by reference laboratory: 7/11
	For in-house in vitro susceptibility testing: 3/11 use VITEK 2 and 1/11 uses yeast one sensititre plates
Characterization of filamentous fungi	
8 and 9. Do you routinely perform antifungal susceptibility testing on any/all of the fungal isolates obtained from CF specimens	Seven (63.6%) of nine respondents do not routinely perform antifungal susceptibility testing on any/all of the fungal isolates from CF specimens
	Two (18.2%) do, stating 'MIC tested on isolates from BAL samples' and 'Yeasts if treatment clinically indicated'

Table 1 continued

Survey question	Survey response
If so, 10. Which Mycology Reference Laboratory is? Do they charge for this service? How much per specimen? What is their turnaround time?	Six laboratories use the Bristol Reference Laboratory and one uses the Manchester Reference Laboratory. Five reported that they are charged for the service, whilst one comment stated that some tests are not charged. None of the respondents knew the cost per specimen. The turnaround time responses varied from 5 days to 2 weeks, depending on the isolate
11. Which antifungal agents would you like tested? (either in-house or via Mycology Reference Laboratory)	Ten (90.9%) would like voriconazole tested, 9 (81.8%) indicated ambisome and itraconazole, 7 (63.6%) fluconazole and caspofungin, 5 (45.5%) posaconazole, 4 (36.4%) micafungin, 2 (18.2%) anidulafungin and isavuconazole, and one chose abelcet. Additional comments were: 'we test amphotericin not the formulations', and 'it depends on what we are sending and what is available in our formulary'
12. Do you perform any further fungal characterization on the isolates? (e.g. molecular typing)	None of the 11 of the respondents perform any other fungal characterization test (e.g. molecular typing) on the isolates
13. What is your laboratory policy on the storage/preservation of CF fungal isolates?	Two (18.2%) laboratories reported (18.2%) the all fungal isolates were preserved, 3 (27.3%) reported that nothing was preserved and the remainder (6) left additional comments including storage of unusual isolates, storage of new isolates, keeping clinical isolates for a limited time due to storage capacity, keeping by specific request (beads for 2 years) and those that have been referred to the Reference Laboratory
Would you be willing to archive your CF fungal isolates in a CF strain repository for sharing with others?	Nine (90%) laboratories reported they would be willing to archive CF fungal isolates in a CF strain repository for sharing with others. The others responded negatively, citing very limited on-site capacity; however, one responded that they would unwilling to archive on site but happy to send to a central fungal repository
Promoting best practice	
14. Would your laboratory have the capacity to handle additional requests for fungal workup from the CF clinical team?	Seven (63.6%) laboratories reported they would have the capacity to handle additional requests for fungal workup from the CF team, and 2 (18.2%) stated they would not have capacity. Comments from the respondents highlighted that funding would be the main barrier to taking on additional requests
Would you be willing to change your CF fungal workup if presented with 'Best Practice Guidelines'?	All eleven respondents stated they would be willing to change their CF fungal workup if presented with 'Best Practice Guidelines'
	Lack of financial resources was reported as the main issue with changing practice and one comment stated, 'CF samples are expensive and not properly resourced'
19. Would you support the establishment of a National CF Mycology Reference Service/Laboratory? (This could take the form of a 'Virtual Reference Laboratory', with several specialist Mycology laboratories providing individual specific assays under a service-level agreement.)	Seven of the ten respondents said they would support this and one person would not. Comments given were, 'not sure if there is a need for this', 'possibly if supported by evidence' and 'undecided—(current Mycology Reference Lab do a good job and unsure of the need for duplication)'

dextrose agar with chloramphenicol and cycloheximide at 37 °C and erythritol agar at 27 °C, should be employed to optimally recover fungal pathogens from CF respiratory specimens. Initiatives such as the MucoFong programme are extremely valuable as they present an evidence base for laboratories to move forward confidently in the knowledge that they are providing an optimal service for the patients.

