Clinical Infectious Diseases

The Evidence Supporting the Revised EORTC/ MSG Definitions of Invasive Fungal Infections

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SUPPLEMENT ARTICLE

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The Evidence Supporting the Revised EORTC/MSGERC Definitions for Invasive Fungal Infections

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Keywords. invasive fungal infections; aspergillosis; candidiasis; definitions; ICU.

Invasive fungal infections (IFIs) in much of the developed world are associated with medical progress. In addition to the millions of individuals worldwide living with human immunodeficiency virus who constitute a significant host group, these infections are also common among other immunocompromised individuals as a reflection of technologic and therapeutic advances that have led to an enlarging susceptible host population. Examples include novel chemotherapy and other antineoplastic treatments, the aggressive use of glucocorticosteroids for a variety of underlying conditions, the rapidly expanding use of monoclonal antibodies and other biologic agents for the treatment of autoimmune disorders, and the extensive use of intravascular catheters and other medical devices that can provide a nidus for IFIs in otherwise immunocompetent individuals [1, 2]. With the exception of the endemic mycoses, cryptococcosis, and isolated mold infections, collectively, IFIs were relatively uncommon before 1950. Since that time, IFIs have been increasingly encountered in hospitalized and immunocompromised individuals, burgeoning to a point that these are dominant causes of undifferentiated fever, pulmonary infiltrates, skin lesions, and central nervous system disorders in a significant proportion of these patients.

Notably, the accurate diagnosis of IFI has lagged behind that caused by conventional pathogens such as bacteria and viruses. This is, in part, due to poor noninvasive diagnostics for many disorders (eg, invasive aspergillosis, mucormycosis) and the relative insensitivity and slowness of culture-based methods for others (eg, the poor performance of blood cultures in the diagnosis of invasive candidiasis). Given the interest in carefully studying these disorders for epidemiologic and therapeutic purposes, it became increasingly important to accurately describe the diagnosis of the most common IFIs so as to develop greater homogeneity among clinical trial participants.

First published in 2002, the genesis of the original EORTC and the Mycoses Study Group (MSG) definitions for IFIs arose from the need to have consensus definitions for proven, probable, and possible infection among patients with cancer and recipients of hematopoietic stem cell transplants [3]. These definitions were established using the combination of host, clinical, and mycologic criteria and were intended for use in epidemiologic and clinical research, but explicitly not day-to-day for clinical decision-making. Despite the admonition that these definitions were not devised to guide clinical practice, they were adopted by the larger practicing community, gradually creeping into the routine practice of clinical medicine to inform decision-making as it related to targeted antifungal therapy [3].

The first revision of these guidelines was published in 2008 [4]. This revision was necessary in order to address shortcomings in the original version of the definitions and to incorporate emerging diagnostic methodologies. A key issue addressed in this revision included the elimination of the “possible” category for IFI, as this allowed many dubious cases to be included in the definitions. The first revision also broadened the host population beyond those with hematologic malignancies and stem cell transplant recipients to include solid organ transplant recipients and patients with primary immunodeficiency. The revision also includes definitions for less common IFIs [4].

In the most recent (2020) iteration of these EORTC/MSG Education and Research Consortium definitions, there have been further refinements to this evolving document [5]. The update focuses on 9 topical areas that pertain to hosts, fungal diagnostics, and pathogens. This version of the definitions is limited to the proven and probable categories, as no consensus was reached about the possible category. The host definition now includes the pediatric age group, innate immunologic disorders, and CD4 lymphopenia, among other conditions. Fungal diagnostics continue to evolve with improved sensitivity

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and specificity, resulting in more rapid and accurate diagnosis. These diagnostics have greatly enhanced the utility of the definitions. Finally, the document now includes definitions for *Pneumocystis jirovecii* infection [5].

In the almost 2 decades since publication of the initial document, the EORTC/MSG definitions for IFIs have established a standard toward defining a more homogeneous population for purposes of conducting clinical trials and for epidemiologic investigations. While not specifically designed for use in clinical practice, these definitions are often used to assist in decision-making regarding targeted antifungal therapy.

The main purpose of this *Clinical Infectious Diseases* supplement is to provide much of the supporting data upon which the recently published revised EORTC/MSGERC definitions for IFI are based [5]. Among the 9 sections covered in revised definitions, data from 6 sections are included herein. These include new understanding pertaining to radiologic diagnoses, updated information on *Aspergillus* galactomannan serologic studies, updated information on *Aspergillus* polymerase chain reaction (PCR) diagnostics, new insights into the utility of 1,3-β-D glucan as a diagnostic modality, data that support diagnostics directly from tissue specimens, and data that support *P. jirovecii* diagnosis. Also included are proposed definitions for IFIs among intensive care unit (ICU) patients. This last category was included among the original 10 groups, but it was removed when the group could not arrive at a consensus for what constituted a susceptible host category for all ICU patients. Nevertheless, these are critically important definitions, and we chose to include them in this supplement in order to provide the type of readership exposure that they deserve.

