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Challenges in laboratory detection of fungal pathogens in the airways of cystic fibrosis patients

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ABSTRACT

Study of the clinical significance of fungal colonization/infection in the airways of cystic fibrosis (CF) patients, especially by filamentous fungi, is challenged by the absence of standardized methodology for the detection and identification of an ever-broadening range of fungal pathogens. Culture-based methods remain the cornerstone diagnostic approaches but current methods used in many clinical laboratories are insensitive and unstandardized, rendering comparative studies unfeasible. Guidelines for standardized processing of respiratory specimens and for their culture are urgently needed and should include recommendations for specific processing procedures, inoculum density, culture media, incubation temperature and duration of culture. Molecular techniques to detect fungi directly from clinical specimens include panfungal PCR assays, multiplex or pathogen-directed assays, real-time PCR, isothermal methods and probe based assays. In general, these are used to complement culture. Fungal identification by DNA sequencing methods is often required to identify cultured isolates, but matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is increasingly used as an alternative to DNA sequencing. Genotyping of isolates is undertaken to investigate relatedness between isolates, pinpoint the infection source, and to study the population structure. Methods range from PCR-fingerprinting and amplified fragment-length polymorphism (AFLP) analysis, short tandem repeat typing, multilocus sequencing typing (MLST) and whole genome sequencing (WGS). MLST is the current preferred method whilst WGS offers best case resolution but currently is understudied.

INTRODUCTION

Chronic airway infections cause major morbidity in cystic fibrosis (CF) [1, 2]. Many studies have defined the role of bacteria particularly of *Pseudomonas aeruginosa*, in acute pulmonary exacerbation events, decline in FEV_1 (forced expiratory volume in 1 second) and lung function decline [3-5]. Filamentous fungi and *Candida* spp. are also increasingly isolated from the airways of CF patients and recognized as potential pathogens [6, 7]. Unlike bacteria, however, a causal relationship between fungal colonization or infection and progressive lung disease has not been established, and uncertainty surrounds the longer term effects of fungal colonization on lung function. There are few data examining the propensity of individual strains to initiate or sustain colonization, or to cause invasive disease.

A number of factors have resulted in this gap in knowledge of the clinical impact of fungal colonization, compared with their bacterial counterparts. One of these is the difficulty in defining disease as opposed to fungal colonization (often interpreted as infection) in CF. The European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) definitions [8] focus on hematology-oncology patients and do not take into account the contribution of airway inflammation in lungs already damaged by CF, which act as a nidus for trapping fungi. Secondly, there are few standardized methods, culture-based or otherwise, to detect fungi in the respiratory tract that have been validated for routine use, hence the epidemiology of fungal colonization is largely non-comparable between studies.

This article will discuss the challenges associated with the detection and identification of fungi in respiratory tract specimens from CF patients. It will review culture- and molecular-based methodologies, and the relevance of genotyping studies in understanding fungal colonization in CF. Serological diagnosis for detection of an infection or sensitization to fungi however is not discussed.

FUNGAL COLONIZATION: AETIOLOGY AND SIGNIFICANCE

Colonization of CF airways with *Aspergillus, Scedosporium and Exophiala* spp. amongst other fungi is increasing [6, 7, 9]. *Aspergillus fumigatus* is the most common fungus (> 50%) and is associated with allergic bronchopulmonary aspergillosis (ABPA) in ≈ 10% cases, yet the clinical relevance of *A. fumigatus* in the absence of ABPA is unclear [6, 7]. Other *Aspergillus* species, *e.g. Aspergillus terreus,* also colonize the airways. A 2-year study in the United Kingdom [10] and a Dutch survey over 5 years [11] noted that *A. fumigatus* colonization was not associated with more severe lung function decline; however, a longitudinal Canadian study [12] and a 12-year French study [13] reported the reverse. The impact of colonization by *Candida* spp. on lung function decline has also been contradictory [14] underscoring the need for standardized study methodologies.