The current study highlights the following:

(1) that current UK laboratory practice, in general, follows the current guidelines, but that the scope and diversity of what is currently being delivered by laboratories far exceeds what is detailed in the guidelines;
(2) there is a lack of standardization of fungal tests amongst laboratories, outside of the current guidelines;
(3) as a result, both the UK CF Trust Laboratory Standards for Processing Microbiological Samples from People with Cystic Fibrosis [4] and the US Cumulative Techniques and Procedures in Clinical Microbiology (Cumitech) Guidelines 43 Cystic Fibrosis Microbiology [5] need to

Table 2 Comparison of UK CF Trust Laboratory Standards for Processing Microbiological Samples from People with Cystic Fibrosis and the US Cumulative Techniques and Procedures in Clinical Microbiology (Cumitech) Guidelines 43 Cystic Fibrosis Microbiology, for the examination of fungi from patients with CF

CF Trust Consensus Guidelines	US Cumitech Guidelines
Fungal infections have become more prevalent in people with CF in recent years. Infection with *Aspergillus* spp. has long been recognized as a problem in CF, usually presenting as allergic bronchopulmonary aspergillosis. Recently, it has been suggested that *Aspergillus* infection can cause respiratory exacerbations by stimulating a fungal-associated bronchitis that responds to specific antifungal therapies. Other fungi are increasingly recognized as complications of CF care, e.g. *Scedosporium apiospermum* and *Wangiella* (now called *Exophiala*) *dermatitidis*	Routine culture for fungi is not recommended for CF patients, although the organisms may grow on bacterial isolation media. When fungal isolation is attempted, it is recommended that antibacterial agents with antipseudomonal activity be incorporated into the medium
Sabouraud medium should be used to enhance the recovery of fungi from respiratory samples of people with CF	Allergic bronchopulmonary Aspergillosis (ABPA) laboratory diagnosis may be aided by: serum immunoglobulin E (IgE) concentration of 500 IU/ml and the presence of IgE antibodies to *A. fumigatus* and/or IgG antibodies to *A. fumigatus*
The addition of appropriate antibiotics reduces contamination rates with *P. aeruginosa*	*Aspergillus* spp. should be isolated on fungal media containing gentamicin at 30 °C for 3 weeks in ambient air. Identification should adopt conventional identification methods. Susceptibility tests are not applicable
Plates should be incubated at 35–37 °C in air and examined after overnight incubation and after at least another 24 h. Prolonging cultures up to 7 days and at different temperatures (e.g. 22 °C) may increase yield	The major fungal pathogen in CF patients is *Aspergillus*. It can grow on several of the selective bacterial media. *Aspergillus* can chronically infect or colonize CF patients. When *Aspergillus* is first detected in a bacterial culture, it should be identified to the species level and reported. After the initial isolation, the frequency of identification and reporting should be based on clinician expectations and needs. When fungal cultures are specifically requested for CF patients, selective fungal media are required because of the potential for bacterial over-growth, especially with *P. aeruginosa*. Selective fungal media containing gentamicin, amikacin, or ciprofloxacin should be used because of their activity against strains of *P. aeruginosa* and because media containing these antimicrobials will enhance the recovery of moulds from CF respiratory specimens. Although *Candida* species are frequently recovered from CF respiratory specimens, there is no evidence that these organisms play a role in chronic CF lung disease. Therefore, *Candida* should not be reported for respiratory cultures from CF patients
Correct identification of *Aspergillus* species is important as some are resistant to amphotericin, e.g. *A. versicolor, A. nidulans, A. lentulus*. Itraconazole-resistant *Aspergillus fumigatus* has been described, and multiple triazole resistance has also been reported. If a patient has had prior treatment with azoles, susceptibility testing may therefore be warranted	
In people with CF, the repeated isolation of *Aspergillus* spp., in spite of long-term treatment with antifungal drugs (e.g. itraconazole or voriconazole as steroid sparing treatment for ABPA), may indicate the need for referral of isolates to a reference laboratory for susceptibility testing	

be updated to reflect both new methodological innovations, as well as better knowledge of fungal disease pathophysiology in CF;

(4) there is a need for clinical medicine to decide upon a stratification strategy for the provision of new fungal assays with added value to guide physicians for an optimal management of CF patients;

(5) there is a need to rationalize what assays may be performed at local laboratory level and those which are best served at National Mycology Reference Laboratory level and;

(6) further research is required in developing laboratory assays, which will help ascertain the clinical importance of 'old' fungal pathogens, as well as 'emerging' fungal pathogens.

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Compliance with Ethical Standards

Human and Animal Rights No human or animal subjects were involved with this publication which as such did not require ethical permission to be undertaken.

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