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SPECIAL REPORT

ECIL-3 classical diagnostic procedures for the diagnosis of invasive fungal diseases in patients with leukaemia

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Invasive fungal diseases (IFDs) continue to cause considerable morbidity and mortality in patients with haematological malignancy. Diagnosis of IFD is difficult, with the sensitivity of the gold standard tests (culture and histopathology) often reported to be low, which may at least in part be due to sub-optimal sampling or subsequent handling in the routine microbiological laboratory. Therefore, a working group of the European Conference in Infections in Leukaemia was convened in 2009 with the task of reviewing the classical diagnostic procedures and providing recommendations for their optimal use. The recommendations were presented and approved at the ECIL-3 conference in September 2009. Although new serological and molecular tests are examined in separate papers, this review focuses on sample types, microscopy and culture procedures, antifungal susceptibility testing and imaging. The performance and limitations of these procedures are discussed and recommendations are provided on when and how to use them and how to interpret the results.
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Introduction

Invasive fungal diseases (IFDs) continue to cause considerable morbidity and mortality in patients with haematological malignancy.[1–5] Diagnosis of IFD is difficult, with the sensitivity of the gold standard tests (culture and histopathology) often reported to be low.[6] Therefore, physicians frequently rely on a constellation of clinical signs, imaging, culture, histopathology and adjunctive tests to establish a diagnosis.[7,8]

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Many of the classical diagnostic procedures found in the modern mycology laboratory are based on methods in use since the early days of the specialty, refined through experience rather than through controlled comparative studies. However, given the difficulty of diagnosing IFD and the increasing relevance of knowing the species and antifungal susceptibility of the causative organisms, it is important to optimize their isolation and microscopic identification in relevant samples. Insufficiency of the conventional diagnostic tests may at least in part be due to sub-optimal sampling, processing of samples or interpretation of the results.[9,10]

Although the various serological tests, that is, *Aspergillus* galactomannan Ag, *Cryptococcus* Ag, β-glucan and *Candida* mannan Ag and antimannan Ab have or will be dealt with in detail in separate papers,[11] the aim of this review is to provide an overview of the classical diagnostic options including imaging, microscopy, culture and susceptibility testing and their optimal use. A summary of these recommendations is provided in Tables 1 and 2. The recommendations were derived from published data generated in a number of different populations and were adapted to patients with haematological cancer when appropriate in the context of clinical relevance. However, the majority of these recommendations can be extrapolated to other patient categories as well.

Direct examination

Direct microscopic examination of samples is important for two reasons:[12] (i) it provides rapid information about the presence of fungi and other pathogens and may allow sufficient identification to guide management and (ii) it is more sensitive than culture for a number of samples.[13,14] Nevertheless, the result is only as good as the quality of the sample allows and thus obtaining optimal patient specimens is mandatory.

Sample types

Respiratory samples. The confirmation of many fungal infections is based on respiratory samples. Increasing the

Table 1 Recommendations regarding clinical specimens, processing and interpretation for leukaemic patients at risk of fungal infections

Test	*Recommendations*
Direct examination	Specimen and processing Sputum: at least three sputum samples. BAL fluid is preferred over sputum. *P. jirovecii*: liquefaction of respiratory samples should be used and special staining is required. *C. neoformans*: India ink should be used. Tissue biopsy: sufficient tissue for culture as well as histopathology (placed in fixative) should be obtained. Concentration: respiratory and fluid samples should be concentrated by centrifuging at $\geqslant$1000 g for at least 10 min. Cytocentrifugation is an alternative method. Stain Optical brighteners: are recommended for all samples. Silver staining is recommended for tissue sections when fungal infection is suspected. Direct immunofluorescent staining is recommended in patients with suspected *Pneumocystis* infection. India ink staining of CSF samples is recommended in addition to Gram staining.
Culture	Culture should always be attempted. Specific mycological media (for example, Sabouraud for moulds and yeasts and Chromogenic agar for yeast) are recommended. Fungi isolated from sterile sites should be identified to the species level. Surveillance cultures are recommended in patients with prolonged granulocytopenia receiving azole prophylaxis and should be identified to the species level.
Interpretation	Recovery of moulds from sputum in symptomatic patients with prolonged granulocytopenia (for example, allo-SCT, induction therapy for leukaemia) should be regarded as a possible indicator of fungal pneumonia). Definite diagnosis of invasive pulmonary aspergillosis should be attempted whenever possible. Positive blood cultures with yeast, dimorphic fungi and certain moulds (incl. *Fusarium* spp. and *Scedosporium* spp.) should be regarded as a sign of invasive fungal disease/disease and treated accordingly. Catheter-related infection should be suspected particularly when catheter drawn cultures are positive earlier than simultaneous peripheral cultures. Focal infections should be investigated with biopsy samples for histopathology, direct microscopy and culture. Positive results document proven infection whereas negative results do not rule out infection.
Susceptibility testing	Susceptibility testing is recommended (1) for invasive isolates /isolates from normally sterile sites, (2) in invasive and mucosal infections failing therapy and (3) for isolates otherwise considered clinically relevant in patients exposed to antifungals. Susceptibility testing of yeast and moulds should be performed in laboratories by staff with medical mycology training and regularly receiving samples for antifungal susceptibility testing.

Abbreviation: BAL = bronchoalveolar lavage.

number of sputum samples examined increases the sensitivity of detection, with three samples providing optimum yield in invasive aspergillosis.[15] Bronchoalveolar lavage (BAL) fluid provides a more representative sample from the lower respiratory tract and allows computed tomography (CT) scan abnormalities to be directly sampled, but the overall sensitivity of culture and microscopy for the diagnosis of invasive aspergillosis in the haematology population is 50% at best.[6,15–18] Sensitivity is increased in patients with more severe and advanced disease.[19] Microscopy provides approximately 20% greater diagnostic yield than culture.[13,14]

Induced sputum samples have a sensitivity of 55–94% for the detection of *Pneumocystis jirovecii* in HIV patients[20] and are suitable for the majority of such patients, provided measures are taken to safeguard staff and other patients from other respiratory pathogens. However, the incidence and prevalence of *P. jirovecii* pneumonia is much lower in the haematology population and the organism burden is also lower.[21] Consequently, the use of BAL, which in most studies has a higher sensitivity is recommended for leukaemic patients to optimize the diagnosis. PCR may also improve the sensitivity in these patients, but further discussion of this is outside the remit of these guidelines.