Also intriguing is the relative high frequency of *Scedosporium* spp., which rank second amongst CF-associated fungi (8.5-17.4% prevalence); the intrinsic pathogenicity of the major species, *Lomentospora* (formerly *Scedosporium*) *prolificans*, *Scedosporium aurantiacum* and *Scedosporium apiospermum* is recognized [6, 7, 11, 15, 16]. Risk factors for airway colonization /infection by *Scedosporium* and their impact on lung function are not yet defined, although one study found no association between colonization and more severe reduction in FEV_1 [16]. Colonization can be transient or persistent, and clinical disease is uncommon, ranging from allergic bronchopulmonary mycosis, fungus ball formation to disseminated infection [17-19]. Since colonization poses a risk for infection post lung transplantation with high mortality (> 50%) [6, 20], some centers are reluctant to offer transplantation in this setting. The *Scedosporium* species distribution in CF also differs with geographic region, with *S. apiospermum* being the most common species globally. However, in an Australian study using culture methods selective for non-*Aspergillus* fungi, *S. aurantiacum*, *L. prolificans* and *S. apiospermum*/*S. boydii* (formerly *Pseudallescheria*

boydii), were isolated in equal frequencies [7]. Species identification is necessary for epidemiological studies and because there are species-specific differences in antifungal susceptibility [21].

Data describing the clinical epidemiology of other non-*Aspergillus* fungi in CF, including novel dematiaceous fungi, *e.g. Acrophialophora fusispora,* are likewise emerging [6, 22-24]. Blyth *et al*. [7] using culture methods showed their collective prevalence to be 3.2-8.3%. Kondori *et al.* [22] employed different selective media and identified the black fungus, *Exophiala* in 19% of CF respiratory samples in Sweden, whilst the prevalence of these fungi in a study from Belgium was lower (5.8%) [23]. In France, the detection of *Geosmithia* species as potential pathogens is notable [24]. These findings and those of others remind us that the range of fungal pathogens and their relative prevalence not only varies widely with geography but are influenced by the lack of standardized methods for mycological examination of CF sputum samples, whether culture-based or using molecular approaches [25]. Studies exploring the pathogenic potential of these fungi hinge on good study design and reliance on reproducible methods to recover fungi.

CULTURE-BASED DETECTION METHODS

Culture-based methods remain the standard and key approach for identifying fungi from CF airways (Figure 1). Somewhat surprisingly, whilst mycology laboratory accreditation programs are common, most countries lack guidelines for processing respiratory samples. In countries such as the UK where national guidelines do exist, these have been shown to be insensitive for detecting medically important fungi [26, 27]. In CF studies, a number of alternative methods have been routinely employed and a multi-center study looking at prevalence of fungi using different culture protocols has highlighted the need for a standardized approach [25]. There are a number of variables that can affect the likelihood of

recovering fungi from a respiratory specimen; these include; media, quantity of inoculum, prior processing, incubation temperature, length of observation, and type of clinical sample.

Standard bacteriological media have been demonstrated to be inferior for detecting fungi compared to fungal media [28, 29], however, differences can be detected in fungal recovery rates even between standard fungal growth media, such as Sabouraud dextrose agar (SDA) and Potato dextrose agar (PDA) [27]. Some laboratories now incorporate selective media in their protocols to encourage growth of specific fungi, such as erythritol-chloamphenicol agar (ECA) for *Exophiala dermatitidis* [30] and SceSel+ medium for detecting *Scedosporium* in CF patient samples [7]. The quantity of inoculum added to culture plates has a direct effect on the likelihood of recovering fungi [26, 27]. A large comparative survey of CF centers and mycological laboratories in the UK and France revealed that, whilst all laboratories treated sputum samples with an equal volume of a mucolytic agent, the volumes cultured ranged from 0.001 - 500 µl, with some laboratories concentrating the sputum before culture [25]. One study to directly compare the effect of different sputum processing methods on the recovery of fungi in CF initially compared undiluted sputum to homogenized sputum, and whilst 7% of their fungal positive samples were only positive with undiluted sputum, overall they found that homogenizing of sputum resulted in a greater number of fungal-positive patients, a higher diversity of fungal isolates and a higher number of fungal colonies. They then extended their study to include serial dilutions of the homogenized sputa and additional selective media, and found a greater diversity of fungal species could be detected [31].

Incubation temperature and the length of time a culture is observed for will also affect the likelihood of recovering fungi. The comparative CF study revealed that incubation temperatures ranged from 25 to 37°C and incubation times ranged from 7 days to 6 weeks [25]. Although no recommendations as to optimum temperature or incubation time were

given, the use of selective media and prolonged incubation times may be preferred for the detection of some organisms such as *E. dermatitidis* although larger studies are required to confirm this observation.

