British Society for Medical Mycology best practice recommendations for the diagnosis of serious fungal diseases

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Invasive fungal diseases are an important cause of morbidity and mortality in a wide range of patients, and early diagnosis and management are a challenge. We therefore did a review of the scientific literature to generate a series of key recommendations for the appropriate use of microbiological, histological, and radiological diagnostic methods for diagnosis of invasive fungal diseases. The recommendations emphasise the role of microscopy in rapid diagnosis and identification of clinically significant isolates to species level, and the need for susceptibility testing of all *Aspergillus* spp, if treatment is to be given. In this Review, we provide information to improve understanding of the importance of antigen detection for cryptococcal disease and invasive aspergillosis, the use of molecular (PCR) diagnostics for aspergillosis, and the crucial role of antibody detection for chronic and allergic aspergillosis. Furthermore, we consider the importance of histopathology reporting with a panel of special stains, and emphasise the need for urgent (<48 hours) and optimised imaging for patients with suspected invasive fungal infection. All 43 recommendations are auditable and should be used to ensure best diagnostic practice and improved outcomes for patients.

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Introduction

Invasive fungal diseases are a worldwide health problem, not only in immunocompromised patients and those undergoing intensive-care treatment, but also increasingly in patients with chronic disorders such as chronic lung diseases.[1–5] Despite the discovery of new antifungal agents and formulations, the morbidity and mortality of invasive fungal diseases is high.[1,6,7] Therefore, early recognition and diagnosis of mycoses have become a major focus for improvement of the management and outcome of these infections.[5,8,9]

In 2003, a working group of the British Society of Medical Mycology (BSMM) proposed quality-of-care standards for patients with invasive fungal infections.[10] These standards attempted to provide guidance for microbiology and histopathology laboratories and radiology and clinical specialists for improved use of available diagnostic tests for the management of invasive fungal diseases. Subsequent audits of these standards identified areas for improvement.[11,12]

Inclusion of antigen testing and radiology within the consensus definitions for invasive fungal infections by the European Organization for Research and Treatment of Cancer (EORTC) and Mycoses Study Group (MSG) lead us to include best practice guidance for the use of antigen and molecular testing, and more detailed radiology diagnostics, in this document.[9,13] The European guidelines for diagnosis and management of candida diseases were likewise considered, whereas guidance for antifungal treatment was omitted since clinical guidelines have been published.[14] We define best practice recommendations for the microbiological (panel 1), histopathological (panel 2), and radiological (panel 3) diagnostic investigation for diagnosis of serious fungal diseases, including serology, molecular diagnostics, and susceptibility testing.

Microbiology best practice

Direct microscopy, for many sample types, provides an important diagnostic benefit that is greater than culture alone. Another advantage of microscopy is the rapid availability of results—often within 2–4 h of a specimen's arrival in the laboratory.[15] Rapid processing and reporting is important, because delayed diagnosis of an invasive fungal infection can be lethal.

Microscopy can distinguish whether an infection is caused by a septate mould (*Aspergillus* spp) or non-septate mould of the order Mucorales (members of the families Mucoraceae, Cunninghamellaceae, Saksenaeaceae, Mortierellaceae, and Syncephalastraceae), which affects the choice of antifungal treatment. Direct microscopy is especially important for diagnosis of non-septate fungi, because these fungi are poorly recovered by culture, partly as a result of damage during refrigeration or homogenisation of tissues. Many other distinctive fungi can be provisionally identified by direct microscopy (capsule of *Cryptococcus* spp, spherules of *Coccidioides* spp, or small intracellular yeasts of *Histoplasma capsulatum* or *Talaromyces* [*Penicillium*] *marneffei*). Direct visualisation of fungi in diagnostic sterile fluids or tissues likewise aids confirmation of cases of invasive fungal disease when a fungal growth alone could be a result of culture contamination.[9] Centrifugation and staining (Gram stain, India ink, or optical brighteners) of liquid specimens concentrates fungal elements and increases the probability of detection. Microscopy can be useful for several specimens, which are discussed below.

First, blood cultures should be done in all cases in which systemic fungal infections are suspected, with a large volume of blood and recommended commercial systems and media.[16,17] Gram stain of routine blood cultures is suitable for detection of the most prevalent

fungaemia pathogens (*Candida* spp and *Cryptococcus* spp), but is unsuitable for speciation and identification of moulds.[18] The clinical usefulness of microscopy can be improved by the use of peptide nucleic acid fluorescent in-situ hybridisation (PNA-FISH), which can identify *Candida* species that are likely to be fluconazole-resistant

Panel 1: **Microbiology best practice recommendations**

Microscopy and stains

- Fluids from usually sterile sites and bronchoalveolar lavage (BAL) from patients with suspected infection should be examined by direct microscopy with suitable methods for fungal detection*
- Adequate tissue for histology and culture should be ensured before direct microscopy is done on the rest of the sample
- Optical brighteners are recommended for microscopy on all samples from immunocompromised patients
- Direct fluorescent-antibody staining, PCR, or both is recommended for patients with suspected pneumocystis infection
- India ink staining of cerebrospinal fluid samples from immunocompromised patients is recommended in addition to Gram staining if cryptococcus capsule antigen (CRAG) testing is not available on site

Culture and identification

- Bronchoscopy fluids should be cultured in suitable media to support fungal growth*
- Yeasts cultured from urine samples should be identified to species level and reported for all critical care and immunocompromised patients*
- All clinical isolates of aspergillus from patients who will receive antifungal treatment should be identified to species complex level, by referral to a specialist laboratory if necessary*
- All fungi (yeasts and moulds) obtained from sterile sites, including blood and continuous ambulatory peritoneal dialysis fluids, and intravenous line tips should be identified to species complex level by referral to a specialist laboratory if necessary. Bronchoscopy fluid and paranasal sinus material is regarded as sterile in this context for all fungi except *Candida* spp*
- All aspergillus isolates from patients who have allergic bronchopulmonary aspergillosis, aspergilloma, or acute or chronic aspergillosis should be susceptibility tested for antifungals used for treatment (eg, voriconazole or itraconazole) if therapy is initiated; isolates should be stored for 6 months in case additional susceptibility testing is needed at a later date
- Any amount of fungi cultured from vascular-device tips should be identified to species level and reported

