Volume dependency for culture of fungi from respiratory secretions and increased sensitivity of Aspergillus quantitative PCR

Marcin G. Fraczek,1,2 Marie B. Kirwan,1,3 Caroline B. Moore,1,2,3 Julie Morris,4 David W. Denning1,2,3 and Malcolm D. Richardson1,2,3

1Institute of Inflammation and Repair, Education and Research Centre, Manchester Academic Health Science Centre (MAHSC), University of Manchester, Manchester, UK, 2Mycology Reference Centre, Education and Research Centre, University Hospital of South Manchester (UHSM), Manchester, UK, 3National Aspergillosis Centre, University Hospital of South Manchester (UHSM), Manchester, UK and 4Medical Statistics, Education and Research Centre, University Hospital of South Manchester (UHSM), Manchester, UK

Summary

Diagnosis of aspergillosis is often difficult. We compared fungal yields from respiratory specimens using the Health Protection Agency standard culture method (BSOP57), a higher volume undiluted culture method Mycology Reference Centre Manchester (MRCM) and Aspergillus quantitative real time polymerase chain reaction (qPCR). Sputum, bronchial aspirate and bronchoalveolar lavage (BAL) samples (total 23) were collected from aspergillosis patients. One fraction of all samples was cultured using the MRCM method, one BSOP57 and one was used for qPCR. The recovery rate for fungi was significantly higher by MRCM (87%) than by BSOP57 (8.7%) from all 23 specimens. Sputum samples were 44% positive by MRCM compared to no fungi isolated (0%) by BSOP57. Bronchial aspirates were 75% positive by MRCM and 0% by BSOP57. BAL samples were positive in 20% by MRCM and 10% by BSOP57. qPCR was always more sensitive than culture (95.6%) from all samples. In general, over 100 mould colonies (81 Aspergillus fumigatus) were grown using the MRCM method compared with only one colony from BSOP57. This study provides a reference point for standardisation of respiratory sample processing in diagnostic laboratories. Culture from higher volume undiluted respiratory specimens has a much higher yield for Aspergillus than BSOP57. qPCR is much more sensitive than culture and the current UK method requires revision.

Key words: Aspergillus, sputum, bronchoalveolar lavage, culture, qPCR.

Introduction

Aspergillus fumigatus is a major contributor to the morbidity and mortality of immunocompromised patients and those with primary diagnoses of asthma, cystic fibrosis (CF) and chronic pulmonary aspergillosis, affecting millions worldwide.1 Despite impacting significantly on the disease burden of these patients, culture yield from standard bronchoscopic and sputum samples is low with 1 of 1000 microbiology laboratory samples or 2.1 patients of 10 000 admissions growing A. fumigatus.2 Standard culture has a low sensitivity and can be slow whereas Aspergillus antigen detection and quantitative real time polymerase chain reaction (qPCR) offer the opportunity for faster return of results and higher sensitivity.3 Often presumptive clinical diagnosis is required in the absence of culture.

The need for precise and prompt identification of fungal lung disease is not a new phenomenon and the need for improved diagnostic performance that can
guide antifungal therapy in real time is widely recognised. Early identification and subsequent earlier antifungal treatment significantly improves survival. Standard fungal detection techniques employed include direct microscopy and culture. Additional tools include specific IgG and IgE antibody detection and serum galactomannan antigen testing, which has ~20% sensitivity for invasive aspergillosis (IA) in non-haematological populations. Culture has a critical role in the clinical diagnosis of respiratory infection and is still a major diagnostic tool, hence the standardisation of methodology as recommended by Public Health England (formerly the Health Protection Agency (HPA)) in the UK for both sputum and bronchoalveolar lavage (BAL) samples, namely BSOP57 or B 57.

Very few comparative studies of diagnostic yield of fungi from respiratory samples have been done to date. Bakare et al. [12] analysed diagnostic yield in CF patients and Pashley et al. [13] compared fungal culture methodology in chronic obstructive pulmonary disease patients (COPD). The primary objective of this study, however, was to compare the fungal diagnostic yield from various respiratory samples obtained from aspergillosis patients and directly compare the BSOP57 methodology with our respiratory sample processing method and qPCR. This study provides a reference point for standardisation in respiratory sample processing which is required in Europe and possibly all over the world to prevent variations in relative organism prevalence rates between laboratories and to improve fungal diagnosis.

