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The prevalence of *Aspergillus fumigatus* in early cystic fibrosis disease is underestimated by culture-based diagnostic methods

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ABSTRACT

Aspergillus fumigatus is the most common fungus infecting/colonising people with cystic fibrosis (CF) and can negatively impact clinical status. Diagnostic laboratories rely on culture to detect *A. fumigatus* which is known to be less sensitive than molecular approaches. Therefore, *A. fumigatus* colonisation in the CF population may be underestimated.

Sputum ($n = 60$) from 25 children with CF were collected and *A. fumigatus* was detected using routine culture (CM1), enhanced culture (CM2) and ITS1 qPCR. The prevalence of *A. fumigatus* in this young CF population was 68% by qPCR and only 16% by CM1. CM1, CM2 and qPCR detected *A. fumigatus* in 8%, 22% and 53% of samples, respectively. qPCR had a 94.2% and 77.4% increased odds of detecting *A. fumigatus* over CM1 and CM2, respectively.

Molecular methods proved superior for detecting *A. fumigatus* in CF sputum. *A. fumigatus* is likely more prevalent in early CF disease than is currently reported.

1. Introduction

Patients with cystic fibrosis (CF), a life-shortening inherited disease, suffer from recurrent and chronic pulmonary infections. *Aspergillus fumigatus* is the most common fungus isolated from CF airway specimens and the reported prevalence varies significantly, ranging from 11 to 58% (Valenza et al., 2008; Coughlan et al., 2012; Reece et al., 2017; Engel et al., 2019) depending on the method of detection used and the patient population. The most common manifestation of *A. fumigatus* infection in CF is allergic bronchopulmonary aspergillosis (ABPA) and historically *A. fumigatus* colonisation in the absences of allergic symptoms was considered insignificant. However, chronic *A. fumigatus* colonisation has been associated with significantly lower Forced Expiratory Volume in 1 s (FEV_1) % predicted (Amin et al., 2010) and itraconazole treatment of *A. fumigatus* colonised patients was shown to reduce *A. fumigatus* bioburden in the airways, reduce exacerbations, stabilize lung function and reduce mosaic patterns on CT scans (Coughlan et al., 2012; Chotirmall et al., 2008), which may suggest a pathogenic role for *A. fumigatus* in CF. Numerous some studies have now concluded that CF patients chronically colonised with *A. fumigatus* have a more rapid decline in FEV_1 (Saunders et al., 2016) and in general experience a poorer lung function than non-colonised patients (Noni et al., 2015). Furthermore, *A. fumigatus* co-colonising with bacteria has been shown to cause poorer clinical outcomes in CF patients (Amin et al., 2010). *A. fumigatus* has a negative impact in CF and improved detection may be important in enhancing the clinical outcomes of patients with CF.

Identifying the microorganisms in clinical samples and determining their antibiotic sensitivity are cornerstones of CF care and these are largely achieved using culture-based methods in diagnostic laboratories. Culture-based detection techniques have proven to be broadly useful in providing phenotypic and antimicrobial resistance information, although there are inherent limitations. It has previously been reported that culture methods are not as sensitive as molecular methods such as PCR for detecting microorganisms from CF sputum (Alfaresi and Mahboub, 2017). Culture-based methods exclude the growth of fastidious, rare and unknown microorganisms. In mixed species infections, fast growing organisms can inhibit or obscure slower growing organisms (Schlaberg et al., 2012; Burns and Rolain, 2014). *P. aeruginosa*, the most common bacteria infecting CF patients, in its mucoid form is known to invade agar plates, obscuring the presence of other microorganisms (van Belkum et al., 2000). In particular, pyocyanin produced

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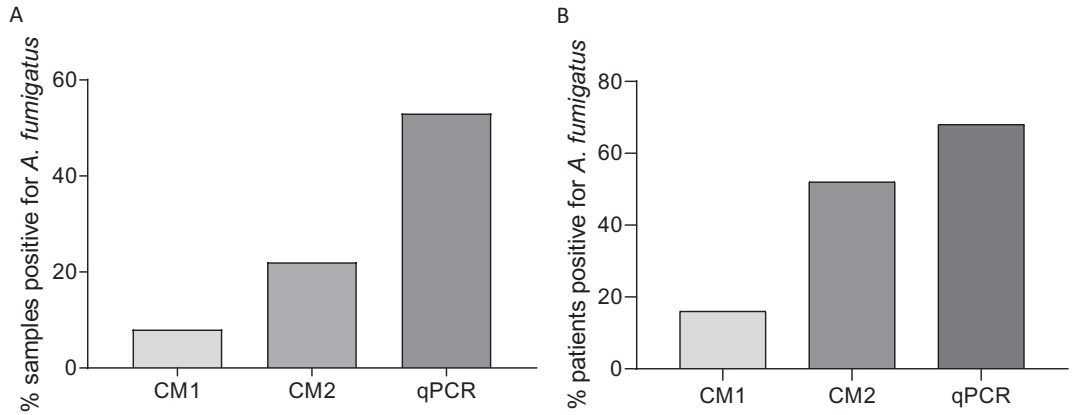