Respiratory specimens

- At least three sputum samples should be obtained for detection of respiratory fungi, unless a validated PCR method is used
- BAL fluid is recommended for diagnosis of invasive fungal disease in patients with haematological disease
- Respiratory and fluid samples should be concentrated by centrifugation at 1000 g or greater for at least 10 min or with the cytocentrifuge before microscopy
- Respiratory samples should be liquified, especially for detection of *Pneumocystis jirovecii*
- Isolation of *Aspergillus* spp from respiratory samples should be reported with interpretative comments according to patient risk group and likelihood of invasive, chronic, or allergic disease

Cerebrospinal fluid (CSF) specimens

- All CSF specimens that are from immunocompromised patients, from those with sarcoidosis or cancer, or who show abnormal concentrations of glucose, protein, or leucocytes without an adequate explanation should be cultured and antigen tested for *Cryptococcus neoformans*; all bacterial plates should be incubated for a minimum of 5 days and fungal media incubated at 30°C for up to 21 days

Fungal serological and molecular testing

- Serum samples from immunocompromised patients with presentations consistent with cryptococcal meningitis for whom a CSF specimen is not available (eg, cases in which lumbar puncture is contraindicated) should be tested for *Cryptococcus* spp antigen (CRAG)
- Galactomannan screening of serum (two times per week) from patients with haematological malignancies at high risk of invasive aspergillosis should be considered in those not receiving mould-active prophylaxis; optical density (OD) index threshold of 0·5 has a high negative predictive value, enabling invasive aspergillosis to be excluded
- Galactomannan testing of BAL from patients at high risk of invasive aspergillosis should be considered, although the current OD index cutoff of 0·5 might change
- β-D-glucan screening of serum from patients at high risk of invasive fungal disease should be considered; a negative result has a high negative predictive value, enabling invasive fungal disease to be excluded
- PCR screening of serum for aspergillus from patients at high risk of invasive fungal disease should be considered; a negative result has a high negative predictive value, enabling invasive fungal disease to be excluded
- Combination testing with aspergillus PCR plus another antigen test improves the positive predictive value and diagnosis of invasive fungal disease
- Patients with pulmonary cavities of uncertain cause (with or without an aspergilloma) should have serum samples tested for antibodies to aspergillus
- Patients with suspected allergic bronchopulmonary aspergillosis should have serum samples tested for total IgE and aspergillus-specific IgE

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Antifungal drug-susceptibility testing

- Isolates of *Candida* spp from sterile sites, or from patients not responding to therapy at a minimum should have their susceptibility tested against fluconazole
- Isolates of *Aspergillus fumigatus* should have their susceptibility tested against antifungal agents used locally for treatment (eg, itraconazole and voriconazole) if antifungal treatment is given

Therapeutic drug monitoring

- No indications for therapeutic drug monitoring of amphotericin B or the echinocandins; measurement of fluconazole concentrations is rarely necessary
- Therapeutic drug monitoring of itraconazole, voriconazole, and posaconazole is usually needed. Specifically, voriconazole monitoring is needed in most patients, and certainly in children, including repeat monitoring after dose changes and shift from intravenous to oral treatment; dose optimisation during long-term therapy needs such monitoring
- Flucytosine monitoring is recommended for all patients receiving treatment

Clinical requests and reporting

- All test requests should state whether the patient is immunocompromised*
- All intravascular devices should be removed promptly if clinically feasible after diagnosis of candidaemia
- All new fungaemia, positive results of microscopy on sterile tissues or fluids, and positive cryptococcal antigen and galactomannan results should be telephoned or actively communicated by laboratory staff to clinicians within 2 h of their availability

*Previously published BSMM standards[10]

in 100% of cases within 2 hours of a positive Gram stain.[19–21] Another rapid technique for identification of fungi directly from blood cultures is matrix-assisted laser desorbtion/ionisation time-of-flight mass spectrometry (MALDI-TOF), but MALDI-TOF is less sensitive and specific than PNA-FISH.[22]

Second, discrimination between colonisation and infection is difficult when yeasts are seen in respiratory samples, since colonisation is common with candida, and pulmonary infection is rare. For moulds, sensitivity of sputum microscopy can be increased with larger sample volumes and repeated sampling, and usually represents disease in at-risk patients.[23,24] Overall sensitivity of culture and microscopy of bronchoalveolar lavage (BAL) fluid in invasive aspergillosis is about 50% in high-risk patients with haematological disease. Sensitivity of fungal culture is increased in patients with severe and advanced infection.[23,25–28] BAL is the preferred specimen for *Pneumocystis jirovecii* microscopy in patients without AIDS, whereas in those with AIDS, induced or routine sputum might be as sensitive as BAL.[29–32] Microscopy of respiratory samples is also helpful for diagnosis of cryptococcosis and infections with endemic fungi. For diagnosis of fungal sinusitis, microscopy of sinus biopsy tissue can be done rapidly with a wet potassium hydroxide preparation, which has a sensitivity of 78%, when compared with histological examination in a study of aspergillus sinusitis.[33]

In a review of cryptococcal meningitis, India ink staining of CSF gave an overall sensitivity of 60% in HIV-positive and HIV-negative immunocompromised patients.[34] For candida meningitis, microscopy gives a positive result in about 40% of Gram-stained CSF.[35] Microscopy of undiluted, centrifuged urine can rapidly detect the presence of fungi and tubular casts.[36] However, the importance of urine microscopy for guidance of the management of invasive fungal diseases is unclear in at-risk patients. Because the presence of fungus in tissue is diagnostic for proven fungal infection, biopsy of infected tissue should be attempted whenever possible.[9] Tissue samples should be kept moist during transport, processed promptly, and cultured in fungal media (up to 14 days or longer if positive on microscopy or evidence of possible endemic fungal infection exists).[14] Formal histopathological examination is preferable to rapid smears.[33,37,38]