Materials and methods

Study population, recruitment and ethics

We prospectively recruited five patients attending a follow-up outpatients’ appointment at the National Aspergillosis Centre (NAC), Manchester, UK. This national centre is co-located with the Mycology Reference Centre Manchester (MRCM). All patients were scheduled for a diagnostic bronchoscopy and were expressly selected and enrolled in the study because they were highly likely to have Aspergillus in their airways (i.e. a pretest probability of 100%). They were not taking any antifungal therapy on the day of bronchoscopy, or in the previous month. Bronchoscopists were all highly experienced.

Patients were recruited under the auspices of the large Fungal Exposure and Colonisation in Respiratory Disease Research Ethics Committee (reference: 07/Q1403/70). Recruitment was performed in accordance with ICH Good Clinical Practice guidance.

Sputum, bronchial aspirate and BAL sample collection

A total number of 23 specimens were processed from the five patients (Table 1). These included pre and post bronchoscopy sputa, a bronchial aspirate, BAL wash 1 and BAL wash 2 samples. A pre bronchoscopy sputum (morning sputum) was obtained from four of five patients. During bronchoscopy, after initial entry into the main bronchi trap specimens (bronchial aspirates) were taken (four of five patients) and an initial wash of 5–20 ml of 0.9% saline was performed in all patients (here denoted as BAL wash 1). Based on chest radiography, the lung lobe with a consolidation or shadowing was instilled with 10–120 ml of 0.9% saline for the BAL (BAL wash 2). The volume instilled was judged by the bronchoscopist based on the patients’ clinical condition and oxygen saturation. A second sputum sample was obtained post bronchoscopy from all patients. During bronchoscopy, an air sampler collected air within the theatre to exclude any contamination. Collected patients’ samples were immediately transferred to the MRCM laboratory and classified into two groups: (I) sputum samples and high viscosity BAL/bronchial aspirate samples including those with volume <5 ml, and (II) low/moderate viscosity BAL/bronchial aspirate samples with a total volume >5 ml. All samples were processed by both the Department of Clinical Microbiology (DCM), University Hospital of South Manchester and by the MRCM as described below and shown in Fig. 1.

Sputum, bronchial aspirate and BAL sample processing in MRCM

In this study, we directly compared the BSOP57 protocol (below) to the higher volume undiluted respiratory sample processing method (MRCM) and to Aspergillus qPCR. All work was carried out in a class II microbiology safety cabinet, decontaminated before use to prevent cross-contamination. Moreover, to control for any false positive colonies on the culture plates, an air sampler collected air within the cabinet during the procedure onto a fungal culture plate, which was incubated along with the test plates. Because sputum, BAL and bronchial aspirate samples differed in terms of viscosity and volume they were processed using different approaches (Fig. 1). Colony forming units (cfu) on culture plates were counted.
### Table 1 Patient details, culture and qPCR results.

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diagnosis</strong></td>
<td>ABPA, bronchiectasis</td>
<td>Previous IA after non-small cell carcinoma, COPD and TB</td>
<td>Aspergillus bronchitis</td>
<td>ABPA, asthma, bronchiectasis</td>
<td>Aspergillus bronchitis, COPD</td>
</tr>
<tr>
<td><strong>Sample description</strong></td>
<td>DCM (cfu)</td>
<td>MRCM (cfu)</td>
<td>qPCR (Ct)</td>
<td>DCM (cfu)</td>
<td>MRCM (cfu)</td>
</tr>
<tr>
<td>Pre-bronch. sputum</td>
<td>Ca+8, Af, 35 Ca</td>
<td>33.9</td>
<td>N</td>
<td>1 Af</td>
<td>32.8</td>
</tr>
<tr>
<td>Bronchial aspirate</td>
<td>N</td>
<td>3 Af</td>
<td>31.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BAL wash 1 (2–8 ml)</td>
<td>N</td>
<td>2 Pen (pt), N (st)</td>
<td>38.0 (pt), &gt;38 (st)</td>
<td>N</td>
<td>3 Ed1</td>
</tr>
<tr>
<td>BAL wash 2 (8–16 ml)</td>
<td>N</td>
<td>N (both pt and st)</td>
<td>37.2 (pt), &gt;38 (st)</td>
<td>N</td>
<td>12 Ed1</td>
</tr>
<tr>
<td>Post-bronch. sputum</td>
<td>N</td>
<td>3 Af</td>
<td>33.2</td>
<td>N</td>
<td>6 Y</td>
</tr>
</tbody>
</table>

