Fungal culture and sensitisation in asthma, cystic fibrosis and chronic obstructive pulmonary disorder – what does it tell us?

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Abstract

Collectively asthma, chronic obstructive pulmonary disorder (COPD) and cystic fibrosis (CF) are very common, important causes of disease and ill health. Filamentous fungal colonisation of the airways can occur in all three disease groups, although the clinical relevance is unclear. Allergic bronchopulmonary aspergillosis (ABPA) is a well-recognised severe complication of airway colonisation associated primarily with *Aspergillus fumigatus.*

Fungal colonisation may have a deleterious effect without fulfilling all the diagnostic criteria of ABPA; however, a lack of standardisation in processing respiratory samples hampers comparisons. Whilst mycology laboratory accreditation programs are common, most countries have no national standard guidelines for processing respiratory samples. Fungal recovery from sputum in CF, asthma and COPD can be around 40%, 54% and 49% respectively. Isolation of fungi from sputum has been associated with reduced lung function in asthma and CF, although no such associations have been found in COPD. It is unclear whether fungal colonisation contributes to lower lung function or is a marker of more severe lung disease and aggressive therapy.

Fungal sensitisation may contribute to the persistence of active respiratory symptoms; however, the exact prevalence is unclear. Sensitisation to *A. fumigatus* has been associated with reduced lung function in asthma, COPD and CF. It has suggested that both skin prick tests and specific IgE measurement by the ImmunoCAP system should be used in diagnoses of allergy, due to discordance in test results; however, there is currently no widely adopted consensus as to which fungi to test for.

Keywords *Aspergillus fumigates*; allergy; atopy; colonisation; respiratory disease; fungi.

Background

Asthma, chronic obstructive pulmonary disorder (COPD) and cystic fibrosis (CF) are important causes of disease and ill health, affecting >300 million [1], >64 million [2] and 70,000 [3] worldwide respectively. Filamentous fungal colonisation of the airways can occur in all three disease groups. Allergic bronchopulmonary aspergillosis (ABPA) is a well recognised severe complication of airway colonisation associated with a florid hypersensitivity reaction primarily to *Aspergillus fumigatus*, although other *Aspergillus* species have been implicated including *A. niger*, *A. flavus*, and *A. nidulans* [4]. Prevalence estimates of ABPA are 0.7-3.5% in asthma [5] and 7-9% in CF [6]. ABPA has been recognised as a complication of COPD [7, 8]; however, prevalence rates are not established. Other fungal genera have been associated with similar clinical and radiological features, referred to as Allergic bronchopulmonary mycoses (ABPM), including *Penicillium, Candida*, *Fusarium*, *Schizophyllum*, and *Stemphylium* [4, 9].

Fungal colonisation and sensitisation may have a deleterious effect without fulfilling all criteria necessary for a diagnosis of ABPA; however, a lack of standardisation and non-transparent methodology in terms of how respiratory samples are processed hampers comparisons. Fungal sensitisation affects 3-10% of the population, although exact prevalence is uncertain [10]. Fungal sensitisation is common in asthma, particularly in severe disease where prevalence estimates can be 70% [11], and in CF [12, 13], but less common in COPD, with reported rates of 8.5-13% [7, 14].

Culturing fungi from respiratory samples

Whilst mycology laboratory accreditation programs are common most countries lack guidelines for processing respiratory samples. In the UK most clinical microbiology laboratories follow the national standard method set out in BSOP57 [15], however, a number of studies have suggested this method is insensitive for detecting medically important fungi. Studies in COPD [16] and aspergillosis [17] have directly compared the BSOP57 protocol to in-house protocols using higher concentrations of sputa, and found significantly higher recovery of yeast and fungi with the in-house protocols. In CF a number of alternative methods are routinely employed and a multi-centre study looking at prevalence of fungi using different culture protocols has highlighted the need for a standardised approach [18]. There are a number of variables that can affect the likelihood of recovering fungi from a respiratory specimen including; media, quantity of inoculum, prior processing, incubation temperature, length of observation, and type of clinical sample.