Expectorated sputum samples are frequently used for microbiological investigations of respiratory infections. Induced sputum (IS) has the advantage of higher density of cell recovery and is less invasive than bronchoalveolar lavage (BAL) [32], whereas BAL fluid specimens have the advantage of avoiding contamination with flora of the oropharynx. Fungal recovery rates can vary depending on the respiratory sample analyzed. A study comparing *A. fumigatus* recovery rates using a high volume undiluted culture method found only 20% of BAL samples were culture positive, compared to 44% of sputum samples and 75% of bronchial aspirates [26]. The bronchial aspirate represented the sample collected during bronchoscopy after initial entry into the main bronchi, and the BAL samples were washes with 0.9% saline. Higher culture rates from sputum and bronchial aspirates could suggest samples from the upper airways contain more *Aspergillus* than distal samples, although sample dilution may be a factor [26].

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)-based identification of fungi is increasingly used to complement, or substitute for traditional phenotypic fungal identification [33-36]. However, its wider application is limited by the lack of quality controlled reference fungal spectral libraries in commercial databases to compare spectra from unknown fungi and, necessitating the construction of sufficiently robust in-house reference spectra [33, 34]. Finally, Laiger *et al*. have coined the term "culturomics" to describe a novel high-throughput culture method [37, 38] for examining gut microorganisms. However, to date there have been no studies that have used this approach to study fungi in the lung in CF.

In summary, it is clear that different approaches used to process respiratory samples and culture fungi impact significantly on rates of fungal detection and in identifying species diversity, and without standardization, comparisons between studies are hampered. This is further complicated by clinical studies that send respiratory samples to external microbiology laboratories and do not state in the methods section how the samples were processed or what culture media and incubation conditions were used.

MOLECULAR APPROACHES TO FUNGAL DETECTION

Given the limitations of histopathology and morphological methods (as above) to provide timely diagnosis and indeed, to provide species identification of many of the fungi encountered in CF, numerous molecular techniques have been developed to assist fungal detection (Figure 1). Polymerase chain reaction (PCR)-based methods have grown in popularity to rapidly detect fungi directly from clinical specimens and to identify cultured isolates. The myriad of the assays developed to identify cultured fungal isolates based on DNA sequencing is detailed in recent reviews [39-41]. Here we focus on the techniques used to detect fungi directly from respiratory tract specimens including the use of panfungal PCR assays, multiplex or pathogen-directed assays, and technologies using real-time PCR (RT-PCR), isothermal methods and probe based assays.

Panfungal PCR

Multiple panfungal PCR assays are published [42-44]. These are mainly directed at amplifying parts or the whole fungal internal transcribed spacer (ITS) regions (ITS1, ITS2 or both), the current universal fungal DNA barcode [45] followed by DNA sequencing and comparison of the obtained DNA sequences against a quality controlled database, for example the ISHAM–ITS database via http://www.isham.org, http://its.mycologylab.org or

Ref-Seq at GenBank at http://www.ncbi.nlm.nih.gov/refseq/ [46, 47]. Performance is best when tested on tissue or sterile site specimens [42, 44] and the assay mostly, complements culture. The clinical utility, however, when applied to non-sterile samples including BAL fluid and sputum is limited due to amplification of commensal fungi eg. *Candida* species. In the context of CF, where often more than one fungus is colonizing the airways, the assay is also limited by inability to detect mixed infections although the predominant species may be detected. The recent developments in next generation sequencing (NGS) technologies [48] and their applications in airway metagenomic studies [49] open up a visible solution to this problem. The choice of PCR target may further be problematic; although many fungi can be identified by ITS-directed panfungal assays, others including certain dematiaceous fungi, *Aspergillus* and *Scedosporium* species require analysis of other loci such as the D1/D2 28S rRNA and the *β*-tubulin gene for accurate detection [50-52]. For these reasons, panfungal PCR in its simplest form is not widely used to directly detect fungi in respiratory tract specimens.