Fig. 1. Percentage of samples and patients positive for *A. fumigatus*. The percentage of CF sputum samples positive for *A. fumigatus* (A) and the percentage of patients positive for *A. fumigatus* (B) as detected by culture method 1 (CM1), culture method 2 (CM2) and qPCR.

by *P. aeruginosa* has been shown to inhibit *A. fumigatus* growth (Kerr et al., 1999). Additionally, culture-based methods have been reported to misidentify important CF pathogens; for example *A. fumigatus* has been misidentified as *Neosartorya pseudofischeri* (Khare et al., 2014), *A. lentulus* and *A. udagawae* (Balajee et al., 2006). This together with the relatively recent discovery of a complex microbiome in the CF airway (Coburn et al., 2015) highlights the need for a paradigm shift in how we approach infection diagnostics in CF.

We hypothesize that routine culture-based diagnostic methods underestimate the prevalence of *A. fumigatus* in children and young adults with CF. We aimed to compare culture and molecular-based methods for the detection and quantification of *A. fumigatus* from sputum of children with CF to determine the true prevalence and bioburden of *A. fumigatus* in our patient population.

2. Methods

2.1. Patient details

Patients were < 20 years of age (ranging 1 to 19 years) and had a confirmed diagnosis of CF. Sputum samples (n = 60) from 25 patients with CF were collected as part of routine diagnostic procedures between October 2014 and March 2015. Ethics for the use of these samples in research was granted by the St James Hospital and the AMNCH joint ethics committee (2006/37/06).

2.2. Culture methods

Samples were homogenised in an equal volume of Sputasol (Oxoid, Hampshire, UK) and incubated at 35 °C for 15 min shaking. ***Culture method 1 (CM1).*** A sterile swab dipped into the homogenised sample was streaked across Potato Dextrose Agar (PDA) (Fannin, Dublin, Ireland) and incubated at 35 °C for 5 days. ***Culture method 2 (CM2).*** A serial dilution (10^0 to 10^{-2}) of each sample was performed in triplicate and 50 µl was plated on Malt extract agar (MEA) (Fannin, Dublin, Ireland). Plates were incubated at 35 °C for 7 days. Colonies were counted and CFU/ml calculated.

2.3. Identification and quantification by qPCR

A. fumigatus was detected and quantified using primers and probes for the internal transcribed spacer 1 (ITS1) region (Walsh et al., 2011). All primers and probes were supplied by Eurofins Genomics, Ebersberg, Germany. The master mix was composed of 10 µl of TaqMan Gene Expression (Biosciences, Dublin, Ireland), 0.4 µM of each primer, 0.16 µM of each hydrolysis probe, and 4 µl of DNA in a final reaction volume of 20 µl. Cycling was performed on QuantStudio™ 5 Real Time PCR System (Applied Biosystems, Dublin, Ireland), with an initial hold at 95 °C for 15 mins, followed by 45 cycles at 95 °C for 15 s, and 60 °C for 1 min, with a C_T of 35 being the threshold for positivity. A negative control consisting of master mix without DNA was included in each qPCR run alongside the negative DNA extraction control. DNA extracted from 10-fold serial dilutions (10^1 to 10^6 CFU/ml) of the reference strain, AF293, was used to generate a standard curve and to act as a positive control. A *ctrA* plasmid was amplified in duplicate on each qPCR run as a reaction control. Each sample was run in duplicate and samples were considered as positive if both duplicates showed Ct values below 35. The amplification efficiency of the qPCR was calculated from the slope of the standard curve using the following equation; $E = 10^{(-1/\text{slope})}$.