Rapid and easily reproducible staining methods typically used in routine microbiology or cytology laboratories

***Panel 2*: Histopathology best practice recommendations**

Specialised stains

Specialised stains should be done in parallel with standard stains if mycosis or another infection is to be assessed or excluded*

- Standard stain: haematoxylin and eosin (H&E) on histopathology slides; Giemsa or Papanicolaou on smears
- Triple set of stains: Ziehl–Neelsen stain for acid-fast organisms; Gram stain for bacteria, fungi, and others; Grocott silver stain, or periodic acid–Schiff, to highlight fungi

Reporting of results

Report fungal morphology (yeast or hyphae), including the following

- Whether a yeast is small, medium, or large
- Whether a yeast has cross walls or septa (ie, is splitting rather than budding)
- Whether a hyphal form has usual width, or has a dilated, bizarre shape
- Whether H&E-stained fungi are pigmented and brown, or are unpigmented and colourless or pale blue

Positive results should be telephoned to clinicians immediately*

*Previously published BSMM standards[10]

Panel 3: Radiology best practice recommendations

Patients with leukaemia, and patients who have undergone haemopoietic stem cell or solid organ transplantation

All patients with leukaemia and patients who have undergone haemopoietic stem cell transplantation, who are, or who have been, profoundly neutropenic (<500 neutrophils/mL) with any of the following should have a high-resolution (or spiral) or, preferably, multidetector CT scan of the entire thorax within 48 h, with immediate consultant review*

- A new cough, chest pain, or haemoptysis
- An abnormal chest radiograph
- A new positive culture of an *Aspergillus* spp or other mould from any site
- Microscopic evidence of hyphae in any invasive sample
- Unresolved temperature after 5 days of antibiotics, antifungals, or both
- Positive fungal biomarkers (ie, galactomannan, β-D glucan)

All transplant recipients who test positive by microscopy, PCR, galactomannan, or culture of *Aspergillus* spp or other mould should have a CT scan of the chest (as above) within 48 h*

Immunocompromised patients with new neurological features

All immunocompromised patients with new neurological features (eg, change in mental status, seizure, stroke, or persistent headache) or possible or proven meningitis should have MRI of the brain within 48 h (or if not possible, a contrast-enhanced CT scan, but not a non-enhanced CT scan)*

Suspected invasive fungal sinus infection

All patients with suspected invasive fungal paranasal sinus infection should have a non-contrast CT scan within 48 h

Suspected disseminated fungal infection

Patients undergoing investigation for disseminated fungal infection should have an MR or dual-phase CT scan of the abdomen

There should be a low threshold for repeat scanning in patients with suspected cerebral and hepatosplenic fungal infection

Suspected pneumocystis infection in patients without HIV

In patients not infected with HIV who have possible pneumocystis pneumonia, a CT scan of the chest is important to make differential diagnoses, in combination with respiratory sample testing for *Pneumocystis jirovecii*

*Previously published BSMM standards[10]

include Gram stain, India ink, optical brighteners, Papanicolaou stain, methenamine silver, and immunofluorescent stains, but large comparative studies of methods have not been done. Gram stain smears show fungi in most cases because most fungi are Gram-positive. This method is used routinely for blood cultures and shows yeasts and other fungi. For CSF samples, Gram staining in a large study had a sensitivity of 85% for culture-confirmed cryptococcal meningitis.[39] For diagnosis of cryptococcal disease, India ink is a more specific stain than Gram stain since India ink creates the characteristic halo effect defining the capsule.[34,40] Use of optical brighteners (Calcofluor white or Blankophor) needs a fluorescence microscope. Optical brighteners can greatly enhance detection of fungal structures in respiratory and other specimens (figure 1).[41–44] For diagnosis of *P jirovecii* infection, direct immunofluorescence is a sensitive method (86–97%) for BAL and induced sputum.[45–49] However, results of several studies have shown that PCR is more sensitive and has a higher negative predictive value than microscopy.[50,51] Both stains can be used for detection of *P jirovecii*, but are less sensitive than immunofluorescent staining.[42,46,52–56] For detection of *Coccidioides* spp, Papanicolaou staining, rather than Blankophor, is recommended.[57,58]

Isolation of aspergillus from respiratory samples can suggest infection, allergy, colonisation, or laboratory contamination. The positive predictive value of such isolates for invasive pulmonary aspergillosis varies according to immune status.[23,59] Although BALs are useful for deep lung infections, these specimens might not always be obtainable, in which case three sputa specimens should be sent for microscopy and culture.[23] Nasal swabs growing aspergillus have proved to predict invasive aspergillosis in acute leukaemia, but are not recommended as a routine diagnostic method.[60] Positive respiratory cultures can represent allergic or chronic pulmonary aspergillosis, both of which are responsive to antifungal therapy.[23,61]

Visualisation of fungi by direct microscopy helps to confirm that the organism is in the sample rather than contamination. Repeated isolation of the same species in two or more samples is associated with increased likelihood of invasive pulmonary aspergillosis, as is the quantity of fungi by culture, although these findings have not been standardised.[62–65] Isolation of *Aspergillus flavus* seems to be consistently associated with an increased risk of invasive aspergillosis, and *Aspergillus niger* is associated with a decreased risk.[23,59,65] A positive serum aspergillus IgG might also increase the clinical significance of aspergillus cultured from respiratory specimens.[66,67]

However, these tests only provide indications, and the presence of these organisms in respiratory cultures needs to be interpreted in view of underlying disease, risk of fungal infection, and other diagnostic findings. Reporting comments should help to guide clinicians about the clinical significance of the isolate. Storage of clinically significant isolates enables susceptibility testing to be done if needed. Such testing might be requested months after initiation of antifungal therapy, so isolates should be kept for at least 3 months.