Multiplex PCR and organism-specific PCR assays

Multiplex PCR involves the amplification by PCR of two or more loci simultaneously in one reaction using species-specific primers based on target sequence data. There are few reports with regard to the use multiplex PCR to detect fungi in CF airways. Nonetheless, Harun *et al*. [53] developed an ITS/28S rDNA-directed assay to detect three species groups of *Scedosporium* directly from sputum specimens (*S. aurantiacum*, *L. prolificans* and *S. apiospermum*/*S. boydii*). The PCR assay was tested on a small number (n = 29) of sputum samples from CF patients (n = 11), which were known to contain *Scedosporium* spp. by culture. The multiplex PCR assay was positive for 18/29 (62%) samples or 7/11 (63.6%) patients with overall specificity of 97.2%, PPV 78.3% and NPV 94.1%. Competition between primers for the target sequences and reagents requires strict primer design to avoid false

positive and false negative results. False negative results may also result from insufficient target DNA and PCR inhibition [54]. However, this simple technique can be tailored to complement culture where a particular fungus is clinically suspected to be present.

A number of RT-PCR assays for specific pathogens have also been published for fungal detection in CF but their wider application requires further study. Castelli *et al*. [55] evaluated two separate ITS-directed RT-PCR assays to detect *L. prolificans* and *S. apiospermum* in mice. A total of 141 blood, serum and lung samples were analyzed. Assay sensitivity for both assays was > 95.5% for lung, > 81% for serum and least 54% for blood; this assay has not yet been applied in the routine diagnostic setting. Recently, Steinmann *et al*. [56] validated a commercially available real-time PCR assay (Primerdesign^{TM}, UK) for detection of the *Rasamsonia argillacea* complex from CF respiratory samples, targeting the calmodulin gene. In this study, the authors compared the PCR with the culture of 234 respiratory samples. PCR reliably detected the three main pathogenic species, *R. argillacea, R. piperina* and *R. aegroticola*. Four patients studied (2.6%) were colonized with *R. argillacaea* species complex. Three of the four were culture positive [56] and the novel RT-PCR was found to be sensitive and specific for these fungi. In a study examining the utility of an *Exophiala*-specific PCR assay to detect/identify *E. dermatiditis* from fungal culture [22] where this species was found to be a frequent colonizer in CF patients (18/97; 19%) in Sweden. Further, an *Aspergillus*-specific PCR assay has been employed by Baxter *et al*. [57] to detect *Aspergillus* in sputum with greater sensitivity (74%) compared with culture (37%) as well as with *Aspergillus* galactomannan (46%), assisting with classification of *Aspergillus*–related syndromes in CF.

A number of commercial assays are available to detect and identify *Aspergillus* species. The MycAssay (Myconostica, Manchester, UK) targets the 18S rDNA gene for the detection of 15 different species including *A. fumigatus*, *Aspergillus flavus*, *Aspergillus*

terreus and *Aspergillus niger* [58]. The new AsperGenius^{R} multiplex assay (PathNostics, Maastricht, the Netherlands) has allowed for sensitive detection of a number of pathogenic *Aspergillus* species directly from BAL fluid and includes the detection of a number of genes associated with azole resistance [59]. However, at the time of writing, the assay has not been evaluated using sputum specimens from CF patients.

Oligonucleotide arrays

Also employing a broad-range fungal detection approach, but unlike the typical "panfungal" PCR assay, at least one oligonucleotide array platform has been reported to have high sensitivity (almost 100%) for the direct detection of fungi in CF sputum [60]. The principle of using microarray formats is to provide species identification based on incorporation of species-specific probes. Bouchara *et al*. [60] constructed such an array by incorporating ITS-targeted species-specific probes to detect 20 fungal species of *Aspergillus, Candida, Scedosporium* and melanized fungi. The array was evaluated in comparison with culture on 57 sputum samples from 39 CF patients. For 16 specimens, the results of the microarray corresponded with those of culture, while for 33 sputa, the array detected more fungal species than culture, and the reverse found for eight. The advantage of oligonucleotide arrays is that multiple fungal species may be simultaneously identified. Drawbacks are the high acquisition costs and need for batch testing for efficiency.

Other molecular assays

An increasing number of publications have focused on accurate identification of various fungi in CF airways, encompassing rolling circle amplification (RCA), restriction fragment length polymorphism (RFLP) analysis, semi-automated repetitive sequence-based PCR (rep-PCR). Although not yet applied routinely, many of these techniques take into account the

continuing re-organization of fungal taxonomy of fungal species recovered from CF lungs and are important in informing future clinical applications.