A diagnosis of proven invasive sinusitis requires histopathological evidence of tissue invasion. Microscopy of sinus biopsy material can be done rapidly by making a wet potassium hydroxide preparation and this had a sensitivity of 78% in comparison with histological staining of the same samples in a recent study on *Aspergillus* sinusitis.[22]

Although positive culture of nasal and throat swabs can provide evidence of fungal colonization (usually with *Candida* species) or support for the diagnosis of oropharyngeal candidiasis, direct microscopy of such swabs adds little to the culture result and is not recommended for routine practice. The presence of *Candida* species in BAL fluid, similarly, does not correlate with invasive lung infection.[23]

Cerebrospinal fluid. In a recent review of nine studies of cryptococcal meningitis in 848 patients, India ink gave an overall sensitivity of 57% (much higher in HIV-positive patients).[24] However, it should be noted that cryptococcal Ag assays are more sensitive and should be used where this infection is suspected.

Gram stain for yeasts is positive in approximately 40% of cases in *Candida* meningitis.[25]

Tissue. Proven IFD is defined by the presence of fungus in tissue for most cases of mould infection[26] and so biopsy material should be taken whenever possible. The diagnostic yield of microscopy has not been well defined in patients with invasive mould infections, but only approximately 50%

Table 2 Recommendations regarding imaging options of the chest and extrapulmonary sites

Imaging	*Recommendation*
Imaging of the chest	
Chest X-ray	Perform digital chest radiography in two planes in the upright position. Do not rely on normal chest X-rays.
CT scan	Perform CT early, owing to the much higher sensitivity as compared with chest X-ray. First choice is thin-section multi-slice CT (slice thickness *ca* 1 mm), pitch factor 1.5–2. Alternative option is HRCT (that is, incremental high-resolution CT) using slice thickness of approximately 1 mm and slice distance 10 mm (increment, that is, gap). Neither i.v. contrast enhancement nor electrocardiogram-trigger is necessary. However, digital image reading is strongly recommended.
MRI	MRI is superior to CT in the characterization of abscesses in the late phase of pneumonia. Recommended MR-sequences include: ultrafast T2 weighted inversion-recovery (for fluid and infiltrate detection), gradient echo-T1 weighted with fat-saturation with and without contrast enhancement (for detection of inflammation).
Imaging of extrapulmonary sites	
Brain, sinuses and orbita	Coronal CT is necessary for imaging of the paranasal sinuses and is superior to plain radiography especially for investigation of the ethmoidale and sphenoidale sinuses and the bony barriers. MRI is first choice for brain imaging (that is, central nervous system), orbital infection, and exclusion of those involvements.
Abdomen	CT is first choice for abdominal imaging, except for specific imaging of the liver in chronic disseminated candidiasis where MRI is superior.

Abbreviations: CT = computed tomography; MRI = magnetic resonance imaging.

of microscopy-positive samples are culture positive in invasive aspergillosis[27] and culture is positive in around 40% of cases of zygomycosis diagnosed by histopathology.[28] However, rapid direct microscopy appears less sensitive than formal histology[22] and while able to demonstrate the presence of fungal elements in a much shorter period of time, is less helpful in showing their invasiveness within tissues and their relation to tissue structures. Hence, it is extremely important to ensure that sufficient material is processed for histological examination before impression smears and 'squash' preparations are made.

Urine. Urine microscopy is able to rapidly identify the presence of fungi and casts in the urine. *Candida* casts seem specific for upper tract infection and, in a rabbit model of disseminated candidiasis, occurred in 46% of the animals, usually within the first 3 days.[29] However, the sensitivity in patients with haematological malignancy is not defined and the value of urine microscopy in guiding the management of IFD is unclear in this population.

Recommendations.

1. Sensitivity of sputum samples is increased with increasing numbers of samples. At least three sputum samples should be collected for the detection of respiratory moulds.
2. BAL fluid is recommended for the diagnosis of IFD in patients with haematological malignancy. Microscopy should be performed on all BAL samples in this population. For the diagnosis of *P. jirovecii* infection, special staining is required and thus the investigation should be specifically ordered when necessary (see below). The detection of *Candida* species should be interpreted as of low significance.
3. India ink has a sensitivity of approximately 60% in this population and provides rapid diagnosis of cryptococcal meningitis. However, it is recommended that cryptococcal Ag assay should also be performed.
4. Microscopy of impression smears or squash preparations of tissue biopsy material can provide rapid evidence of fungal infection, but it is less sensitive than histology. Sufficient tissue for the latter placed in fixative (and for culture not placed in fixative) must be ensured before direct microscopy is performed.

Processing

Concentration. Although not formally studied, concentration of fungal elements, suspended in a liquid sample, will increase the probability of their detection. This is usually achieved by centrifugation and making a smear of the deposit for Gram staining or resuspension of the deposit in a smaller volume of fluid containing a stain, such as India ink. The numbers of lymphocytes detected in centrifuged BAL samples have been shown to increase with increased rotation speed ($\geqslant$1200 vs 500 r.p.m.) and duration of centrifugation ($\geqslant$10 vs 5 min).[30] An alternative is the use of the cytocentrifuge, which has been used in a number of studies of diagnostic fungal microscopy.