qPCR, quantitative real time polymerase chain reaction; ABPA, allergic bronchopulmonary aspergillosis; IA, invasive aspergillosis; COPD, chronic obstructive pulmonary disease; TB, tuberculosis; DCM, Department of Clinical Microbiology, University Hospital of South Manchester (BSOP57: routine laboratory); MRCM, Mycology Reference Centre Manchester; Ct, Aspergillus qPCR cross threshold value (Ct ≤ 38 considered positive; Ct > 38 considered negative); cfu, colony forming unit; N, no fungal growth; Af, A. fumigatus; An, A. niger; Pen, Penicillium spp.; Ca, C. albicans; Ed, E. dermatitidis; Rt, Rhodotorula spp.; Y, yeast other than C. albicans; Conf, confluent; ND, not done; BAL wash 1 & 2, volumes collected after procedure; pt, results obtained from pellet; st, results obtained from supernatant.

1High viscosity BAL samples, processed as group I samples (see Materials and methods and Fig. 1).
Upon reception of the specimens, samples were processed in the following manner. Sputum and high viscosity BAL/bronchial aspirate samples including those with volume < 5 ml and low/moderate viscosity BAL/bronchial aspirate samples with a total volume > 5 ml. Samples in the (I) group were divided into three fractions. One was used for DNA extraction and quantitative real time polymerase chain reaction (qPCR), one was cultured in MRCM and one was processed in DCM using BSOP57. Samples in the (II) group were centrifuged. Both the pellet and the supernatant were divided into three fractions. One was used for DNA extraction and qPCR, one was cultured in MRCM and one was processed in DCM using BSOP57.

Figure 1 Sputum and bronchoalveolar lavage (BAL) sample processing approach used in this study. Upon reception in the laboratory, samples were classified into two separate groups: (I) sputum samples and high viscosity BAL/bronchial aspirate samples including those with volume < 5 ml, and (II) low/moderate viscosity BAL/bronchial aspirate samples with a total volume > 5 ml. Samples in the (I) group were divided into three separate fractions. One was used for DNA extraction and quantitative real time polymerase chain reaction (qPCR), one was cultured in MRCM and one was processed in DCM using BSOP57. Samples in the (II) group were centrifuged. Both the pellet and the supernatant were divided into three fractions. One was used for DNA extraction and qPCR, one was cultured in MRCM and one was processed in DCM using BSOP57. MRCM, Mycology Reference Centre Manchester – grey box; DCM, Department of Clinical Microbiology, University Hospital of South Manchester (routine laboratory; BSOP57) – white box.

Upon reception of the specimens, samples were processed in the following manner. Sputum and high viscosity BAL/bronchial aspirate samples including the ones with a total volume lower than 5 ml (sample group I, Fig. 1) were divided into three even fractions – one was used for DNA extraction and qPCR, one was transferred to the DCM for routine culture and microscopy (BSOP57; below) and one was cultured in the MRCM using a higher volume undiluted sputum/BAL processing method. Low and moderate viscosity BAL and bronchial aspirate samples with a total volume higher than 5 ml (sample group II, Fig. 1) were transferred to a 50 ml falcon tube and separated by centrifugation at 10,000 g for 10 min (Jouan B4i Multifunction Centrifuge; Thermo Scientific, Basingstoke, UK) in the MRCM laboratory. It was impossible to quantitatively measure viscosity and so obtain absolute standardisation. However, the laboratory scientists responsible for study sample processing were highly experienced and able to assess accurately by inspecting the samples which would centrifuge easily and those which needed treatment with N-acetyl L-cysteine (below). All samples (from both sample groups) were divided into fractions as equally as possible, despite the fact that some samples, especially the ones with high viscosity, were difficult to manipulate. Each sample was separated on a Petri dish using a Pasteur pipette. The volume was recorded for each separated fraction.
Since some samples were of high viscosity, the accuracy equalled $\pm 100$ µl for each fraction.