Delhaes *et al*. [61] Lackner *et al*. [62] and applied PCR-RFLP to separate the major *Scedosporium* species with good accuracy but the requirement for a large number of restriction enzymes renders the technique time-ineffective for a busy laboratory. RCA is a simple and robust alternative method using padlock-probe technology and isothermal amplification to detect and differentiate between closely-related species. Two ITS-directed assays have been successfully applied to identify *Aspergillus* and *Scedosporium* species [63, 64] and a semi-automated repetitive sequence-based PCR using the DiversiLab mold Kit (bioMérieux, Marcy L'Etoile, France) for separating species within the *S. apiospermum* species complex, *S. boydii* and *Lomentospora prolificans* [65, 66], as well as for species identification and strain differentiation within the *R. argillacea* complex (see also below) [67].

Finally, with regards to molecular approaches for fungal detection, a microbiome, proteome, or metabolome perspective paves the ways for the future. This is comprehensively detailed in the article by Botterel *et al*., in this same issue of *Mycopathologia.*

FUNGAL GENOTYPING IN THE SETTING OF CYSTIC FIBROSIS

Fungal genotyping to compare fungal strains to determine their genetic relatedness has applications in both population genetic studies and in the tracking of isolates for example in suspected case clusters. Many protein- and molecular-based methods have been applied and range from species-specific methods, such as short Tandem Repeat (STR) typing and multilocus sequencing typing (MLST) to methods that encompass multiple species such as multilocus enzyme electrophoresis (MLEE), random amplification of polymorphic DNA (RAPD), PCR-fingerprinting, amplified fragment-length polymorphism (AFLP), and in the

future, whole genome sequencing (WGS) single nucleotide polymorphism (SNP) analysis.

In CF, genotyping may be applied to determine transmission patterns of fungal infection both from environmental sources to patients and amongst patients in a clinical setting, as well as in studies of persistence of fungal strains and population dynamics of fungi. This allows for the determination of relationships between environmental and clinical isolates, and importantly, the detection of transient or persistent colonizing fungal strains, or re-colonization/re-infection with a different fungal strain, and identification and monitoring of the progression from colonization to infection within a given patient. Finally genotyping can help to determine if fungal colonization and/or infection in a patient is due to a single or multiple strains. The discriminatory power of determining relatedness differs between methods, with the most discriminatory method being WGS-SNP analysis, followed by STR typing, MLST, AFLP/PCR-fingerprinting/variable number of short tandem repeats (VNTR) and inter-simple-sequence repeat PCR (ISSR-PCR)/RAPD and multilocus enzyme electrophoresis (MLEE) (Figure 1).

Multilocus enzyme electrophoresis (MLEE)

MLEE evaluates polymorphisms in housekeeping enzymes of fungi. Enzymes are extracted and electrophoretically separated on a polyacrylamide gel, identifying isotypes of the enzymes investigated. The isotypes are the result of homologous amino acid substitutes, which are transferred from parental strains, allowing for inference of phylogenetic relationships between strains. MLEE was used early on to genotype *S. apiospermum* strains from CF patients analyzing 14 enzyme patterns, corresponding to 27 polymorphic sites and 43 iso-enzymes. This study found that carboxyl esterase, superoxide dismutase and malate dehydrogenase were the most polymorphic enzymes allowing the separation of 17 strains into 6-11 MLEE groups [68]. However, MLEE does not directly examine the genetic basis of strains, and as such, changes due to nucleotide substitutions, which do not lead to amino acid

changes will be missed, resulting in homologous enzyme patterns for genetically different strains. This technology is also laborious, and at least 10 enzymes need to be studied.

Random amplification of polymorphic DNA (RAPD)

RAPD uses arbitrary short primers to analyze randomly amplified DNA fragments. As no prior knowledge of the underlying sequence of a studied fungal species is needed, it is widely used for strain typing, to detect sequence polymorphisms to differentiate between strains. RAPD has been used to investigate the population structure of *S. apiospermum* in CF patients, initially employing 20 primers. None of these when used alone could separate all studied strains. Most discriminatory primers were the primers GC70, UBC-701 and UBC-703, but only when used in combination [68]. When applied to 129 sequential and multiple isolates from nine CF patients in three different French hospitals, no common genotype between patients was found. Five of the nine patients were colonized by a single isolate type and the others by a predominant genotype accompanied by one or two other genotypes, which were closely related to the predominant genotype reinforcing the theory of possible microevolution [69]. RAPD using the primers GC70 and GC80, as well as the microsatellite specific primer M13 has also been shown to indicate the presence (or not) of relatedness of two *S. apiospermum* infections suspected to be nosocomially linked but finally refuted [70]. In another study, RAPD analysis using the primers UBC 701 and UBC 703 demonstrated genetic identity between isolates collected from the sputum of one CF patient, chronically colonized by *S. apiospermum,* before lung transplantation and from clinical specimens including vitreal fluid in fatal disseminated infection following transplantation [71]. More recently, RAPD analysis using the primers OPA-10, OPA-11 and OPBG-01 showed that disseminated *Rasamsonia argillacea sensu lato* infection following lung transplantation for CF was the result of recrudescent reinfection from a previously colonizing strain [72]. Methodological disadvantages with RAPD are the absence of standardization and low inter-