Liquefaction. Although recommended for sputum processing, the impact of liquefaction on microscopy for fungal elements is not well defined. However, the diagnostic yield of respiratory samples for *P. jirovecii* was doubled when dithiothreitol was used for liquefaction before centrifugation and microscopy.[31]

Recommendations.

1. Although the optimal speed and duration of centrifugation has not been fully defined for fungal detection, respiratory and fluid samples should be concentrated by centrifuging at $\geqslant$1000 g for at least 10 min. Cytocentrifugation can be used as an alternative method.
2. Liquefaction of respiratory samples may be of benefit for the detection of all fungi and should be used for the investigation of *P. jirovecii*.

Stains
Stains used for direct sample examination are those which are rapid and suitable for a routine microbiology or cytology laboratory. These include Gram stain, optical brighteners (such as Calcofluor White and Blankophor), India ink, Papanicolaou stain, methenamine silver and immunofluorescent stains (for *P. jirovecii*). Whichever stain is used, it should be remembered that the sensitivity and specificity of direct microscopy is dependent on the quality, quantity and type of the specimen, the quality of the microscope and the expertise and mycological experience of the operator.[32]

Comparisons of staining methods for moulds in respiratory samples have involved small numbers in most cases and make conclusions about relative performance difficult to draw. In a study of calcofluor white vs methylene blue stains, *Aspergillus*-like hyphae were detected by calcofluor white in 56% of culture-positive sputum samples from cystic fibrosis patients vs 24% of those stained with methylene blue.[33] A non-comparative study of respiratory samples (mostly BAL fluids) from a mixed population including transplant recipients and granulocytopenic patients found a sensitivity of 88% for the detection of *Aspergillus*-like elements by Blankophor and a specificity of 99% in comparison with 76% sensitivity for culture.[34] The whole staining and microscopy procedure took a maximum of 12 min but requires fluorescence microscopy.

Many fungi stain well with Gram stain. A study of 2635 CSF samples found a sensitivity of 85% for patients with culture confirmed cryptococcal meningitis.[35] However, 68% of the total cases of meningitis diagnosed in this large study were cryptococcal, suggesting a bias in favour of HIV-infected patients, in whom India ink would also be expected to have a high sensitivity. Hence, it is not clear which stain is the more sensitive. However, the characteristic appearances of cryptococci in India Ink render this stain more specific.

The performance of various stains in the detection of *P. jirovecii* varies in different studies with direct immunofluorescent staining appearing the most sensitive for BAL and sputum/induced sputum (86–97%),[36–39] methenamine silver and Papanicolaou stains have similar sensitivities (79–100%; 80–100%) and each is superior to the other in different studies.[40–45] Calcofluor white appears less sensitive for this organism (60–77%).[37,45,46] It is possible that satisfactory performance is dependent on volume of sample provided. However, one study has shown that the detection rate for direct fluorescent Ab staining in samples of induced sputum of 0.5–$<$2.0 mL was not significantly different from that in samples $\geqslant$2.0 mL (18% vs 19%).[47]

It should be noted that PCR assays also have consistently high sensitivity for this.

Recommendations.

1. Optical brighteners provide a rapid and sensitive method for the detection of most fungi encountered in patients with haematological malignancies and are recommended for respiratory samples, pus, tissue samples and fluids from sterile sites from these patients when a fluorescence microscope is available.
2. Direct immunofluorescent staining is more sensitive than other stains in most studies for detection of *Pneumocystis* and is recommended as an option when this infection is suspected.
3. India ink staining of CSF samples is recommended for this patient group in addition to Gram staining.

Culture

Culture is important in order to achieve species identification and allow susceptibility testing.

Specimen types
Blood culture. Specific fungal media are available for several automated blood culture systems and depending on the blood culture system used may be important in order to detect all *Candida* species[48–51] or other organisms such as *Histoplasma* spp.[52] Lysis centrifugation blood culture systems have been recommended for detection of fungaemia because of better performance when compared with older blood culture systems. The system is labour intensive and associated with a higher risk of contamination. A recent study failed to detect any superiority when compared with modern automated blood culture techniques in a high-risk setting and hence this system is not recommended unless already routinely used.[53] There is no consensus regarding the optimal incubation time. Some *Candida* species, such as *C. glabrata*, require longer incubation time than *C. albicans*.

For aspergillosis and zygomycosis blood cultures are of limited utility.[54–56] In contrast, disseminated infections because of *Fusarium* spp. and *Scedosporium* spp. are associated with the recovery of the pathogen in blood cultures in approximately 50% of cases.[57,58]

CSF. Culture is highly sensitive (98%) in patients with *Cryptococcus* meningitis.[12,59] Culture allows diagnosis in cases where the Ag test is falsely negative, and allows further microbiological characterization and susceptibility testing.[12] It is also a marker of response to treatment.

CSF culture is less often positive in cases of *Candida* meningitis (80%)[25] and central nervous system aspergillosis or candidiasis, characterized by parenchyma rather than meningeal involvement.[60] Thus, diagnosis of central nervous system aspergillosis is often presumptive based on compatible clinical and radiological signs and documented invasive aspergillosis in other sites.[54]

Biopsies. Tissue biopsies, when available, have important diagnostic value. However, for some specific pathogens (for example, Zygomycetes), homogenization of the biopsy material reduces the culture yield and inoculating culture plates with slices of minimally manipulated tissue is preferable.[61] Importantly, tissue samples need to be sent to the microbiology laboratory without formalin fixation as opposed to those sent for histopathological examination.