In sample group I, one third of the sputum and high viscosity BAL/bronchial aspirate samples were used for DNA extraction. The sample was pretreated with the BBL MycoPrep Specimen Digestion/Decontamination Kit (N-acetyl L-cysteine) for Processing of Mycobacterial Specimens (Becton Dickinson, Oxford, UK) in accordance with the MycXtra (Myconostica, Manchester, UK) protocol and DNA extraction was carried out from the pellet. The supernatant containing 1x phosphate-buffered saline (PBS) was discarded. DNA extraction on a negative sterile water sample (500 µl; Sigma-Aldrich, Gillingham, UK) was carried out along with the test sample. A volume of 100 µl of the same sterile water was also cultured on a Sabouraud dextrose (SAB) plate to exclude false positives. The MycAssay Aspergillus kit (Myconostica) and the SmartCycler system (Cepheid, Sunnyvale, CA, USA) were used for the qPCR. Both negative DNA extraction sample and internal qPCR sample were also analysed. Data are presented as Aspergillus Ct values, where Ct $\leq$ 38 was considered positive and Ct $> 38$ negative (MycAssay Aspergillus may also generate positive results for Penicillium spp.). One third of the same sputum sample was transferred to the routine laboratory (BSOP57) for culture and microscopy (as described below). The remaining volume of the sample was cultured in the MRCM on a SAB agar plate supplemented with 50 µg ml$^{-1}$ chloramphenicol and incubated at 30 °C for 72 h. Empty plates were left in the incubator for an additional 48 h. This sample fraction was not treated with any reagent before culture.

Following centrifugation of low/moderate viscosity BAL and bronchial aspirate samples (sample group II, Fig. 1), the pellet and the supernatant were treated in a similar manner. The pellet was re-suspended in 1 ml of sterile 1x PBS and separated into three fractions by pipetting. Approximately 450 µl of one fraction was used for DNA extraction (MycXtra) and qPCR (MycAssay). ~450 µl was transferred for routine processing (DCM) of which only a loopful was cultured on SAB agar (BSOP57; details below) and ~100 µl was cultured in the MRCM. Similarly, the supernatant was fractionated – 1 ml was used for DNA extraction. ~100 µl was cultured in the MRCM and the remaining volume (various) was transferred to the routine clinical microbiology laboratory (BSOP57; below). Negative controls were carried out as above. All sputum and BAL culture samples were tested for minimum inhibitory concentration for itraconazole, voriconazole, posaconazole and amphotericin B using a broth microdilution method as previously described.

**Sputum, bronchial aspirate and BAL processing in DCM**

Routine processing adheres to the HPA BSOP57 method, normally performed with one exception in DCM – the plated amount was 10 µl (an increase of 10-fold). Briefly, upon receipt of a sputum specimen, the sample was homogenised by mixing for 10 s with an equal volume of a 0.1% dithiothreitol solution (Sputasol; Oxoid, Basingstoke, UK). The sample was subsequently incubated at 37 °C for 15 min and diluted 500-fold in sterile water. A volume of 10 µl (not 1 µl as in the original BSOP57 method) of the diluted specimen was then inoculated onto a SAB agar plate (for fungi) and incubated at 30 °C for 48 h before the results were read. A BAL sample was centrifuged at 1200 g for 10 min, most of the supernatant was discarded and the pellet was re-suspended in 0.5 ml of the remaining volume. A quantity of 10 µl (10-fold increase as opposite to the original BSOP57 method) of the specimen was inoculated onto a SAB agar plate using a sterile inoculation loop in accordance with BSOP57 and QSOP52, apart from the volume increase. The plate was incubated at 30 °C and the results were read after 48 h.

**Statistical data analysis**

Simple McNemar’s tests and paired t-tests were used to compare positivity rates and Ct values, respectively, between methods at different stage points and between stages within methods. Comparisons between methods over all stages/times and between combined stages within methods (involving multiple samples per patients) were made using logistic and linear regression modelling with generalised estimating equations, which appropriately accounted for multiple patient samples.

**Results**

A total of 23 specimens were collected from five patients. All patients were women with a mean age of 60.5 years (range 47–74 years). The main pulmonary and Aspergillus diagnoses are shown in Table 1. None was on any antifungal therapy at the time of sample collection nor several weeks before.