Candiduria might be a manifestation of contamination from the perineum or genital tract, urinary tract infection, colonisation of urinary catheter, or a sign of candidaemia or disseminated infection.[67] Clinically significant candiduria rarely occurs in otherwise healthy individuals.[68] However, in patients with high risk of invasive candidosis (immunocompromised, low-birthweight, premature babies, and intensive care and burns patients), any appearance of yeasts in urine should be identified to species level and reported to guide appropriate antifungal treatment if necessary.[69] Speciation might also lead to diagnosis of unexpected infections due to *Cryptococcus neoformans* or resistant *Candida* spp.

To assess the clinical significance of candiduria in catheterised patients, re-culture of urine after catheter removal is advisable. If the repeat urine culture is negative, antifungal treatment is usually not warranted.[69] By contrast, persistent candiduria can mean upper urinary tract infection, and further renal imaging is needed to exclude renal obstructions.

Diagnosis of fungal meningitis such as cryptococcosis is well established and discussed in the serology and microscopy sections.[34,70,71] Fungal media should be kept for long-term CSF culture (up to 21 days) at 30°C.[72] Candida meningitis yeasts can be seen with Gram stain and cultured on Sabouraud agar. Rare causes of fungal meningitis include *Coccidioides* spp (best test is CSF antibody), *H capsulatum* (best test is histoplasma antigen), and *Aspergillus* spp (best tests are galactomannan and PCR).[73,74]

Two key advances in the diagnosis of invasive fungal diseases are antigen testing and molecular techniques. International study groups have published and revised consensus diagnostic criteria for invasive fungal diseases, including serological testing.[9,13] Although intended for use in clinical research, these definitions have clear potential to affect patient management, and might contribute to improved outcomes.[75–77] *C neoformans* and *Cryptococcus gattii* shed a capsule antigen (CRAG) that can be detected in the CSF and serum of infected patients with commercial assays.[70,71,78–81] The simple lateral-flow device for serum, plasma, urine, and CSF CRAG detection is used in many laboratories for screening and diagnosis.[80] In symptomatic patients with HIV, CRAG detection is highly sensitive and specific.[70] The usefulness of monitoring CRAG titres in serum in patients without HIV continues to be debated, but is unreliable in HIV-positive patients.[34,82] However, increasing or persistently high CRAG titres might herald therapeutic failure or relapse, and might be useful to distinguish immune reconstitution from persistent cryptococcal infection.[83]

Galactomannan (aspergillus antigen) detection in body fluids is more sensitive than culture for diagnosis of invasive aspergillosis. In serum, the sensitivity is variable (17–100%), having a 0·5 cutoff ratio with the highest sensitivity in patients with haematological disease who are not on antimould prophylaxis.[84,85] For screening, the key factors that affect accuracy of aspergillus antigen testing are the prevalence of invasive aspergillosis and effect of antifungal prophylaxis. The positive predictive value of the test increases from 31% in a population with 5% prevalence to 69% when the prevalence is 20%.[86] As a result, galactomannan screening is unlikely to be beneficial and cost effective if the probability of invasive aspergillosis before testing is low, and should be reserved for high-risk populations (patients undergoing allogeneic-stem-cell transplantation, those with acute myeloid leukaemia, and those undergoing aggressive chemotherapeutic regimens for relapsed disease). A meta-analysis[87] supports the 0·5 cutoff and shows that overall sensitivity is 78% and specificity is 81% in neutropenic patients. This moderate-quality evidence supports the recommendation that serial screening of blood specimens from high-risk patients is appropriate when invasive aspergillosis prevalence exceeds 7% and no antimould prophylaxis is given. Additionally, galactomannan detection is widely used in BAL specimens with evidence that galactomannan values of OD index of 0·5–1·0 have decreased predictive values compared with results of greater than 1·0.[88] The test has diagnostic merit in patients who are undergoing lung transplantation or who are in intensive care.[89–91]

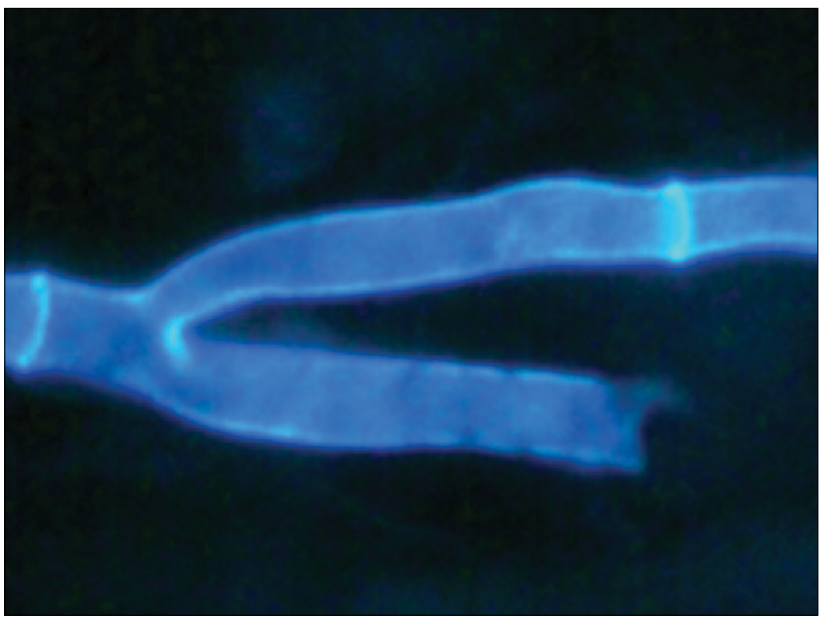

***Figure 1*: Calcofluor white staining of *Aspergillus* spp hyphae**
Fluorescence microscopy (x100) showing Calcofluor white staining of centrifuged bronchoalveolar lavage from a patient with acute myeloid leukaemia presenting with febrile neutropenia and cough. Bright fluorescent dichotomous branching septate hyphae can be seen, which were later confirmed by culture to be *Aspergillus fumigatus*.