2.4. Data analysis

Culture and qPCR data were analysed using GraphPad Prism 6 and two-tailed Chi-squared (Fisher's exact) tests with additional odds ratio used to test statistical significance of positivity ratios. Two-way ANOVA with Tukey's multiple comparisons test was performed to compare bioburden data. P values < .05 were considered significant.

3. Results

3.1. Comparison of culture and molecular methods for detecting A. fumigatus

There was a substantial difference in *A. fumigatus* detection with CM1, CM2 (the enhanced culture method) and qPCR identifying *A. fumigatus* in 8%, 22% and 53% of samples, respectively (Fig. 1A). This translated to 16% of patients positive for *A. fumigatus* by CM1, 52% by CM2 and 68% by qPCR (Fig. 1B). Of the 32 samples that were positive for *A. fumigatus* by qPCR only 8 of those were positive by CM2 and 4 by CM1 (Table S1). Of the total 13 samples positive by CM2, only 1 of those was positive by CM1 (Table S1). There were 28 CM1 negative samples and 24 CM2 negative samples that were qPCR positive (Table S2). Five qPCR negative samples were CM2 positive and 1 qPCR negative sample was CM1 positive. Twelve CM1 negative samples were CM2 positive (Table S2).

Fisher's exact test was performed to determine significant associations between *A. fumigatus* positivity detected using the three different detection methods (Table 1). CM2 had a 74.2% increased odds of detecting *A. fumigatus* positive samples compared to CM1 (p = .0339) while qPCR had a 94.2% and 77.4% increased odds of detecting *A. fumigatus* than CM1 (p < .0001) and CM2 (p = .0003), respectively. Overall, qPCR was a superior detection method when compared to culture detection methods for *A. fumigatus*.

Table 1
Odds ratios for detecting *A. fumigatus* employing CM1, CM2 and qPCR.

Comparison	Odds Ratio (95% CI)	p value
A. fumigatus		
CM1 vs CM2	0.2582 (0.07889, 0.8453)	*0.0339*
CM1 vs qPCR	0.05844 (0.01879, 0.1818)	*< 0.0001*
CM2 vs qPCR	0.2263 (0.1019, 0.5024)	*0.0003*

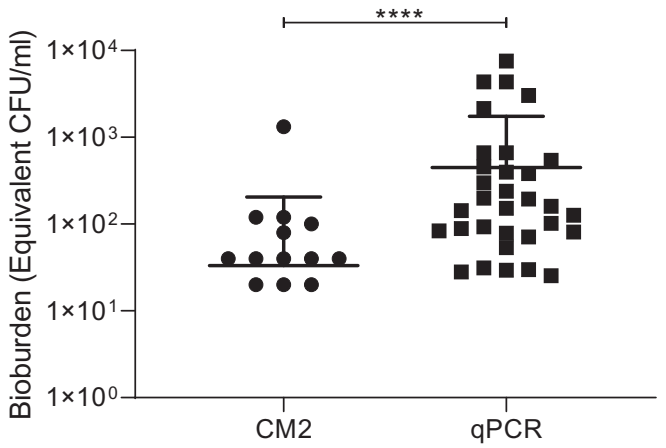

Fig. 2. Bioburden of *A. fumigatus* in CF sputum. Equivalent colony forming units per ml (CFU/ml) of *A. fumigatus* by CM2 and qPCR. Experiments were carried out in triplicate and error bars represent standard deviations. **** $p < .0001$.

We determined the bioburden of samples that were positive for *A. fumigatus* by culture and/or qPCR. The enhanced culture method, CM2, detected significantly lower average bioburden of *A. fumigatus* ($p < .0001$) than qPCR (Fig. 2) suggesting that the bioburden of *A. fumigatus* in the CF airway may be higher than previously considered.

4. Discussion

Studies have shown that molecular techniques out-perform culture-based techniques for the detection of even the most common CF pathogens (van Belkum et al., 2000) and the use of culture independent molecular detection methods has greatly expanded our understanding of the complexity of CF airway disease. Many CF pathogens identified by molecular methods can also be identified by more extensive culture methods (Sibley et al., 2011). Molecular techniques can detect non-culturable microorganisms independently of their growth status which is important during antimicrobial therapy (Jordan and Durso, 2005). PCR methods do have their limitations and do not provide important phenotypic information such as antimicrobial resistance profiles that culture-based methods do. However, new next generation sequencing methods may bridge this gap in the future as whole genome sequencing can now provide detailed resistance profiles and indeed detail on the mutations inferring resistance (Garcia-Rubio et al., 2018). Azole resistant strains of *A. fumigatus* have been reported in numerous countries yet resistance testing of *A. fumigatus* clinical isolates is not part of routine diagnostic laboratory practice (Hamprecht et al., 2018; Seufert et al., 2018).