In total, 104 mould cfu were identified from all 23 samples, 103 (99%) only by the higher volume MRCM
method and one by BSOP57 (1%) \((P = 0.005)\) (Table 1). Of these, 82 were identified as *A. fumigatus*, 20 as *Penicillium* spp. and two as *Aspergillus niger*. Altogether 95 discrete yeast cfu were grown – 94 (98.9%) on plates by the MRCM method compared with only one (1.1%) from BSOP57 \((P < 0.001)\). Of those enumerated, 72 cfu were *Candida albicans*, 15 *Exophiala dermatitidis*, two *Rhodotorula* spp. and six unidentified *Candida* spp. Two MRCM plates showed confluent yeast, which might have hindered the recovery of moulds. In general, various yeast and moulds were observed from 20 of 23 samples (87%) with the MRCM culture method (1–33 mould cfu and two confluent yeast spp.) \((P < 0.001)\), whereas only two of 23 (8.7%) samples were culture positive for fungi with BSOP57 (one sample with one cfu of *A. fumigatus* from BAL wash 1 and one sample with *Candida* spp. from pre bronchoscopy sputum) (Fig. 2a).

*Aspergillus* qPCR proved to be positive in 22 of 23 (95.6%) samples compared to nine (39%) *A. fumigatus* culture positive samples by the MRCM method \((P = 0.001)\) and one (4.3%) with BSOP57 \((P < 0.001)\) (Fig. 2a) (Ct values for all qPCRs are presented in Table 1). Pre and post bronchoscopy sputum samples \((n = 9)\) were always positive by *Aspergillus* qPCR (100%) compared with 4 of 9 (44.4%) by MRCM method for *A. fumigatus* \((1–8\) cfu) (pre bronchoscopy 4/4 vs. 3/4, \(P = 1.0\); post bronchoscopy: 5/5 vs. 1/5, \(P = 0.12\) respectively) (Fig. 2b). The higher *A. fumigatus* culture rate from pre bronchoscopy (75%) was not significantly different from post bronchoscopy specimens (40%) for both moulds and yeast \((P = 0.27)\).

All four bronchial aspirate samples were *Aspergillus* qPCR positive (100%) and three \((75%)\) were *A. fumigatus* culture positive \((1–33\) cfu) with the MRCM method \((P = 1.0)\), but not with BSOP57 \((0%)\) \((P = 0.125)\) (Fig. 2c). Furthermore, a few colonies of *Penicillium* spp. and yeast were cultured. Histopathology analysis was performed on a bronchial aspirate sample from patient 1, which showed hyphae consistent with *Aspergillus* spp. and Charcot Leyden crystals, but the specimen was culture negative by BSOP57.

Between 2 and 8 ml of BAL wash 1 and 8–16 ml of BAL wash 2 were collected after the bronchoscopy from the five patients (instilled volumes were 5–20 and 10–120 ml for BAL wash 1 and BAL wash 2 respectively). Due to high viscosity and/or low volume, three BAL wash 1 and two BAL wash 2 samples were treated as sputum samples for culture and DNA extraction (Fig. 2d). Nine of 10 BAL samples were *Aspergillus* qPCR positive (90%), however only two samples were *A. fumigatus* culture positive \((20\) cfu for BAL wash 1 and 12 cfu for BAL wash 2) with the MRCM method \((20%)\) \((P < 0.001)\), and both were from patient 4. BAL wash 1 was also culture positive for *A. fumigatus* with BSOP57 from the same patient \((1\) cfu; 10%) \((P < 0.001)\). A few BAL wash 1 and 2 samples were also *Penicillium* spp. \((14\) cfu total) and yeast positive \((29\) cfu total). All control culture plates and control qPCR reactions were negative for all samples tested.

Overall, the higher volume undiluted MRCM culture method for *A. fumigatus* was successful for five of five patients (100%) as well as the *Aspergillus* qPCR (100%). BSOP57 was successful only in one patient (20%), generating a single *A. fumigatus* colony \((P = 0.125)\) (Fig. 2e). We were also able to identify other fungi by application of the MRCM culture method, which may or may not be as clinically important as detection of *A. fumigatus*.

No triazole resistance was found in any *Aspergillus* isolate.