All bacterial media are considered inferior for detecting fungi compared to fungal media [15]. There are many types of fungal growth media, including Sabouraud dextrose agar (SDA) and Potato dextrose agar (PDA). SDA was designed to cultivate dermatophytes and other fungi, and is frequently used by clinical mycology laboratories. PDA is commonly used to culture plant pathogenic fungi, and is recommended in many environmental mycology laboratories. Isolation rates of yeasts and *A. fumigatus* from sputum were found to be higher on PDA than SDA, particularly for *A. fumigatus* [16], although differences in the antibiotics in the media make it unclear whether: *A. fumigatus* grows preferentially on PDA; the higher concentration of chloramphenicol in the SDA plates inhibits *A. fumigatus*; or the fluconazole in the PDA enhances *A. fumigatus* recovery. A study in CF [19] compared non-selective conventional media (Columbia blood agar) to a fungal specific culture media using two media (SDA and Medium B+) and obtained recovery rates of 18% with conventional media versus 78% with the selective mycological approach. Unfortunately other major differences between the techniques make the relative influence of media unclear (the conventional approach used diluted sputum cultured at 37°C for 48 hours; the selective approach used undiluted sputum cultured at 22°C for three weeks). In CF some laboratories are now incorporating selective media to encourage growth of specific fungi such as erythritol-chloamphenicol agar (ECA) for identifying the black yeast *Exophiala dermatitidis* [20] and SceSel+ medium for detecting *Scedosporium* [21].

The UK BSOP57 guidelines were designed for identifying bacteria and fungi. For routine microbiological investigations sputum is homogenised with an equal volume of a suitable mucolytic agent, diluted 1:500 in water, and 1 µl inoculated onto SDA. For specific mycological investigations 1 µl of the undiluted homogenised sputum is inoculated [15]. A study comparing the recommended microbiological and mycological methods to a method whereby sputum plugs are removed from saliva to minimise oropharyngeal contamination [22] and approximately 150 mg directly plated onto culture plates, found isolation of *A. fumigatus* was significantly higher using the research approach compared to the standard for mycology ($P<0.001$), whilst the microbiological method failed to detect any colonies [16]. Another study comparing the microbiological method to an approach where total sputum (saliva and plugs) is directly cultured similarly found that the in-house method resulted in significantly higher fungal recovery rates [17]. A recent comparison of culture methods used in several CF centres 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 µl to 500 µl; with some laboratories concentrating the sputum before culture [18].

BSOP57 recommends culturing at 35 - 37°C for two days for standard microbiology and five days for mycological investigations, unless *Paracoccidioides brasiliensis* is clinically indicated when culture may be prolonged to up to 6 weeks [15]. Daily observations for *A. fumigatus* colonies over 7 days found that whilst the majority were observed within 2-5 days, a few required 7 days of incubation. The comparative study by Borman *et al*. [18] revealed that incubation temperatures ranged from 25 to 37°C and incubation times ranged from 7 days to 6 weeks.

Sputum samples are frequently used for microbiological investigations of respiratory infections, including fungi. Induced sputum has the advantage of higher density of cell recovery and is less invasive than bronchoalveolar lavage (BAL) [23], whereas BAL has the advantage of avoiding contamination with the oropharynx. Fungal recovery rates vary depending on respiratory sample analysed ranging from 20% with BAL, 44% with sputum and 75% with bronchial aspirates [17].

It is clear that different approaches used to process respiratory samples and culture fungi have drastic effects on fungal detection, and without standardisation comparisons between studies or between institutes are hampered. The current UK standard culture protocol is clearly insensitive and should be reviewed. Whilst molecular approaches may be suitable for detecting and identifying fungi, it is still beneficial to obtain an active culture to confirm the presence of a living fungus and to enable anti-fungal susceptibility testing and/or strain typing investigations.

Testing for fungal sensitisation

The diagnosis of fungal allergy is based on patient history and *in vivo* and *in vitro* testing. The most common tests used are skin prick tests (SPT) and specific serum IgE tests. The SPT is a simple diagnostic tool that can be useful for screening for sensitisation, but it is not without limitations. The majority of SPT positive individuals are also positive by specific IgE, giving the SPT a high negative predictive value (95%); however a significant proportion of individuals with positive IgE tests are SPT negative [6]. Intradermal tests are more sensitive than SPT [24]; however, these are rarely performed and carry a higher rate of false positives [25]. *In vitro* measurement of specific IgE antibodies are more costly than SPTs. The immunoassay capture (ImmunoCAP) system for specific serum IgE testing has higher sensitivity than radioallergosorbent testing (RAST) with comparable specificity [25]. It has been suggested that both SPTs and specific IgE measurement by the ImmunoCAP system should be used in diagnoses of allergy due to discordance in test results [26]; although of the two, specific IgE testing is the more sensitive [27, 28, 26]. Comparing SPT and specific IgE for six fungi,

O'Driscoll *et al* [26] found discordant results in 23% of patients tested. Individual fungal allergen concordance rates varied from 14% with *Botrytis* to 56% with *Alternaria*. For *Aspergillus,* IgE testing alone missed 19% of those sensitised and SPT alone missed 28%.