Cross-reactivity and false positives arise as a result of dietary and medical factors, reducing the specificity of the test in serum and BAL specimens. Piperacillin–tazobactam was a source of false positives, but the problem has been resolved.[92,93]

1,3-β-D-glucan (BDG) is a carbohydrate moiety in the cell walls of many fungi, and is produced in vivo during infection by several important fungal organisms (*Aspergillus* spp, *Candida* spp, and *P jirovecii*, but not by *Cryptococcus* spp or species of the order Mucorales). The BDG test is indicated for the presumptive diagnosis of invasive fungal disease, and seems to be sensitive with a good negative predictive value (ie, excludes infection).[94,95] This test might have a role in some care pathways, making use of the high negative predictive value, but positive results will always necessitate further investigations. False positive results can occur, resulting from gauze dressings, dialysis, and some bacteria.[95,96]

The test is useful for diagnosis of pneumocystis pneumonia, especially when a respiratory sample cannot be obtained.[95]

Commercially available ELISAs for detection of candida antigen (mannan) and anti-mannan antibodies are available for diagnosis of candida infection, but little clinical assessment has been done, and testing might not detect some candida species.[97] On the basis of the poor quality of evidence, we do not recommend testing for mannan and anti-mannan antibodies at present, although other guidelines provide some indication for use.[14]

Detection of aspergillus antibodies (mainly *Aspergillus fumigatus*) is useful for diagnosis of several forms of aspergillosis in immunocompetent patients. Absence of comparative studies of methods restricts highly specific recommendations; in UK laboratories, passive diffusion, counter immunoelectrophoresis, in-house and commercial ELISAs, and semiautomated fluorescent immune assay systems are all used.[98] Despite these caveats, increased concentrations of IgG against aspergillus (often called aspergillus precipitins) are useful to confirm chronic pulmonary aspergillosis and aspergilloma. Patients with allergic bronchopulmonary aspergillosis in asthma and cystic fibrosis and those with aspergillus bronchitis might likewise have increased concentrations of IgG antibodies. A raised total serum IgE (>1000 IU/mL), an increased concentration of *A fumigatus* IgE, or both are essential criteria for diagnosis of allergic bronchopulmonary aspergillosis.[99]

Molecular or nucleic acid amplification tests have the potential to improve diagnosis of invasive fungal diseases, but are not implemented in most diagnostic laboratories. Scarcity of standardisation and absence of fully assessed commercial systems mean that PCR testing was not included in the 2002 or revised 2008 EORTC and MSG diagnostic criteria. However, publication of standards for aspergillus and candida assays and protocols for whole blood and serum ensure efficient DNA extraction, amplification, and standardised methods.[100–103] Agreement about international standards and the availability of external quality-control schemes have enabled robust validation of analytical performance, clinical use has been partly assessed, and prospective controlled trials clearly show screening of some patient groups to be useful.[104,105] The usefulness of PCR has been reviewed for diagnosis of candidosis and invasive aspergillosis in meta-analyses.[106,107] As a result, moderate evidence supports use of molecular assays for blood specimens for diagnosis of candida and aspergillus infections in immunocompromised patients. Data for pan-fungal assays are scarce, and these assays are still being assessed. Aspergillus PCR has also been applied to BAL fluid and sputum specimens, but insufficient data exist to recommend PCR as a sole diagnostic technique.[108–110]

For diagnosis of pneumocystis pneumonia, PCR in-house assays have been used for more than 20 years, and commercial assays are available.[51,111] Pneumocystis PCR is more sensitive than staining methods and, when used on deep respiratory specimens, showed excellent diagnostic value and was sufficient to confirm or exclude diagnosis of disease in high-risk patients. More data for performance in other specimens (sputa or blood) and in different patient populations are needed.

Galactomannan, BDG, and aspergillus PCR all have a high negative predictive value and are ideally suited for screens to exclude diagnosis of invasive aspergillosis. Positive predictive values are suboptimum since disease prevalence is low. A combination of biomarkers increases confidence in a diagnosis, and results of a multicentre randomised trial[105] showed that a combination of galactomannan testing and PCR increased sensitivity of invasive aspergillosis diagnosis and enabled more rational use of antifungal agents. These findings have been supported by real-life observational studies reporting that a combination of galactomannan and PCR leads to accurate detection of invasive aspergillosis infection, and that diagnosis precedes development of overt disease, enabling earlier initiation of antifungal treatment.[112,113] Results of retrospective studies[114,115] also suggest that the combination of a molecular and an antigen-based test is best for diagnosis, but which commercial antigen assay is best is uncertain.

Culture, microscopy, PCR, and antigen-test positivity are reduced by effective antifungal therapy, such that screening assays are not appropriate for patients receiving adequate prophylactic or empirical antifungal therapy.[116] Persistently positive tests in a patient receiving ostensibly appropriate therapy might suggest resistance, low antifungal concentrations, inadequate source control, or a sequestered site (ie, intravascular device or aspergilloma). Blood-culture sensitivity for *Candida* spp, especially *Candida albicans*, is reduced by fluconazole, and presumably by other effective agents. In serum, aspergillus antigen detection is adversely affected by itraconazole and other drugs, but is affected to a lesser extent in BAL fluids.[116–119] Aspergillus PCR sensitivity in BAL fluids is reduced by dual antifungal therapy, but pneumocystis PCR does not seem to be diminished within the first 7 days of therapy, although more data are needed.[120,121] Antifungal therapy takes years to reduce the sensitivity of aspergillus IgG or IgE testing, and takes days or weeks to reduce circulating BDG.[122,123]

Antimicrobial susceptibility testing is clinically useful if resistance is associated with therapeutic failure, the test is done in a timely manner, and resistance is sufficiently common to warrant testing. The method has a major effect on the test result, and validated and standardised assays for antifungal susceptibility testing such as the European Committee for Antimicrobial Susceptibility Testing (EUCAST) or Clinical and Laboratory Standards Institute (CLSI) reference methods are therefore recommended.[124] Some commercial assays, including Etest (bioMerieux SA, Marcy L'Etoile, France) and Sensititre YeastOne system