Urine. Although easy to perform the diagnostic value of urine cultures is uncertain because of difficulties in interpretation of the result.[62] A positive culture may

represent contamination by perineal yeast flora, bladder/catheter colonization or true infection. Contamination should be considered if a subsequent sample is culture negative. But differentiation between colonization and infection is difficult. The presence of WBCs, the quantification of colonies, and the presence of pseudo-hyphae are not reliable.[62] Therefore, interpretation of the presence of yeasts in urine should be done taking the clinical context into account.[63] However, candiduria in febrile neutropenic patients may be a marker of disseminated infection.[62]

I.v. catheter tips. When central venous catheters are removed in the setting of sepsis, it is important to send the catheter tip for culture along with a peripheral blood culture.[64,65] The Maki's roll technique with semi-quantitative culture has become standard for microbiological diagnosis of catheter-related infection after catheter removal although it has not been specifically studied for *Candida*.[65] The clinical relevance of a positive culture of an i.v. catheter tip in the absence of candidaemia remains uncertain.[66]

Choice of media and species identification

Standard bacteriological vs special fungal agar media. Selective fungal media are preferred in order to allow detection of fungi in polymicrobial specimens.[9] Sabouraud agar is suitable for detection of moulds while a chromogenic agar is superior when *Candida* is suspected.[9,67] Some fungal species have an optimal growth temperature of 25–30 °C rather than 37 °C. A minimum of 5 to 7 days of incubation is needed for BAL fluids and samples from normally sterile body fluids to ensure the detection of *Aspergillus* and slow-growing fungi like *Cryptococcus*.[12]

Identification to the species level. Species identification is important in relation to the selection of antifungal treatment (see below), for epidemiological investigations of possible outbreaks and for interpretation of the culture results as some species are more likely to be causative agents of invasive infection than others. Fungi isolated from sterile sites should always be identified to the species level.[12] For non-sterile specimens, *Aspergillus* and *Candida* spp. should be identified to the species level as later infection may arise from the colonizing flora.[12,68]

In addition to the germ tube test, which is specific for *C. albicans* and *C. dubliniensis*, several commercial tests for rapid species identification of common *Candida* species including latex agglutination tests and the rapid trehalose test have been developed and allow identification within minutes of *C. albicans*, *C. dubliniensis, C. krusei* and *C. glabrata*.[69–71]

Role of molecular and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification. - Recent phylogenetic studies have uncovered several new species of yeasts and filamentous fungi responsible for human invasive infections. Some are difficult to identify by classical mycological procedures but possess susceptibility patterns different from the species they phenotypically resemble. For example, *A. lentulus* is multidrug resistant in contrast to *A. fumigatus* and *C. palmioleophila* is fluconazole resistant but echinocandin susceptible in contrast to *C. guilliermondii*. Molecular identification, using nucleic acid techniques like PCR, is of particular interest because it is rapid and yields objective and portable data.[72] Recent recommendations have been proposed concerning the best molecular targets to be used depending on the group of fungi to be identified.[72,73] Another promising tool is the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry instrument, which allows species identification and separation of closely related species at low cost within a few hours.[74–78] The implementation of molecular identification assays or identification using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in laboratories serving large haematology centres is increasingly/urgently needed.

Recommendations.

1. Culture should always be attempted.
2. Specific mycological media (for example, Sabouraud) should be used to optimize growth and avoid suppression by concomitantly present bacteria. For detection of slow-growing fungi, incubation time should be extended to 3 weeks where fungal elements have been seen on microscopy, or where *Cryptococcus* species or endemic fungi are suspected.
3. For *Candida* spp., chromogenic media are recommended as an option for better detection of mixed infection and rapid presumptive identification for some species (for example, *C. albicans*).
4. Fungi isolated from sterile sites should be identified to the species level.

Clinical significance of culture results

As yeasts are part of our normal mucosal microflora, moulds are ubiquitously found in the environment and culture is not a highly sensitive method, interpretation of culture results should take the type and quality of the specimen into account. A positive culture for moulds or yeasts from a normally sterile site (with clinical or radiological abnormal signs consistent with infection) obtained by a sterile procedure defines a proven IFD.[26] Urine, BAL and sinus samples are excluded from this definition.

Airway samples

Patients with prolonged granulocytopenia (for example, induction chemotherapy for acute leukaemia and recipients of allo-SCT) are at risk of developing fungal pneumonia.[79] The lungs may be involved during a candidaemia but primary *Candida* pneumonia is a rare event.[23,80] However, contamination of airway samples with saliva containing *Candida* is not uncommon and thus a diagnosis of primary *Candida* pneumonia cannot be made on the basis of a positive airways sample. Lung infiltrates visible on chest X-ray (CXR) or thoracic CT scan may be caused by bacteria, viruses, moulds (for example, *Aspergillus* spp. and Zygomycetes) or non-infectious diseases like cryptogenic

organizing pneumonia, GVHD, bleeding and drug or radiation toxicity. A positive culture for mould from an airway sample may represent transient colonization, invasive disease or stages in between. The positive predictive value for invasive disease is highly related to the underlying disease and reaches 60% in the high-risk patients after allo-SCT.[81–83] Without performing a transbronchial or transthoracic lung biopsy, it may be difficult to distinguish between colonization and infection.[84] However, because of the severity and risk of a pulmonary mould infection, positive culture results for fungi from BAL fluid (with the exception of *Candida* spp.) are interpreted as an indication for treatment in this population by some.[12]

In invasive pulmonary aspergillosis, the diagnostic yield of BAL fluids ranges between 25 and >50%.[6,15–18] It is lowest if the patient is already receiving antifungal treatment or if the sample is not recovered directly from the affected area.[54] BAL examinations may reveal microbial pathogens that need a modification in the antimicrobial treatment in up to 50% of patients. Thus, in a retrospective evaluation of 246 bronchoscopies in 199 febrile patients with haematological malignancies relevant pathogens were found in 118 cases. In 13 samples, both fungi and bacterial pathogens were detected, in 15 samples *Aspergillus* species, in 16 samples *Candida* species and in 2 samples both *Aspergillus* and *Candida*.[85] However, in a recent prospective study, comparing fibreoptic bronchoscopy plus BAL (FO-BAL) vs no FO-BAL, FO-BAL was the only conclusive test in 34% of examined patients and patient management did not change significantly in any of the groups.[86] Detection of fungal DNA or *Aspergillus* galactomannan Ag in BAL fluid may be of even higher value than conventional culture.[6,17,23,87] However, culture is important in order to allow susceptibility testing due an increasing number of reports of azole resistant *Aspergillus*.[88–91]

Recommendations.