**Discussion**

The UK standard culture method (BSOP57) for respiratory samples, and various minor modifications have been used in most clinical laboratories in the UK for several years, but have never been evaluated rigorously for their performance in detecting fungi. In this study, we show an existing problem of underdiagnosis of fungal diseases caused by erroneous or insufficient data provided to the clinicians by diagnostic laboratories. Some previous studies in CF patients\(^{12,17}\) and more recently those on samples obtained from COPD patients\(^{13}\) also showed that the current HPA culture method may underestimate fungal prevalence in patients’ samples. As opposed to previous studies, we directly compared the fungal yield from 23 different respiratory samples obtained from five patients with the history of *Aspergillus* disease by BSOP57 (with a 10-fold increased plate inoculum), the higher volume undiluted MRCM sputum/BAL processing method and qPCR for *Aspergillus*. We found that BSOP57 was grossly sub-optimal for all fungi, especially *A. fumigatus*, compared with the high volume MRCM culture method and a commercial qPCR for *Aspergillus* spp. In general, sputum and bronchial aspirates had a higher culture yield and stronger qPCR signals than BAL samples, suggesting that samples from higher up bronchial tree contain more *Aspergillus* than distal (and diluted) samples. Whether this was due to colonisation, infection or simply transient exposure to fungal spores is not known. However, addressing the reason
for the presence of Aspergillus in these patient’s airways was not the main aim of this study which was to highlight a problem in current fungal culture methodology. All patients enrolled in this study were selected on clinical grounds and due to their high probability of having fungi growing in their airways it is unlikely that obtained results were due to contamination. Moreover, all procedures performed during this study (sample collection, sample processing, etc.) were carried out in a controlled environment (frequent air
monitoring was performed) which was always culture negative.

Our study clearly indicates a correlation between sample processing and the recovery yield of fungi. Moreover, it appears that the dilution factor and volume cultured are very important in detecting fungi in respiratory secretions. The five patients enrolled in the study demonstrate this convincingly, but do not establish a gold standard for the culture of fungi. From only 23 samples collected from these patients anticipated to have aspergillosis we showed that the current standard methodology (BSOP57) is not effective. A shift in methods to improve culture sensitivity in clinical laboratories is required and more work will be needed to establish the most sensitive method, and to establish its specificity.

The strength of this study is the consistent application of multiple microbiological methods to several samples from the same and different patients with airways aspergillosis enabling two sets of comparisons: method comparisons and sample source comparisons. The results are striking. Clearly more patients should be studied and such studies should include patients with IA in the context of neutropenia (angioinvasive) and corticosteroid immunosuppression (non-angioinvasive) as well as patients with severe asthma and chronic pulmonary aspergillosis. Further studies should also include respiratory secretions from healthy volunteers, which we know from our parallel study performed in the NAC (Manchester, UK) and at the University of Innsbruck (Austria) that are not typically sterile from either colonising or contaminated (inhaled) fungal spores and fragments. This was also shown in several other studies. That the MRCM method also yielded many more fungi (Candida spp. and E. dermatisidis in particular) suggests that improved specimen processing methods would be helpful for many fungal species, including dimorphic fungi and other filamentous fungi such as those causing mucormycosis, another notoriously difficult microbiological diagnosis. We did not find any triazole-resistant A. fumigatus among these patients, although this is an increasing problem, and one for which culture is particularly important in detection.

We and others have recently questioned the performance of current UK respiratory culture methods for fungi. Given that culture methods have been in use in clinical laboratories for over half a century, it is extraordinary that so little attention has been paid to optimising diagnostic yield from the respiratory tract. In immunocompromised patients thought to have a life-threatening invasive fungal infection, most authorities and diagnostic algorithms recommend BAL sampling for fungal microscopy and culture, even though the diagnostic yield is low, as well as Aspergillus galactomannan antigen detection. Rather than exposing the patient to a risky and unpleasant bronchoscopy procedure, this study suggests that sputum is more sensitive than BAL for culture and qPCR and should be analysed prior to bronchoscopy, especially if analysed rapidly using qPCR for Aspergillus. Alternatively the yield from bronchoscopy could be increased if the initial bronchial wash and a sample of the post procedure sputum were analysed alongside BAL fluid.