There are more than 74,000 formally described species within the Kingdom Fungi; with global estimates of the number of species believed to be at least 1.5 million, but probably as many as 3 million [29]. The Kingdom can be divided into 8 – 10 phyla, and fungal pathogens have evolved independently and repeatedly throughout the phyla [30]. There are currently 28 fungal species belonging to the Ascomycota and Basidiomycota with allergens officially approved by the Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS; www.allergen.org). A 2008 review of fungal allergens lists 174 allergens from 19 genera of Ascomycota and 30 from 4 genera of Basidiomycota [31]; and yet, the fungi most commonly included in fungal allergy testing panels are all conidia-producing anamorphs (asexual forms) of ascomycetes such as *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* or anamophic ascomycetous yeasts such as *Candida*. Little is known about the allergenicity of the ascospore-producing teleomorphic (sexual) ascomycetes, but studies have suggested that the prevalence of hypersensitivity to basidiospores of may be comparable to conidial ascomycetes [10]. Many studies use only one or two fungal species in their allergy panels, and a European community health survey recommends including two fungi (*Alternaria* and *Cladosporium*) among a panel of seven allergens to identify atopic individuals within the general population [32]. More comprehensive fungal respiratory health studies may include five [33] to seven [26] fungal allergens, notably all from the Ascomycota, however, some studies have suggested relatively high rates of sensitisation in asthma patients to other non-ascomycetous fungi including *Rhizopus* and *Mucor* [6].

There are no standardised fungal extracts for diagnostic testing. The problems encountered in producing fungal extracts have been reviewed by Crameri *et al.* [34] and include: different patterns of allergens from different isolates of the same species; instability of extracts due to protease content; and, variations caused by differences in culture conditions, medium used to grow the fungus, and the extraction process. Even reagents from the same commercial supplier can be subject to batch to batch variations.

Fungal allergens can be metabolic products secreted externally or cytoplasmic and structural components released following cell lysis. Some are conidial allergens whilst others are present only in hyphae. Many are capable of cross-reactions, with only a few of the major allergens being unique and not sharing sequence homology with other known allergens. According to a review paper on fungal allergens, more than 75% of mould-sensitised patients are monosensitised, with *Alternaria* being the most common fungus to which people

are sensitised, and predominantly through sensitisation to the species-specific major allergen Alt a 1 [35]. Conversely, mono-sensitisation to *A. fumigatus*, *Cladosporium herbarum*, *Penicillium chrysogenum*, and *Saccharomyces cerevisiae* is very rare in fungal sensitised individuals (<1%) [36], although mono-sensitisation to *A. fumigatus* in severe asthma [26] and COPD [14] is more common. The majority of fungal allergens represent cross-reactive structures covering different protein families such as manganese superoxide dismutase (MnSOD), enolase, heat-shock proteins, proteases, ribosomal and peroxisomal proteins; however, the major allergens of *A. fumigatus* (Asp f1), *A. alternata* (Alt a1), and *Malassezia sympodialis* (Mala s1) are species-specific proteins [35]. The clinical relevance of cross-reactivity is unclear but may be responsible for false-positive diagnoses in serological investigations, and may play a role in exacerbation of allergic complications related to sensitisation in atopic eczema and ABPA [35]. The species-specific major allergens are unlikely to be suitable on-their-own as specific recombinant allergens as they do not reach the sensitivity of total fungal extracts being recognised by 60–90% of the atopics sensitised to the corresponding fungus [37]. Specific *A. fumigatus* allergens have been evaluated for their diagnostic performance in serologic studies and may be useful in discriminating between ABPA and *Aspergillus*-fungal allergy in CF [38, 39] and asthma [40, 41].

Clinical relevance

Fungal sensitisation plays an important role in the development, persistence, and severity of asthma. Whilst most of the evidence originates from associations with exposure and loss of asthma control, direct causal associations with asthma development are becoming apparent; these have been linked to a number of fungal genera including *Alternaria, Cladosporium* and *Aspergillus* [6]. IgE sensitisation to *A. fumigatus*, in the absence of ABPA, has been associated with reduced lung function in asthma [42, 28, 43], COPD [14] and CF [44]. Observational cohort studies in CF have found significant drops in lung function in patients with *Aspergillus* sensitisation compared to controls over 2 years [45] and 12 years [46].