(Trek Diagnostic Systems Ltd, East Grinstead, UK), match the EUCAST and CLSI criteria for several antifungal agents, but other tests might have variable reliability.[124] The automated VITEK2 system (bioMerieux SA, Marcy L'Etoile, France) for analysis of candida is not concordant with the EUCAST azoles breakpoints, and might not identify echinocandin susceptibility reliably for some *Candida glabrata*.[125,126] Genotypic resistance might be a better marker of resistance than phenotypic testing, especially for echinocandin resistance in candida. Our recommendations for susceptibility testing are fluconazole and flucytosine for *Candida* spp, and itraconazole and voriconazole for *A fumigatus*, but testing should also be guided by local epidemiological data for antifungal resistance.[127–129] Routine testing of all isolates is unnecessary unless periodical surveillance of susceptibility is done as part of antifungal stewardship.[14] Only isolates from severe infection, isolates that need long-term therapy, or isolates cultured during antifungal therapy need susceptibility testing.

Therapeutic monitoring of antifungal agents

The pharmacokinetics of antifungal agents can vary between patients for various reasons (unpredictable absorption, compliance, metabolism, elimination, or drug–drug interaction), leading to inconsistent serum concentrations. Therapeutic drug monitoring guidance has been published by the BSMM and is recommended for some antifungal agents to monitor therapeutic serum concentrations (for itraconazole and posaconazole) or to avoid toxicity (for flucytosine and voriconazole).[130–132] Indications for therapeutic drug monitoring of amphotericin B or echinocandins do not exist, because little variation between individuals occurs, and plasma concentrations do not relate to tissue concentrations, efficacy, or toxicity.[132]

Requests for, and reporting of, laboratory tests

With increasing demand for hospital diagnostic services and the importance of appropriate and timely management of immunocompromised patients, sufficient clinical details should crucially be provided with the request for investigations.[133,134] The requester should state whether the patient is immunocompromised for laboratory and radiology departments to prioritise, assist with appropriate test selection, and rapidly communicate positive results. All new fungaemia, positive fungal microscopy (sterile tissues or fluids), and cryptococcal antigen and galactomannan results should be provided to the clinical team within 2 hours of their availability.[135]

Histopathology best practice

These recommendations (panel 2) apply to all relevant samples received by cellular pathology departments. Detection of fungi in tissue (surgical or fibreoptic scope biopsy specimens, needle aspiration specimens, dab imprints, or autopsy tissues) often provides definitive diagnosis of invasive fungal disease. Speed is crucial for an early diagnosis of invasive fungal disease, and assessment of haematoxylin and eosin (H&E) stains of tissues before deciding whether to use specialised fungal stains frequently results in delays for patients. Specialised stains should be done in parallel with standard stains. Hyphae and yeasts are often invisible on standard sections stained with H&E stain or Gram stain alone.[136]

Screening of tissues for fungal and other infections

If an infection is to be assessed or excluded, a triple set of histochemical stains should be done in addition to initial standard stains (H&E on histopathology slides; Giemsa or Papanicolaou on smears) to optimise identification of infectious agents. This triple set of histochemical stains consists of: Grocott (methenamine) silver (GMS) stain or periodic acid–Schiff (PAS), to highlight fungi; Ziehl–Neelsen stain for acid-fast organisms; and Gram stain for bacteria and fungi. These infection stains should be used routinely in samples from immunocompromised patients (HIV; glucocorticoid therapy; malignant disease, including leukaemia; cancer chemotherapy; solid organ or bone marrow transplantation; congenital immunodeficiency; and immunosuppressive agents such as anti-tumour necrosis factor immunomodulators or methotrexate).

For visualisation of fungi in tissue, GMS stain is more sensitive than PAS, but has a signal-to-noise recognition problem because it stains tissue reticulin and the lysosomes of inflammatory cells. Additionally, the morphology of the tissue adjacent to the fungi can be better visualised with PAS than with GMS. For differentiation of some fungi, mucicarmine (cryptococcus capsule) and Fontana–Masson stain (dematiaceous fungi) are useful.[137] Moulds of the order Mucorales might need long staining times, but other fungi can become overstained, so good control sections are needed. In one study, different stains were assessed in an animal model of aspergillus keratitis, and GMS stain with an H&E counterstain gave the best results.[138] At least one specialised stain for fungi is recommended for tissues from immunocompromised patients concurrently with H&E and other relevant stains.

Recognition of common histological reactions to fungal infections

Fungi induce varied reactions in tissues in immunocompetent and immunocompromised hosts. Nonetheless, general clues suggest fungal infections (their presence, and sometimes the type). Both yeast and filamentous fungi can produce mixed granulomatous reaction purulent centres within granulomas. Several yeasts (*Histoplasma* spp and *Coccidioides* spp) characteristically induce granulomas with caseation necrosis, mimicking mycobacterial infections. Moulds of the order Mucorales, and candida, and aspergillus species are typical, but not exclusive, causes of vascular invasion and secondary thrombosis with infarction and