In this study the routine culture method used by clinical laboratories only detected *A. fumigatus* in 8% of samples (and 16% of patients) whereas the enhanced culture method increased detection of *A. fumigatus* to 22% of samples in 52% of patients. In response to environmental stresses such as hypoxia and pH, many microorganisms can exist in a viable but non-culturable (VBNC) state which is characterized by loss of cultivability (Li et al., 2014). *A. fumigatus* has previously been shown to enter a resting or dormant state (Aimanianda et al., 2009). VBNC microbes have reduced metabolic activity (Lennon and Jones, 2011) but when stresses are alleviated, they resuscitate and regain their virulence (Baffone et al., 2003). The extended incubation period from 5 to 7 days in this study may provide sufficient time for *A. fumigatus* to recover from the stresses of the CF airway. Furthermore, improved homogenisation has been shown to enhance *A. fumigatus* detection (Baxter et al., 2011) and in the enhanced culture method samples were pipetted for 1 min. Most likely the largest impacting factor on improved detection is the higher volume of sample that was tested due to triplicate plating of serial dilutions in the enhanced culture method. In routine diagnostics a single swab is dipped into a homogenised sample and then plated onto agar. We have shown that more rigorous culturing can improve detection of *A. fumigatus* from CF airway samples.

A. fumigatus was detected in 53% of samples (and 68% of patients) by qPCR compared to 22% of samples (52% patients) by CM2 in our study. One recent study, focusing more generally on the *Aspergillus* genus, has shown an increased rate of *Aspergillus* detection by qPCR in a mixed population of children and adults with CF when compared to culture (Guegan et al., 2018). They detected a range of 47.9 to 57.1% positive CF samples employing 2 in-house methods and 2 commercially available multiplex qPCR kits for detecting pan-*Aspergillus* species. The prevalence rate of *A. fumigatus* in CF varies widely depending on the detection method used and the CF patient population studied and thus the true prevalence of *A. fumigatus* in CF is hard to determine. Most studies report prevalence of *A. fumigatus* in adult or mixed CF populations however we recently performed an Irish CF registry analysis and found that *A. fumigatus* colonisation was most prevalent in pre-adolescents and adolescents (Reece et al., 2017). Currently reported colonisation rates based on culture diagnostics are significantly underestimating the prevalence of *A. fumigatus* in CF. Thus the young Irish CF population most likely has a higher prevalence of *A. fumigatus* than previously reported.

qPCR detected significantly higher average bioburden of *A. fumigatus* than CM2 showing the *A. fumigatus* load in the CF airway is also being underestimated. Another study focused on *P. aeruginosa* detection reported that *P. aeruginosa* specific qPCR detected early, low bioburden colonisation of this bacteria in the year previous to culture detection (Blanchard et al., 2018). Therefore, the higher sensitivity of this *A. fumigatus* specific qPCR could potentially detect early, low bioburden *A. fumigatus* colonisation. Numerous studies have now concluded that CF patients chronically colonised with *A. fumigatus* have a more rapid decline in FEV_1 and in general experience a poorer lung function than non-colonised patients (Amin et al., 2010; Saunders et al., 2016; Noni et al., 2015). We have also shown that *A. fumigatus* co-colonising with *P. aeruginosa* (the most common infecting bacteria in CF) is associated with poorer clinical outcomes for CF patients (Reece et al., 2017) and an altered inflammatory response in CF epithelial cell lines (Reece et al., 2018). *A. fumigatus* has also been shown to increase elastase production by *P. aeruginosa* (Smith et al., 2015) and so increase its virulence. Therefore *A. fumigatus* in CF airways may be contributing to disease progression both directly and indirectly by interacting unfavourably with co-colonising pathogens.

5. Conclusion

This study demonstrated that standard culture methods were unable to detect a substantial portion of the *A. fumigatus* positive samples detected by molecular methods. The true prevalence and bioburden of *A. fumigatus* in children and adolescents with CF is most likely underestimated by current culture-based detection methods and thus most published registry studies. Incorporation of molecular detection of *A. fumigatus* into routine clinical laboratories would improve diagnosis and give more representative data on the prevalence of this fungus in CF.

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Declaration of Competing interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105683.

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