laboratory reproducibility limiting its wider use.

PCR-fingerprinting and inter-simple-sequence repeat PCR (ISSP-PCR)

PCR fingerprinting and ISSP-PC involve the amplification of DNA sequences located between hypervariable DNA repeats, either mini- (10-100 repeats) or microsatellite (1-10 repeats) repeats. PCR-fingerprinting uses either a single primer specific to a minisatellite specific core sequence of the wild-type phage M13 (5'-GAGGGTGGCGGCGGTTCT-3') or the microsatellite specific primers $(GTG)_5$ and $(GACA)_4$. The technique has been successfully applied to investigate suspected outbreaks of *L. prolificans* [73], to demonstrate genetic identity of serial isolates of *S. boydii* [74], and in a large study of 146 clinical Australian *Scedosporium* isolates to demonstrate that the highest genetic variability was present within *S. apiospermum* (58%), followed by *L. prolificans* (45%) and *S. aurantiacum* (28%) [61]. ISSR-PCR showed that 84 clinical and environmental *L. prolificans* isolates were divided into 35 genotypes and clearly separated US isolates from Spanish and Australian strains [75]. Both PCR-fingerprinting and ISSR-PCR have suboptimal inter-laboratory reproducibility due to varying experimental conditions precluding their use in controlled multi-laboratory studies.

Repetitive sequence-based PCR (rep-PCR)

In rep-PCR, mulitple loci representing repetitive genetic sequences are amplified followed by separation of amplified fragments by capillary electrophoresis. This methodology has been sued to genotype *Scedosporium* as well as *R. argillacea* isolates form CF patients; in both studies, the results showed that patients were colonized by a predominantly single genetic type for both these fungal pathogens [66, 67].

Amplified fragment-length polymorphism (AFLP)

AFLP analysis is based on the random amplification of DNA fragments throughout the fungal genome after digestion with restriction enzymes, using specifically designed primers to amplify only a subset of the generated restriction fragments. The fragments are typically separated on an automated DNA sequencer [76]. AFLP analysis was used to investigate the relatedness of 12 Australian *L. prolificans* strains including from CF patients, which were part of two presumptive nosocomial case clusters. Identical AFLP patterns were only recovered from serial isolates form the same patient, supported the findings of previous PCR-fingerprinting results, which rejected a nosocomial link [61]. Again, drawbacks of this technique are the lack of inter-laboratory reproducibility and the possible risk that detected polymorphisms between strains could be artificial due to possible incomplete restriction enzyme digestion before DNA amplification,

Multiple locus variable number of tandem repeat (VNTR) analysis (MLVA)

The *A. fumigatus* high resolution MLVA typing scheme, referred to as STR*Af*, is based on the amplification of a panel of nine microsatellites or short tandem repeats (STRs). The amplified fragments are then separated on an automated DNA sequencer. A panel of nine loci STR*Af*24, STAR*Af*2B, STR*Af*2C, STR*Af*3A, STR*Af*3B, STR*Af*3C, STR*Af*4A, STR*Af*4B and STR*Af*4C has been applied to investigate 100 presumably unrelated *A. fumigatus* isolates, identifying 11 to 37 alleles for the different markers studied, and resulting in 96 different allele profiles. Only one single sample proved to be a mixture of at least two different isolates [77]. A similar approach has also been applied to type 115 clinical *A. terreus* isolates using a panel of nine loci (STR*At*-2A, STR*At*-2B, STR*At*-2C, STR*At*-3A, STR*At*-3B, STR*At*-3C, STR*At*-4A, STR*At*-4B and STR*At*-4C, identifying 17 genotypes, which corresponded to three distinct colonizing patterns: (i) pattern 1 comprising a single predominant genotype with a

few other incidental genotypes (ii) pattern 2 consisting of two distinct genotypes and pattern 3 comprising multiple genotypes which maybe transiently present [78]. A 12-locu based STR typing scheme has been applied to genotype *A. flavus* and was able to demonstrate hospital-acquired infection in a Tunisian study [79]. STR typing combines high reproducibility with ready exchange of results between laboratories. However, because the method is based on the use of microsatellite flanking regions, which are species specific, the loci and primers have to be specific for the fungal species under investigation.