One of the implications of this study is that the BSOP57 needs replacing. The BSOP57 high dilution factor appears to be adequate for identification of medically important bacteria because they usually multiply very quickly especially in pneumonia patients and are usually evenly distributed throughout the respiratory samples. Therefore, dilution is recommended to distinguish pathogens from normal flora. This however, does not appear to be true for fungi, especially moulds because of their slower growth rate and inability to multiply into separated cells. It is believed that hyphal fragments may only be present in some parts of the same sample; therefore, diluting the specimen may lead to false negative results. A good option is homogenisation of the sample; however, culture from some specimens may be difficult because of viscosity and high volume, especially for patients with allergic bronchopulmonary aspergillosis, including those with CF. In this case, sonication may be useful and has been shown before to increase fungal culture and PCR yields. The local clinical microbiology laboratory follows a modified BSOP57 method (500-fold dilution followed by inoculation volume enhanced 10-fold, from 1 to 10 μl). In this study, the detection rate for both moulds and yeast was only 4.3%, respectively, suggesting that the original BSOP57 method followed by many UK laboratories may be even less effective than the modified method presented in this study. The BSOP57 methodology for fungal culture still remains unchanged, as published in the most recent issue from September 2012. Whether higher volume undiluted MRCM method is optimal is also not known, but had it been implemented for the five patients we studied, all would have had A. fumigatus cultured. An optimised and standardised processing method for respiratory samples is required not only in the UK but also in other countries in Europe and possibly in the world. Lack of standardisation in the processing method leads to variations in relative organism prevalence rates in samples in many diagnostic laboratories.

qPCR was shown to be a very helpful and fast diagnostic tool for detection of Aspergillus and in this study
it was superior to culture in most cases, with a detection rate of 95.6%. The qPCR on respiratory specimens is currently used in the MRCM on a daily basis, and is much more sensitive than culture for sputum.\textsuperscript{2,2} We realise that the positive \textit{Aspergillus} qPCR results may however be derived from dead fungal material, especially if the patients have been given antifungal therapy, from fungal colonisation or simply from inhaled spores or fungal fragments trapped by the secreted mucus rather than from infection. It is impossible to distinguish these possibilities by qPCR (or culture), although high fungal loads are more likely to represent infection. Moreover, a positive qPCR should always be evaluated by clinicians who also base their decisions on patient’s clinical history, radiology, microscopy, culture or antigen detection. The culture is however often problematic due to the erroneous BSOP\textsuperscript{57} methodology. Improving culture methodology should decrease the disease burden of patients by fast and accurate treatment which is paramount, especially for immunocompromised patients in whom the morbidity and mortality is high and delay decreases survival\textsuperscript{6–7} because of \textit{A. fumigatus} and other pathogenic fungi.

Our results demonstrate an important comparison between the diagnostic yields between fungal culture and qPCR and between different respiratory samples. This small single centre study suggests that improved respiratory fungal diagnostics is required and is easily achievable for culture simply by increasing the volume plated. The current UK standard culture protocol is insensitive for detection of medically important fungi and should be reviewed. The optimal culture method for fungi is not known and may require additional modifications, such as sonication.\textsuperscript{17} In the absence of culture improvements, \textit{Aspergillus} qPCR would dramatically increase the detection of \textit{Aspergillus} from respiratory specimens. This study clearly demonstrates a problem in sensitivity of detection of fungi in clinical settings; however, we realise that our high volume undiluted sample approach and qPCR may possibly result in overdiagnosis especially in patients producing high volume of dense mucus (such as CF) that trap inhaled fungal spores or hyphal fragments but not necessarily lead to development of disease. Further studies are necessary to confirm this theory, resolve the issue around fungal culture and to increase the sensitivity of fungal diagnosis.

Acknowledgments

We thank Julie Martin and Philip Barber for facilitating the collection of specimens at bronchoscopy.

Conflicts of interest

David Denning holds founder shares in F2G Ltd a University of Manchester spin-out company and has current grant support from the National Institute of Allergy and Infectious Diseases, National Institute of Health Research, the European Union and AstaRene.ca. He acts as an advisor/consultant to F2G and Myconostica (now part of Lab21 group) as well as other companies over the last 5 years including T2 Biosystems, Pfizer, Schering Plough (now Merck), Nektar, Astellas, GSK and Gilead Science. He has been paid for talks on behalf of Merck, Astellas, GSK, Novartis, Merck, Dainippon and Pfizer. Malcolm Richardson acts as an advisor for Gilead Science and Astellas Pharma. Caroline Moore has received travel grants from Astellas Pharma, has been paid for talks on behalf of Pfizer and has received grant support from Pfizer. The other authors report no conflict of interests.

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