1. Recovery of moulds from sputum in symptomatic patients with prolonged granulocytopenia (for example, allo-SCT, induction therapy for acute leukaemia) or patients with GVHD should be regarded as a possible indicator of fungal pneumonia).
2. Definite diagnosis of invasive pulmonary aspergillosis should be achieved whenever possible.
3. BAL should be performed in granulocytopenic patients if clinically feasible.[18]
4. Early BAL (before or soon after antifungal therapy) is recommended.

Blood culture and the diagnosis of catheter-related infections

From autopsy studies performed in the 1990s, it became apparent that only around 50% of candidaemias were detected by conventional blood cultures.[10,92–94] Blood cultures were positive in <20% of the cases of chronic disseminated candidiasis (that is, hepatosplenic candidiasis).[95] However, blood culture systems have evolved since then which may have improved their sensitivity. The leukaemic patient is susceptible to fungi, which do not cause invasive infections in immunocompetent hosts including *Fusarium*, *Scedosporium* and *Trichosporon*.[63] On the other hand, recovery of moulds like zygomycetes, *Penicillium* (except for the dimorphic *P. marneffei*) and *Aspergillus* from blood cultures should be regarded as contaminants as these are ubiquitous in the environment and even in the case of invasive infections rarely lead to positive blood cultures. A lysis centrifugation blood culture technique has been explored in order to improve the detection of fungi, but is time-consuming, more prone to contamination and does not appear more sensitive than current automated blood culture systems in recent studies for the majority of invasive fungi.[53,96]

The diagnosis of catheter-related fungaemia is established when the same organism has been detected both in blood cultures and in catheter cultures.[65] For bacteria, study results suggest that the differential time to positivity of catheter blood vs peripheral blood culture taken simultaneously might be an important diagnostic indicator, but this has not been validated for candidaemia.[65,97–100]

Recommendations.

1. Positive blood cultures with yeast, dimorphic fungi and certain moulds (including *Fusarium* spp. and *Scedosporium* spp.) from patients with haematological malignancies should be regarded as a sign of IFD and treated accordingly.
2. Catheter-related infection should be suspected when catheter drawn cultures are positive earlier than simultaneous peripheral cultures.

Other sterile body sites. Every fungal element detected by histological examination or by mycological culture taken by biopsy from a sterile site (for example, liver) should be regarded as significant evidence of IFD. The predictive value of a negative result is, however, low particularly in cases of hepatosplenic candidiasis and in patients receiving antifungal treatment. In such cases, galactomannan Ag testing and molecular tests should be considered.[101]

Recommendations.

1. Biopsy samples for histopathology, direct microscopy and culture are recommended for focal infections.
2. Galactomannan Ag testing on supernatant from tissue homogenate and molecular tests when available and validated, should be considered.[101]

Colonization surveillance

Colonization by *Candida* spp. is an important risk factor for invasive candidiasis in the intensive care unit setting.[102] Thus, colonization of more than two sites, the colonization index and a corrected colonization index have all been studied and validated as predictors of invasive candidiasis.[102–104] No information from prospective studies in granulocytopenic patients is available. However, invasive infection is uncommon in patients not colonized[105] and isolates responsible for infection have been shown to be genotypically identical to the colonizing isolates.[68] *Candida*

infections caused by non-*albicans* spp. are overall more frequent in patients with haematological cancer as compared with other immunocompromised individuals.[106,107] In particular, selection of *Candida* spp. with reduced fluconazole susceptibility (for example, *C. glabrata*, *C. krusei*) has been observed with the use of azole prophylaxis in granulocytopenic patients and is associated with a high mortality.[108–110] Earlier reports found an association between colonization with species other than *C. albicans,* such as *C. tropicalis,* and subsequent IFD.[111,112] Thus, when fungal surveillance cultures consisting of urine, stool and respiratory specimens were analysed from 89 patients with prolonged granulocytopenia, 67% of the patients were colonized with *C. albicans* and 28% with *C. tropicalis* but the majority of invasive infections involved *C. tropicalis* (16/21, 76%) and only 3 (14%) *C. albicans*.[111] No patients were colonized with *Aspergillus* spp., but two developed invasive aspergillosis.

Recommendations.

1. Surveillance cultures are should be considered in patients with prolonged granulocytopenia receiving azole prophylaxis whenever feasible.

Antifungal susceptibility testing

Intrinsic susceptibility pattern and acquired resistance
For *Candida* species, the susceptibility pattern is closely related to the species and therefore, the species identification itself provides sufficient information in $\geqslant$95% of the cases for appropriate selection of antifungal treatment (Table 3). Acquired resistance to the azoles, can be mediated by mutations in the target enzyme, upregulation of target enzyme level and efflux pumps.[113,114] Resistant isolates are mostly seen after long-term exposure to azole compounds.[115] Acquired resistance to the echinocandins is mediated via mutations in hot spot regions of the target genes *FKS*1 and *FKS*2 and apparently involves all three currently licensed compounds.[90,116,117] Such isolates have been increasingly often reported and are most commonly seen after 2–3 weeks of echinocandin treatment.[90,118]