	Size	Other features	Key differential diagnosis
Yeast or yeast-like appearance			
Cryptococcus neoformans	5–15 μm	Clear mucoid capsule surrounding yeast cells	*Histoplasma capsulatum*
Candida glabrata	3–15 μm	No pseudohyphae	*H capsulatum*
H capsulatum	3–5 μm	Intracellular, many; often birefringent	Leishmania, *Talaromyces (Penicillium) marneffei*, *C neoformans*
Pneumocystis jirovecii	3–5 μm	Fine honeycomb	Fibrin exudates, alveolar proteinosis
Paracoccidioides brasiliensis	3–15 μm	Many buds off a single yeast, resembling a ship steering wheel	Blastomyces infection
Sporothrix schenckii	3–15 μm	Can be very scarce; pseudoepitheliomatous hyperplasia	Mycobacterial infection
Chromoblastomycosis*	5–15 μm	Brown pigmented, splitting cells	..
Hyphae only			
Aspergillus spp	5–15 μm	Septate; branching at 45° angle	Less common hyphae—eg, *Fusarium* spp, *Scedosporium* spp (hyaline), *Cladosporium* spp (pigmented)
Moulds of the order Mucorales*	15–100 μm	Usually non-septate; hyphal folding can mimic septation; irregular diameter hyphae; bizarre morphologies	*Aspergillus* spp
Mycetoma*	5–15 μm	Colonies or grains; pigmented or pale; expanded chlamydospores at periphery of colonies; Hoeppli–Splendore reaction; much fibrosis	Bacterial colonies of *Actinomycete* spp
Subcutaneous phaeohyphomycosis*	5–15 μm	Usually brown pigmented (seen in H&E-stained sections)	Mycobacterial infection
Dermatophyte infection*	5–15 μm	Usually hyphae, but may be dimorphic; typically seen in the epidermis and within hair shafts	..
Mixed hyphae and yeasts, unusual morphologies			
Candida spp	3–15 μm	Usually hyphae-like structures and yeasts	..
Coccidioides immitis/*Coccidioides posadasii*	15–100 μm	Large yeast-like structures containing endospores	Tuberculosis

H&E=hematoxylin and eosin. *Many genera and species of fungi.

***Table*: Morphological features of specific fungi important in presumptive identification**

haemorrhage. In skin, epithelial hyperplasia, mixed inflammation, and abscesses within the epidermis are characteristic of cutaneous mycoses. An absence of host cell reaction in immunocompromised patients is characteristic of pulmonary pneumocystis and disseminated cryptococcus infections.

Expectations of histopathology reporting

A skilled histopathologist should fulfil the following best practice (panel 2).[139] The histopathologist should be confident that a fungal infection is present in the tissue sample; describe whether the fungal morphology is yeast, hyphal, or both; and estimate whether a yeast is small, medium, or large (table). They should note whether yeast has cross walls or septa (ie, splitting rather than budding distinguishes *Histoplasma* spp from *T marneffei*); note whether a hyphal form is regularly septated or not (Mucorales are poorly septated), is the usual width of aspergillus hyphae (3–12 μm) or wider, or has a dilated, bizarre shape; and whether hyphae are pigmented, or unpigmented and colourless or pale blue (hyaline) when stained with H&E. The histopathologist should identify to genus level several common fungal infections by morphology such as *Aspergillus* spp, *Cryptococcus* spp, *Pneumocystis* spp, *Histoplasma* spp, *Coccidiodes* spp, Mucorales, and *Candida* spp (table). The accuracy of diagnosis of causation with histology is at best 80%. Concurrent positive cultures are therefore helpful, but are often negative. In-situ hybridisation and PCR might identify the causative species or genus.[140–143] Immunohistochemistry for mycoses is not widely available, and is now replaced by molecular diagnostics.[139]

Discussion of diagnostic and difficult cases with local clinicians, radiologists, and microbiologists is important. If the pathologist is uncertain about the presence or type of infection, then consultation with a histopathologist experienced in fungal infection or a mycologist with access to molecular diagnostics is appropriate. PCR diagnostics can increasingly be done with formalin-fixed paraffin-embedded (FFPE) tissues for fungi.[140–143] In the UK, specimens can be referred to the mycology reference centres, but results should be interpreted in the context of the clinical situation and the fungal morphology on histology. As medicine creates increasing numbers of immunocompromised patients, pathologists encounter more cases in which fungus is present, but genus or species cannot be identified by morphology. Examples are invasive dermatophytes, yeasts, and hyaline (colourless) hyphae that look like aspergillus. Culture of fresh material, PCR, or both with FFPE tissues is most useful in these cases.

Radiology best practice

Imaging has a crucial role in diagnosis and management of patients with suspected invasive fungal disease who are immunosuppressed (panel 3). Invasive fungal diseases should be considered with any new broad-spectrum antibiotic, persistent fever, new pulmonary symptoms, or infiltrates during substantial immunosuppression. Pulmonary infection is most common, but such patients are likewise susceptible to sinus, cerebral, hepatic, splenic, renal, bone, and disseminated fungal infection. The threshold for imaging should be very low when the patient has persistent fever and symptoms and signs of pulmonary infection (cough, shortness of breath, chest pain, bronchial breathing, pleural rub, or pulmonary decompensation), or when biomarkers are positive (panel 3).

Findings of a chest radiographs might seem normal or non-specific in neutropenic patients with invasive pulmonary aspergillosis.[144] The presence of nodules on the chest radiographs is the most specific finding for invasive fungal infection.[145] Overall, chest radiographs are inadequate for investigation of pulmonary invasive fungal diseases in this patient population, except for pneumocystis infection. Pneumocystis pneumonia can seem normal on a radiograph at presentation, followed by typical bilateral, perihilar, diffuse granular, or hazy (ground-glass) opacification, which becomes denser with worsening infection, progressing to areas of consolidation.[146]

Since CT has greater sensitivity and specificity than chest radiography for investigation of fungal infection, it is the investigation of choice, and often leads to a change in clinical management.[147–150] Patients with neutropenic sepsis should have a CT thorax examination within 48 h of the onset of pulmonary symptoms or persistent pyrexia despite broad-spectrum antibiotic therapy for 5 days (panel 3). In neutropenic patients, the thorax is usually imaged in its entirety with multidetector CT (MDCT), unlike the traditional high-resolution CT (HRCT). MDCT has become the investigation of choice for suspected invasive fungal disease, and is associated with increased radiation dose compared with HRCT, although the benefit of identification of small nodules suggestive of idiopathic pulmonary fibrosis (not often seen with HRCT) potentially outweighs the risk.[151] CT findings of pulmonary invasive fungal diseases are diverse in different patient groups. Specifically, the halo sign is transitory and an early feature of invasive aspergillosis (figure 2). Nodules larger than 1 cm are suggestive of invasive fungal disease rather than bacterial or viral infection.[152,153] Areas of consolidation on CT provide locations for targeted bronchoalveolar lavage. For diagnosis of pneumocystis pneumonitis, a CT scan is more sensitive for early detection of infection than chest radiograph. A common CT finding of pneumocystis is ground-glass density, where the lung looks white and contains vascular markings.