ABPA is a well recognised complication of fungal colonisation associated with hypersensitivity. Screening for total IgE and fungal specific IgE and IgG are used as diagnostic criteria for ABPA, however, there is no consensus on the cut-off value that should be used [47]. International consensus groups have proposed diagnostic criteria for ABPA in asthma [47] and CF [48], both proposing a cut-off value for total IgE of 1000 IU/mL (2400 ng/mL). The asthma group acknowledge that some patients meet all other criteria for ABPA but have IgE <1000 IU/mL and these can still be accepted as having ABPA [47], whilst the CF group additionally proposed a minimal diagnostic criteria cut-off of > 500 IU/mL (1200 ng/mL) [48]. In COPD, the criteria used

for ABPA complicating asthma are used [7]. In asthma the cut off value of > 0.35 kUA/L is accepted for *A. fumigatus* specific IgE [47], whilst in CF the criteria suggest immediate cutaneous reactivity or *in vitro* demonstration of IgE antibody, but do not give a cut-off [48]. ABPA has been sub-classified many times and includes the designation ABPA-S (seropositive ABPA), which is often considered as "early stage" ABPA [49]. The newly proposed clinical staging of ABPA in asthma includes a stage 0 for patients who have controlled asthma and a stage 1 for uncontrolled asthma, both of which could be considered ABPA-S as this group felt CB should be considered a complication of ABPA and not a diagnostic criterion [47]. A large study in India found significant overlap in *A. fumigatus* specific IgE and total IgE between ABPA and asthmatics [27]. This was based on a single referral centre and needs to be conducted in further centres representing other geographical regions and ethnicities, however, it has been speculated that ABPA may represent one florid manifestation of a spectrum of fungus-associated airway disease [6].

Severe asthma associated with fungal sensitivity (SAFS) describes patients with severe asthma and fungal IgE who do not meet the criteria for ABPA [50], although due to ambiguity in diagnostic criteria SAFS is more a diagnosis by exclusion than a diagnosis of a specific disease entity [34]. It is unclear whether sensitisation to thermotolerant fungi able to colonise the respiratory tract, such as some species of *Aspergillus* and *Pencillium,* has the same clinical relevance as sensitisation to aeroallergenic fungi such as *Alternaria*, *Cladosporium* and *Botrytis* [51, 33]; leading some researchers to coin the phrase *A. fumigatus*-associated asthma (AFAA) [28]. A clinical trial of the anti-fungal agent itraconazole in SAFS found 60% of patients showed quality of life improvements compared to placebo [52], whereas a 3 month trial of voriconazole in AFAA failed to show improvement in quality of life [53]. With voriconazole there was a reduction in the degree of *A. fumigatus* colonisation in the active group; however, the drug did not totally eradicate the fungus and rates of positive culture returned to baseline within a few months of stopping treatment [53].

In CF incidence of recovery of at least one fungal species from an individual can be around 40% and one of the strongest risk factors associated with isolation of fungi is decreased lung function, even after exclusion of patients diagnosed with ABPA [54]. A 2-year study found no statistical difference in lung function decline between patients colonised with *Aspergillus* and those who were not [45]; however, a 7 year study found the FEV_1 was 3.61% ($P \leq 0.0001$) lower compared with uninfected patients [55] and a 12 year study found a significant association between lung function decline and persistent carriage of *A. fumigatus* [46]. In a study of people with moderate to severe asthma, filamentous fungi were isolated from the sputum of 54% of patients and they had significantly lower lung function than those who were culture negative [33]. In contrast in COPD

where filamentous fungi were recovered from 49% of clinically stable patients, there was no difference in lung function between those who were culture positive to those who were not [14]. It is unclear whether fungal isolation contributes to lower lung function or is a marker of more severe lung disease and aggressive therapy. Longitudinal studies are needed to determine the effect of fungal colonisation on lung function decline, in addition to intervention studies to assess the effectiveness of antifungal agents on suppressing or eradicating fungi.

Summary

Evidence is mounting that fungal colonisation and sensitisation play a role in asthma, COPD and CF; however, variability in the screening tests can complicate diagnosis and estimates of prevalence. Sensitisation to *A. fumigatus* is associated with worse lung function in all three diseases, whilst culture of filamentous fungi is associated with worse lung function in asthma and CF, but not COPD. Assays to determine fungal sensitisation are not without limitations, and the discordance between test results must be taken into consideration. The detection of *A. fumigatus* and other fungi from clinical specimens such as sputum is highly dependent upon the methodology used, and a more sensitive approach that is standardised and universally adopted is a definite need.

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