Resistance in *Aspergillus* has now been reported in Austria, Australia, Belgium, Canada, China, Denmark, France, Italy, Norway, Spain, Sweden, Switzerland, The Netherlands, the UK and the USA despite the fact that susceptibility testing is not performed routinely at most institutions.[119,120] Azole resistance has been demonstrated in clinical as well as environmental isolates of *A. fumigatus* in the Netherlands and Denmark.[88,89,91] In the Netherlands, 6% of the isolates are azole cross-resistant, 94% of which share the same resistance mechanism (L98H mutation in combination with a 34-bp tandem repeat in the gene promoter region) and infections involving such isolates have been diagnosed even in azole naïve patients. In Denmark, 8% of *A. fumigatus* soil isolates harboured this resistance mechanism.[91] Although, most studies so far have reported low rates ($\leqslant$5%) the incidence is increasing at centres monitoring systematically for resistance.[115,121] Echinocandin resistance in *Aspergillus* is poorly understood and rarely reported in the literature.[122]

Antifungal susceptibility test procedures
Reference standards have been developed for the susceptibility testing of fungi by CLSI and EUCAST.[123–125] A number of commercial susceptibility tests have subsequently been marketed and validated against the reference methods.[126–131] Most used is the Etest, which has been prepared and marketed for most licensed compounds. The test is easy to perform and the test-principle is well known among microbiological technicians as it is also used for antibacterial susceptibility testing. However, significant

Table 3 Susceptibility of pathogenic fungi without acquired resistance mechanisms

	Amphotericin	*Echinocandins*	*Fluconazole*	*Itraconazole*	*Voriconazole*	*Posaconazole*	5-FC
Candida							
C. albicans	S	S	S	S	S	S	S
C. glabrata	S	S	I-R[a]	S-I-R[a]	S-I-R[a]	S-I-R[a]	S
C. krusei	S	S	R	I-R	S-I-R[a]	S-I-R[a]	R
C. parapsilosis	S	S-I	S	S	S	S	S
C. tropicalis	S	S	S	S	S	S	S
Cryptococcus	S	R	S[b]	S	S	S	S
Trichosporon	S-I-R	R	I-R	I-R	S	S	R
Aspergillus							
A. fumigatus	S	S	R	S	S	S	R
A. terreus	I-R	S	R	S	S	S	R
Fusarium	S	R	R	R	S-I-R	S-I-R	R
S apiospermum[c]	R	R	R	R	S-I	S-I	R
S prolificans[c]	R	R	R	R	I-R	I-R	R
Zygomycetes	S-I-R	R	R	R	R	S-I-R	R

[a]The wild-type populations of *C. glabrata* and *C. krusei* (that is, isolates without acquired resistance mechanisms) are less susceptible to azoles. Owing to methodological variation azole MIC values span the S, I and R categories for these species leading to random classification. *C. glabrata* and *C. krusei* are not regarded as optimal targets for azoles by EUCAST. Isolates with acquired resistance mechanisms to fluconazole also show elevated MICs to the other azoles.
[b]Hetero-resistance has been reported for *C. neoformans* and fluconazole.
[c]Synergy *in vitro* between azoles and terbinafine.

trailing growth in the inhibition zone is seen for some drug–organism combinations leading to difficult endpoint reading and a significant variation in minimum inhibitory concentration (MIC) determination when implemented in routine clinical laboratories, potentially leading to incorrect susceptibility classification.[9] Other commercial systems are based on the broth dilution system incorporating a colour change in the medium and visual or spectrophotometric reading (Sensititre yeast-one, TREK Diagnostic Systems, Cleveland, OH, USA, Micronaut, MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany, Vitek, bioMérieux SA, Marcy l'Etoile, France).[127,132,133] The semi-automated reading of such systems reduces the inter-technician variation in reading but the range of compounds included is not complete. In general, the agreement between the commercial systems and the reference methods has been satisfactory when evaluated in mycology reference laboratories.[126,127,130–133] However, the tests have not been validated using the current CLSI or EUCAST breakpoints.

Indications for susceptibility testing
Susceptibility testing is indicated for two reasons. First, to provide the basis for selection of appropriate antifungal treatment in individual patient cases and second, for epidemiological reasons in order to continuously follow susceptibility patterns and thereby detect any emergence of resistance at an early stage. Antimicrobial treatment is, in most cases, initiated before susceptibility results are available and thus our treatment guidelines are based on a detailed knowledge of susceptibility patterns generated through epidemiological surveillance and susceptibility testing schemes. For individual patient care antifungal susceptibility testing should be performed in cases of serious/invasive infection and in cases where the susceptibility pattern cannot be reliably predicted based on the species identification alone such as refractory or relapsing infection despite prior antifungal treatment and in centres where acquired resistance is prevalent.[88–90,115,134] Finally, species identification and antifungal susceptibility testing should be considered for surveillance culture isolates from patients at high risk for IFD especially if exposed to antifungals.

Interpretation of susceptibility results
Susceptibility testing provides a MIC, which is subsequently translated into one of three susceptibility categories (1) susceptible (S), (2) intermediate (I) or susceptible dose-dependent (CLSI terminology) and (3) resistant (R).

CLSI and EUCAST have established breakpoints for antifungals and *Candida* spp.[123,135,136] and although originally quite different these have recently been harmonized for most compounds.[137,138] For *Aspergillus* species breakpoints do not yet exist, but susceptibility results can be interpreted as wild type (normal for the species in question) or higher than wild type using epidemiological cut off values.[139–141]

Correlation with in vivo *outcome*
A number of observations illustrate that susceptibility results correlate with *in vivo* efficacy. At the species level it is well known that antifungals, which are not efficacious exhibit high MICs, for example, fluconazole vs *C. krusei* and moulds, echinocandins vs *Cryptococcus*, voriconazole vs zygomycetes, and so on. and this has been incorporated into treatment guidelines.[54,63,142] Second, isolates from clinical failures and with documented resistance mutations have elevated MICs compared with their wild type, for example, *Candida* isolates with *FKS* alterations and echinocandins and *A. fumigatus* isolates with *CYP51A* mutations and azoles.[90,122,143,144] In conclusion, susceptibility classification correlates with the likelihood of success but does not predict outcome *per se* as severity of infection, timing of treatment, underlying disease and virulence of the pathogen also influence outcome.[145]

Recommendations. Susceptibility testing is recommended:

- In IFD.
- In invasive and mucosal infections failing therapy.
- For other isolates considered clinically relevant particularly in patients exposed to antifungals.
- Susceptibility testing of yeast and moulds should be performed in laboratories by technicians with medical mycology training and regularly receiving samples for antifungal susceptibility testing.