Two completely new topical areas in the most recent definitions include the definition of *P. jirovecii* infection and enhanced tissue-based diagnosis of IFI [5]. The enhanced diagnosis of this entity using *P jirovecii* PCR in bronchoalveolar lavage specimens has revolutionized the ability to diagnose accurately this infection in the appropriate host. Enhanced diagnosis of fungal infection based on tissue samples is possible through organism-specific immunostaining and PCR-based diagnostics using a variety of techniques for a host of fungal pathogens.

There are 3 important sections that are not included in this supplement but were part of the revised EORTC/MSGERC IFI definitions. A section on pediatric fungal infections was included in the revised definitions but it is not included here because of a recent update published as a supplement in the *Journal of the Pediatric Infectious Diseases Society* [6]. We refer the reader to this excellent supplement. Similarly, we also did not include data that support the updated definitions for cryptococcosis because a review that includes many of the same authors has recently been published in *Clinical Infectious Diseases* as a major article, and there is little to add to this excellent article [7]. Finally, the endemic fungi are not included in this supplement because there is simply too little new information to justify an additional manuscript.

We believe that the reader of this supplement will find that a deeper dive into the data that describe the rationale supporting these modified definitions will lead to a better understanding of both the importance and the difficulty in achieving consensus definitions for so many key fungal pathogens. This is an area of constant change, driven by improved fungal diagnostics, evolving host characteristics, and continually changing immunotherapeutics. The importance of these definitions in achieving more consistency and homogeneity in defining patients for purposes of inclusion into clinical trials and epidemiologic studies should not be underestimated.

Notes

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SUPPLEMENT ARTICLE

Guidance on Imaging for Invasive Pulmonary Aspergillosis and Mucormycosis: From the Imaging Working Group for the Revision and Update of the Consensus Definitions of Fungal Disease from the EORTC/MSGERC

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Background. Clinical imaging in suspected invasive fungal disease (IFD) has a significant role in early detection of disease and helps direct further testing and treatment. Revised definitions of IFD from the EORTC/MSGERC were recently published and provide clarity on the role of imaging for the definition of IFD. Here, we provide evidence to support these revised diagnostic guidelines.

Methods. We reviewed data on imaging modalities and techniques used to characterize IFDs.

Results. Volumetric high-resolution computed tomography (CT) is the method of choice for lung imaging. Although no CT radiologic pattern is pathognomonic of IFD, the halo sign, in the appropriate clinical setting, is highly suggestive of invasive pulmonary aspergillosis (IPA) and associated with specific stages of the disease. The ACS is not specific for IFD and occurs in the later stages of infection. By contrast, the reversed halo sign and the hypodense sign are typical of pulmonary mucormycosis but occur less frequently. In noncancer populations, both invasive pulmonary aspergillosis and mucormycosis are associated with "atypical" nonnodular presentations, including consolidation and ground-glass opacities.

Conclusions. A uniform definition of IFD could improve the quality of clinical studies and aid in differentiating IFD from other pathology in clinical practice. Radiologic assessment of the lung is an important component of the diagnostic work-up and management of IFD. Periodic review of imaging studies that characterize findings in patients with IFD will inform future diagnostic guidelines.

Keywords. invasive fungal disease; imaging; radiography; aspergillosis; mucormycosis.

Invasive fungal disease (IFD) remains an important cause of morbidity and mortality. A uniform definition of IFD will promote improvement in the quality of clinical studies and aid in the differentiation of IFD from other pathologies encountered in clinical practice. Revised definitions of IFD from the EORTC/MSGERC were recently published and provide clarity on the role of imaging for the definition of IFD [1] (Table 1).

Clinical imaging in suspected IFD has a role in early detection and helps direct further testing. While the presence of specific lesions may increase the likelihood of IFD, the diagnosis of IFD by clinical imaging lacks specificity. The typical radiologic manifestations of IFD include nodules, masses, segmental or subsegmental consolidations, atelectasis, ground-glass opacities, a tree-in-bud pattern, cavities, or pleural effusions [2] (Table 2). However, these radiologic findings can be encountered in other infectious or inflammatory processes of the lungs. In specific clinical scenarios, findings such as the halo sign (HS), reversed HS, hypodense sign (HDS), and air crescent sign (ACS) may have better discrimination between mold infections and nonfungal pneumonias. Determination of the actual specificity of the various radiologic lesions for IFD is hindered by clinical studies that are often not comparable since radiologic and other diagnostic technologies as well as therapies and outcome measurements have changed significantly over the past 2 decades. Furthermore, conclusions regarding

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Table 1. Revised Definitions of the Radiologic Criteria of Lower Respiratory Tract Invasive Fungal Disease