Multilocus sequence typing (MLST)

MLST analysis is based on the amplification of highly variable partial sequences of a set of 7-10 housekeeping genes followed by sequencing. Each unique sequence per MLST locus is assigned an allele type number, and each unique combination of all MLST loci allele types is assigned a unique sequence type number. Comparative analysis of the assigned sequence types is then used to investigate genetic diversity amongst a particular fungal species to identify potential clonality or study its population genetic structure. MLST is highly reproducible as it is sequence based, uses standardized primers and PCR conditions, enabling unambiguous identification of genotypes via the comparison of the generated sequence/allele/sequence type data with public databases.

Relevant to the study of fungi in CF, MLST schemes have been developed for *S. boydii* and *S. apiospermum*, both using the same set of six genetic loci: actin (*ACT*), calmodulin (*CAL*, exon 3-4), second largest subunit of the RNA polymerase II gene (*RBP2*), β-tubulin (*BT2*, exon 2-4), manganese superoxide dismutase (*SOD2*) and elongation factor 1 alpha (*EF1 α*). Results are compared against a public MLST database at htpp://mlst.mycologylab.org. MLST analysis of *S. apiospermum* and *S. boydii* isolates from CF patients have shown that nearly all patients were colonized by different strains, with a very few harboring more than one strain type [80], which confirmed earlier findings of

RAPD studies [69]. No correlation between genotype and geographic origin was found [80]. Attempts to develop an MLST scheme for *L. prolificans* have to date been unsuccessful, due to the low inherent genetic variability in many regions (Cano J and Meyer W, unpublished data). A MLST scheme for *S. aurantiacum* using six genes, actin (*ACT*), elongation factor-1α (*EF1*α), calmodulin (*CAL*), RNA polymerase subunit II (*RPB2*), manganese superoxide dismutase (*SOD2*), and β-tubulin (*TUB*), has been developed, and is available at http://mlst.mycologylab.org (Harun A, Chen S and Meyer W, unpublished data). The discriminatory power of MLST and the genetic loci analyzed depend on the species of interest, necessitating a specific set of genetic loci for each studied species.

Whole genome sequencing (WGS) - Single nucleotide polymorphism (SNP) analysis

With advance in sequencing technologies, whole genome sequencing is increasingly used for population genetic analysis. WGS-based SNP analysis is based on low coverage re-sequencing of strains of a species, which have already annotated genomes. The generated sequences are then aligned to the annotated reference genomes and SNPs are identified and used for phylogenetic and population genetic analysis, *e.g.* as applied for the fungus *Cryptococcus gattii* [81]. Similar approaches are imaginable to be applied for the characterization of fungal species and strains recovered from CF patients. This will not only enable strain typing but will also offer possibilities to investigate possible links with pathogenicity or antifungal susceptibility, and as such will offer new approaches in the future for evidence-based CF patient care.

Summary

Given the interest in fungal colonization and infection studies in CF, a pragmatic approach to the detection, and identification and further characterization of potential fungal pathogens in

CF airways is required. One such approach is shown in Figure 1, which centers around culture because most clinical laboratories are still only able to offer culture methods for fungal detection. To enable important clinical questions to be answered regarding the significance of fungal colonization in CF, standardized methods are essential to implement across laboratories whether they be culture- or molecular-based. With the use of semi-selective or selective media for fungi, many fungi of interest can be cultivated. Molecular methods of identification however gain more and more in importance for fungal identification and to detect a specific fungus or group of fungi in clinical specimens where culture is not able to provide a result, as they are for the tracking of isolates and for studying genetic relatedness.

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FIGURE LEGEND

Figure 1: Suggested culture-, and molecular-based methods for fungal identification and genotyping in a cystic fibrosis setting. This pragmatic approach highlights the yet still central position of culture methods but also indicates alternative molecular techniques, as well as the range of genotyping methods and their discriminatory power and degree of reproducibility.