Imaging pulmonary infiltrates in febrile granulocytopenic patients

Chest X-ray
In febrile patients suffering from granulocytopenia, CXRs show pulmonary infiltrates in $<2\%$ if there are no clinical signs and symptoms a of a lower respiratory tract infection.[146] The sensitivity of supine films is 46% for early detection of pneumonia even if done using digital techniques and therefore the upright position is strongly recommended.[147]

Recommendations.

1. Perform digital chest radiography in two planes in the upright position.
2. Do not rely on a normal CXRs in febrile neutropienia.

CT of the chest
CT detects pulmonary lesions in $>50\%$ of patients with neutropenia, even if the CXR is normal.[148–150] CT findings such as consolidation, air-crescent and nodules with or without a halo sign are classified by the 2008 EORTC/MSG (European Organisation for Research and Treatment of Cancer/Mycosis Study Group) criteria as important signs of fungal pneumonia.[151] Lesion detection is adequate in non-enhanced CTs but the use of i.v. contrast agent can provide additional information concerning vascular relation and perfusion, for example, in haemoptysis.[152] Low-dose CT has been studied in a single retrospective simulation trial and appears to be acceptable but further studies are needed before a firm recommendation on its use can be given.[153]

CT is a sensitive tool for early detection of pneumonia, however, specificity is lower as the characteristic features of

IFD may appear late. Thus, serum galactomannan antigen detection (GM) was an important complementary test in a study of the diagnosis of invasive aspergillosis in this patient group.[154]

Besides early detection of a focus, radiological imaging may provide presumptive diagnosis of the cause of pulmonary disease, for example, typical bacterial, viral[155,156] or fungal pneumonia,[8] non-infectious infiltrates like pulmonary oedema, NSIP (nonspecific interstitial pneumonia) or cryptogenic organizing pneumonia, formerly known as bronchiolitis organizing pneumonia.[157] Overall, CT techniques are clearly superior to conventional radiography and thin-section-multi-slice CT is better than high resolution computerized tomography (HRCT), which is again better than thick-section-CT.[137,138]

Recommendations.

1. Perform CT early, because of the much higher sensitivity as compared with CXR.
2. Thin-section multi-slice CT (slice thickness *ca* 1 mm), pitch factor 1.5–2[ref. 158] is recommended as first choice.
3. HRCT (that is, incremental high-resolution CT) using a slice thickness of approximately 1 mm and a 10 mm slice distance (that is, gap) is recommended as an alternative option.
4. Neither i.v. contrast enhancement nor electrocardiogram-trigger is necessary. However, digital image reading is strongly recommended.

Magnetic resonance tomography of the chest

A comparative analysis of CT and magnetic resonance imaging (MRI) for early detection of pneumonia was done in 33 patients where the CXR was normal.[159,160] MRI reached a sensitivity of 95% (specificity 88%, positive predictive value 95%, negative predictive value 88%). Detection of lesions <1 cm and those close to the heart because of cardiac motion was less optimal as expected.[160] Leutner *et al*.[161] compared MRI and CT for the detection of pulmonary infiltrates, and found them overall equally sensitive with the exception of MRI being superior to CT in the detection of abscesses. The drawbacks are, however, that MRI of the lung is more time consuming, sensitive to respiration artefacts and requires special expertise.

Recommendations.

1. MRI is recommended for the characterization of abscesses in the late phase of pneumonia.
2. Recommended MR-sequences include: ultrafast T2 weighted inversion-recovery (for fluid and infiltrate detection), fat-saturated gradient echo-T1 weighted with and without contrast enhancement (for detection of inflammation).

Imaging of extrapulmonary sites

Paranasal sinuses. As the paranasal sinuses are part of the respiratory tract, sinusitis and pneumonia often coexist.[162] Although the incidence of sinusitis is low (3.4%) during granulocytopenia,[163] it is as high as 30% in the transplant setting.[162] Screening before transplantation has been investigated in 80 patients by CT, revealing 17 cases of acute sinusitis, including two requiring surgery before transplantation.[163] Sonography and conventional radiography is useful for the sinus maxillary and frontal sinusitis.[164] However, infections involving the deeper situated ethmoidal and sphenoidal sinuses, are only sufficiently visualized using coronal CT,[164,165] a technique that is able to display even mild osteodestruction.[166] The diagnosis of invasion of the orbits or brain and estimation of the extent of disease require targeted MRI in addition.

Brain. MRI is the gold-standard for brain imaging.[167] CT can be useful in emergencies (for example, haemorrhage because of thrombocytopenia) and for non-compliant or intensive care patients needing close monitoring.[168]

Abdomen. Bedside ultrasonography is recommended for initial imaging, provided the level of expertise is available, and rapidly demonstrates ascites, fluid collections, biliary tract and parenchymatous organ lesions. The 'bulls's eye' lesion consisting of a hyperechoic nidus is highly suggestive of hepatosplenic candidiasis.[169] However, fungal microabscesses can usually only be detected by sonography after neutrophil recovery. CT, particularly if contrast enhanced, may reveal weak hepatic or splenic hypodense lesions and is superior to sonography (70–90% vs 10–30% detection rate).[170,171] The most sensitive technique for this condition, however, is MR as with T2w hyperintense and T1w hypointense lesions, which accumulate contrast agent early are the characteristic findings.[172,173] Finally, conventional radiography in the lateral position is helpful for the detection of free abdominal air and fluid levels.