Current Criteria for Pulmonary Aspergillosis
Presence of 1 of the following patterns on computed tomography:
Dense, well-circumscribed lesion(s) with or without a halo sign
Air crescent sign
Cavity
Wedge-shaped and segmental or lobar consolidation
Current Criteria for Other Pulmonary Mold Diseases
Similar to above criteria with addition of reversed halo sign

Source: [1]

radiologic manifestations of IFD are drawn on the basis of *possible* or *probable* IFD, which may not necessarily be true IFD [1]. While imaging remains a cornerstone of IFD diagnosis, the choice of the optimal test and the validity of the findings to ensure a confident and accurate diagnosis remain controversial. Our objective in this review was to assess the role of imaging in the diagnosis and management of pulmonary IFD in adults, focusing on invasive pulmonary aspergillosis (IPA) and pulmonary mucormycosis (PM).

RADIOLOGIC TESTING FOR DIAGNOSIS OF IFD

Multiple imaging techniques are useful to support the diagnosis and evaluation of diseases of the chest, including chest X ray (CXR), computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). The principles of these procedures, benefits, and potential harms are discussed below.

CXR

The CXR in immunocompromised hosts is less frequently used today as it suffers from low sensitivity, especially for early detection of pneumonia. The benefit of lower radiation compared with low-dose and ultra–low-dose CT techniques has dropped while availability of CT has increased. Post-processing of digital radiographs, including techniques to limit the visibility of overlying ribs, increase the conspicuity of focal opacities. However, these techniques cannot compensate for the overall lower contrast resolution of CXR compared with CT. Many lesions, especially small nodules (<10 mm), will be indistinguishable from other parenchymal abnormalities; thus, the utility of CXR for the confident diagnosis of opportunistic lung infections is limited [3, 4].

CT

Volumetric thin-section (high-resolution) CT with a slice thickness of approximately 1 mm is the method of choice for lung imaging. Iodine-based contrast agents may be used; potential adverse events include anaphylactic reactions and contrast-induced nephropathy [5, 6]. While a CT-angiogram that requires contrast may be indicated to demonstrate erosion if a typical vessel-related lesion is detected, intravenous contrast is not typically required for the evaluation of IFD because of the inherent very high contrast of lung tissue [7–9].

The radiation dose is an important consideration when referring patients for CT. The radiation dose required for a chest CT is highly dependent on the scanner and acquisition technique. While the standard chest CT delivers radiation doses of around 10–40 times (2–4 mSv) higher than those of conventional CXR (0.1–0.2 mSv), more modern acquisition techniques that use advanced model–based noise reduction algorithms allow for a low dose acquisition that is only on the order of 5 times higher than the dose for CXR (<1 mSv). As many patients with suspected IFD have underlying malignancy and have received chemotherapy or radiation therapy (which is 5000–60 000 times more radiation compared with a diagnostic CT scan), the impact of a possible and late negative side effect of diagnostic radiation seems less relevant [10–12].

Table 2. Computed Tomography Thoracic Imaging Findings

Definition
Nodule: A rounded or irregular opacity, well or poorly defined, measuring up to 30 mm in diameter:
Micronodule: ≤3 mm
Macronodule: 3–30 mm.
Mass: An opacity greater than 30 mm in diameter is a mass without regard to contour, border, or density characteristics.
Consolidation: Appears as a homogeneous increase in pulmonary parenchymal attenuation that obscures the margins of vessels and airway walls. An air bronchogram may be present.
Air bronchogram: A pattern of air-filled (low-attenuation) bronchi on a background of opaque (high-attenuation) airless lung.
Tree-in-bud pattern: Represents centrilobular branching structures that resemble a budding tree. The pattern reflects a spectrum of endo- and peribronchiolar disorders, including mucoid impaction and bronchiolar inflammation. This pattern is most pronounced in the lung periphery and is usually associated with abnormalities of the larger airways.
Halo sign: Pulmonary nodule or mass surrounded by a halo of ground-glass attenuation.
Reversed halo sign: Focal rounded area of ground-glass opacity surrounded by a more or less complete ring of consolidation.
Hypodense sign: Central area of discrete lower attenuation within a nodule or mass.
Air crescent sign: Peripheral crescentic area of cavitation within a nodule (<30 mm) or mass (>30 mm).

CT imaging for the diagnosis of IFD can improve the early diagnosis and management of IPA in immunocompromised patients [1–3, 13–15]. Thin-section CT imaging can reveal distinct lesions early in infection that would not be detected or well characterized by conventional CXR imaging [13–15]. Cross-sectional technologies such as CT and MRI are the only imaging modalities that can detect typical lesions of IPA, such as the HS or the ACS [14, 16]. Moreover, CT has a role in the follow-up of IFD to monitor lesion size, identify potential complications such as vessel erosion or bronchial compression, and detect typical radiomorphologic findings of later stages of infection, which may be linked to therapeutic response and prognosis [17–19].