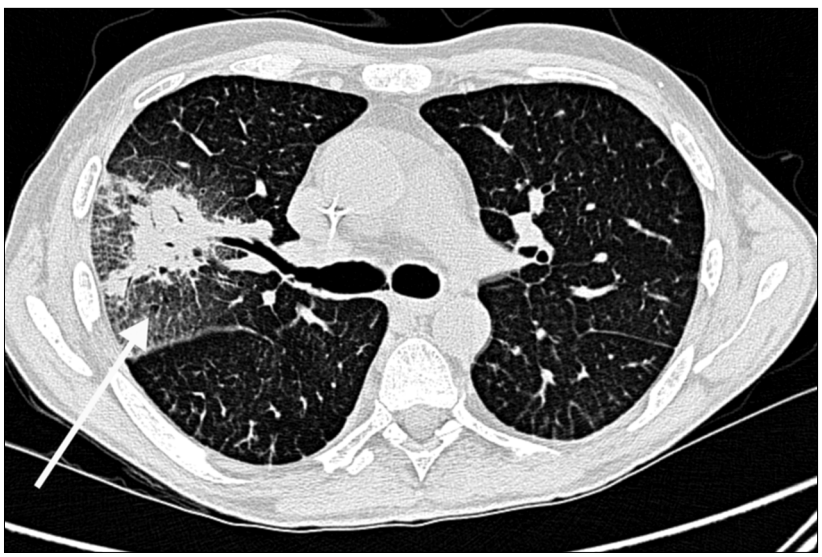

***Figure 2*: Invasive pulmonary aspergillus infection**
High-resolution CT scan of 41-year-old man with acute myeloid leukaemia and neutropenic sepsis. At the level of the carina on lung windows in the upper-right lobe, invasive fungal infection is seen as a mass with a peripheral halo (arrow) of ground-glass opacification.

Imaging of invasive fungal sinusitis and CNS infection

Cross-sectional imaging CT and MRI are crucial to assess patients with possible invasive fungal sinusitis; no role exists for plain radiography. Bone destruction and mucosal thickening seen in immunocompromised patients are most consistent with invasive fungal disease.[154] Once bone destruction has occurred, intra-orbital extension, intracranial extension, or both can occur rapidly, with soft tissue inflammation and abscess formation. As a result, a low threshold should be adopted for repeat imaging because bone destruction can be subtle.[155] MRI with gadolinium enhancement is better than CT for assessment of soft tissue detail in three planes (axial, sagittal, and coronal) for the extent of intracranial and intraorbital extension.[155–157] Invasive fungal infection of the CNS can occur as a result of direct sinus extension or haemogenous spread.[158] MRI with gadolinium enhancement should ideally be used for suspected invasive fungal infection. Brain CT imaging without intravenous contrast is insensitive and might be misleading. MRI findings due to direct sinus infection are dural thickening and enhancement, leptomeningeal enhancement, cerebritis, and cerebral abscess. Haemogenous spread causes cerebral abscesses, which appear as irregular variably enhancing ring lesions at the junction of the grey and white matter, sometimes with vasogenic oedema.[159,160] Cerebral aspergillosis might also appear as cerebral infarction with haemorrhage.[161]

Conclusions

We have updated the previously published BSMM standards of care for patients with invasive fungal infection and present them as 43 best practice

Search strategy and selection criteria

An agreement about the scope of the recommendations was achieved by the authors, on the basis of knowledge, experience, discussion, and advice from expert colleagues. For each recommendation, relevant published evidence was collected from database searches. References in English, French, and German were identified through PubMed searches for articles published from Jan 1, 1971, to Aug 1, 2014. Search terms varied depending on the precise point to be referenced, but several terms were used to ensure comprehensiveness. In subject areas for which many publications exist, systematic reviews and meta-analyses were sought. A summary of the referenced evidence for key recommendations is provided as an appendix.

See Online for appendix

recommendations. These recommendations provide the opportunity for microbiologists, histopathologists, radiologists, and clinicians to implement, assure, and audit best practice for the management of serious fungal diseases. The recommendations emphasise the role of microscopy in rapid diagnosis and identification of clinically significant isolates to species level, and the need for susceptibility testing of all *Aspergillus* spp, if treatment is to be given. We provide information to improve understanding of the importance of antigen detection for cryptococcal disease and invasive aspergillosis, use of molecular (PCR) diagnostics for aspergillosis, and the crucial role of antibody detection for chronic and allergic aspergillosis, and we emphasise the need for urgent (<48 h) and optimised imaging for patients with suspected invasive fungal infection. All 43 recommendations are auditable and should be used to ensure best diagnostic practice and improved outcomes for patients.

Contributors

SS led the British Society of Medical Mycology (BSMM) working group. All authors worked collectively on development of the recommendations described in this manuscript, reviewed the evidence for recommendations, and contributed to writing of the manuscript. All authors have read and approved the final version.

Declaration of interests

SBL declares no competing interests. SS has received honoraria from Pfizer. RAB has served on advisory boards, and has received sponsorship from Merck, Astellas, Gilead, and Pfizer. RAB has received sponsorship from Merck Sharp and Dohme (MSD), Pfizer, and Gilead. RCB received sponsorship from MSD. JRC received an educational honorarium from MSD. CCK has received honoraria from Astellas, MSD, Pfizer, and Gilead. DWD holds founder shares in F2G Ltd, and has grant support from the National Institute of Allergy and Infectious Diseases, National Institute of Health Research and European Union. DWD has been an advisor to F2G, T2 Biosystems, Pfizer, Merck, Nektar, Astellas, and Gilead. He has received honoraria from Merck, Astellas, GlaxoSmithKline, Novartis, Merck, Dainippon, and Pfizer.

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