Overall, sonography is associated with a low resource and radiation burden; contrast enhanced CT covers a wide spectrum of abdominal diagnoses including colitis, and MRI has the highest sensitivity for hepatosplenic candidiasis but is less attractive for critically ill patients because of the difficulties of monitoring vital organ function and treatment limitations in the magnetic field.

Recommendations for imaging of extrapulmonary sites.

1. Coronal CT is superior to plain radiography and is recommended as first choice for imaging of the paranasal sinuses.
2. MRI is recommended as first choice for brain imaging, deeper sinuses and orbital involvement.
3. Bedside ultrasonography is useful for initial imaging of the abdomen, provided the level of expertise is available.
4. CT is recommended as first choice for abdominal imaging. However, it should be noted that MR is superior for specific imaging of the liver in chronic disseminated candidiasis.

Fever of unknown origin

PET-CT scanning (usually performed as ^{18}F-Fluorodeoxyglucose Positron Emission Tomography/CT) is a technique that visualizes pathological local metabolism, for example, in association with neoplasm or inflammation. Preliminary data suggest this technique can be useful identifying the

focus in patients with fever of unknown origin.[174] More data are needed before a recommendation can be made.

Monitoring the course of infection
The timing of follow-up imaging depends on the clinical situation, for example, persisting or relapsing fever, deterioration or haemoptysis. Reduction in size of halos and development of the air-crescent sign is associated with favourable outcome. In contrast to tumour staging, increasing volume of infiltrate, especially if occurring during neutrophil recovery, is not necessarily a sign of treatment failure.[175] As lung imaging is very sensitive, residual findings will be evident even months after the resolution of pneumonia.

Recommendations.

1. Follow-up CT should be performed if new signs/symptoms or findings appear or persist unexpectedly.
2. CT is recommended in a patient with previous infection before further immunosuppressive therapy.

Radiation consideration
Radiation sensitivity is higher in patients younger than 40–50 years of age and in females. Estimates of cancer risk following ionizing radiation such as CT and PET/CT are 1:1250 in children but 1:10000 in adults.[176] Additionally, this patient group have often been exposed to other potential cancer inducing agents. On the other hand, the mortality associated with fungal infection increases when diagnosis is delayed. Radiation burden and likely diagnostic yield should therefore be weighed against the possible cancer risk in each individual patient.

Conclusion

The evaluation of the leukaemic patient at risk of fungal infection is complex. Typical signs of infection may be absent because of neutropenia and concomitant steroid treatment, and the haematological disease itself may mimic classical signs of infection, for example, neutropenic fever, leukaemic infiltrates, lymph node swelling, and so on. As early initiation of antifungal treatment is imperative for a successful outcome, but, when initiated, impairs the sensitivity of subsequent mycological investigations, close surveillance and optimal sampling is important in order to increase the chances of early and correct diagnosis of IFD.

The quality of the result is highly dependent on the sample type, amount and quality as well as of the procedures and expertise in the microbiological laboratory. Hence, optimal approaches to both can potentially improve the diagnostic yield.[9] The diagnosis of fungal invasive disease requires interpretation and understanding of the clinical symptoms, pathogenic spectrum of the various fungi, and relevant specimens, depending on the suspected focus and the possibilities and limitations of the microbiological tests in concert. In Tables 1 and 2 overviews of traditional microbiological techniques and imaging options are summarized in order to facilitate the choice of the most appropriate diagnostic approaches. Furthermore, a list of minimal diagnostics that should be available at an HSCT/leukaemia centre is presented in Table 4.

In conclusion, although a significant number of new tests have been developed for the diagnosis of IFDs over recent decades, classical diagnostics, including imaging, remain a corner stone in the management of IFD in the haematological setting. It is our hope that the recommendations given in this review will assist in their optimal use.

Conflict of interest

MCA has been a consultant for Astellas, Merck, Pfizer, and SpePharm, been an invited speaker for Astellas, Cephalon, Merck Sharp and Dohme, Pfizer, Schering-Plough, and Swedish Orphan and has received research funding from Astellas, Merck and Pfizer. CK has received a research grant from Gilead and honoraria for lectures and/or attendance at advisory boards for Gilead, MSD, Pfizer, Astellas and Astra Zeneca. ED has received funds for speaking from Merck and Schering, for consultancy from Merck and Astellas, and for travel from Merck, Schering,

Table 4 Recommended minimal available diagnostics for an HSCT/leukaemia centre

Technique	*Minimum criteria*
Bronchoscopy and BAL	Should be available within 48 h.
Radiology	Access to CT and MRI should be available within 48 h.
Histopathology	Tissue samples: fungal stains (for example, PAS or Grocott silver methamine) should be routinely performed on tissue samples from these patients.
Microscopy	Wet mounts/direct microscopy: optical brighteners and fluorescent microscopy should be used. Immunofluorescence should be used for PCP.
Culture	Fungal agar should be used for primary culture. Laboratory SOPs should include prolonged incubation where appropriate. Species identification should in the majority if cases be available within 2 days for *Candida* and within 3 days for *A. fumigatus*.
Susceptibility testing	Access should be available to CLSI or EUCAST methodology for all licensed antifungals. Results should be available from receipt of a specimen within 1 week.
Indirect tests	One or several of the following tests: *Aspergillus* galactomannan Ag, B-D-Glucan, *Cryptococcus* Ag, *Candida* mannan Ag and antimannan Ab or PCR should be available according to local epidemiology and expertise. Please refer to specific ECIL-3 guidelines on the use of these tests.

Abbreviations: BAL = bronchoalveolar lavage; CT = computed tomography; MRI = magnetic resonance imaging; PAS = periodic acid-schiff; PCP = pneumocystic (carinii) jiroveci pneumonia; SOP = standard operating procedure.

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