MRI

The principal benefits of MRI, in contrast with CT, are the absence of ionizing radiation and the superior resolution in solid organs [20, 21]. However, there are important drawbacks. First, the relative paucity of protons in the lungs, which consist of 90% air, means that the signal-to-noise ratio is intrinsically low. Second, susceptibility artifacts related to air–tissue interfaces in the lungs cause a rapid diminution of the MR signal; and there is the effect of cardiac/respiratory motion on image quality. The most common contraindication is the presence of implanted devices (ie, pacemakers, certain intracranial aneurysm clips, or hemodynamic support devices) as well as claustrophobia [22, 23].

High-speed gradient systems and sequences with short echo times have increased the role of MRI in diagnosis of IFD as an alternative option, with sensitivity and specificity comparable to those of CT [16, 24–29]. MRI may detect nodules with hyperintense T1-weighted images at early stages that correspond to hemorrhages [30, 31]. At later stages (usually ≥10 days from the onset of symptoms), the target sign, defined as a hyperintense rim area of gadolinium enhancement on T1-weighted images, and the reversed target sign on T2-weighted images are strongly suggestive of IPA [25–27, 30, 31]. Nevertheless, the acquisition of quality MR images requires an elaborate optimization of technique and it requires that patients are able to sufficiently cooperate and hold their breath 20–50 times for 10–20 seconds during table time of 20–30 minutes.

PET/CT IMAGING

The most common clinically used radionuclide in PET imaging is 18-fluoro-2-deoxyglucose (FDG), which is transported into cells and accumulates. One disadvantage of PET imaging alone is that images are of intrinsically poor resolution; however, this limitation has been overcome with the development of PET/CT machines. The principle role of FDG-PET imaging is that many tumor cells have a higher metabolic activity (compared with normal tissue) that, in turn, requires an increased expression of surface glucose transporters [32]. The avidity of FDG uptake is calculated and reported as the standardized uptake value (SUV). An SUV that exceeds 2.5 raises the possibility of malignant disease [33, 34]. FDG is also accumulated in the brain, myocardium, and by activated granulocytes and macrophages [35]. The accumulation in activated granulocytes and macrophages provides a possible role for PET in the search for a focus of inflammatory diseases [36].

Infectious lesions that recruit inflammatory cells (that, like cancer cells, also have a high metabolic rate) may also demonstrate significant FDG avidity, resulting in PET/CT scans that are false-positive for cancer [37–40]. In some cases, such presumed cancer lesions have been attributed ultimately to IPA [41, 42]. A role of FDG-PET as a complement to CT imaging for the initial diagnosis and follow-up of IFD has been evaluated [43–47].

CT FINDINGS ASSOCIATED WITH IPA

CT characteristics of IPA are related to the type or degree of immunodeficiency and the underlying host disease. The influence of degree of immunosuppression on radiologic appearance should not be underestimated since the lesion detected is the reaction of the immune system to the infection. During periods of profound immunosuppression (eg, during neutropenia), the immune reaction may be very limited. During immune recovery/reconstitution, the reaction will increase and lesions may enlarge, which does not necessarily indicate treatment failure.

Well-circumscribed lesions (nodules) represent the main radiologic finding of IPA [48, 49]. Nodules may have certain ancillary signs, such as a surrounding halo of ground-glass attenuation (the HS), that are more suggestive of IPA and that represent certain stages of disease [18, 19, 48–52]. Nonspecific and less common findings of IPA include consolidation, cavitary lesions, pleural effusions, ground-glass opacities, tree-in-bud-lesions, and atelectasis [48, 49, 53, 54] (Table 2).

Nodules

Nodules are defined as round opacities, at least moderately well marginated and no greater than 30 mm in maximum diameter [55] (Figure 1). In patients with hematologic malignancies, nodules are the most frequent lesions on CT and are more commonly observed in IFD compared with bacterial or viral infections [48, 50, 56]. However, nodules are nonspecific for IPA and can be seen in pulmonary malignancies, lymphoma, secondary malignancies, and bacterial infections. Micronodules (discrete, round, focal opacity of at least soft-tissue attenuation and with a diameter no greater than 3 mm) are also frequent at initial presentation [49, 55]. A rather widespread distribution of solitary or a few nodules in multiple lobes is more suggestive of a fungal

infection, while a localized segmental area of multiple, clustered, centrilobular micronodules is more indicative of bacterial or viral pneumonia in immunocompromised patients [57, 58].

IPA is a common cause of pulmonary nodules in solid organ transplant recipients [59–61]. Nodules (measuring 10–30 mm in diameter) were found to be prevalent and suggestive of IFD among liver transplant recipients [59–64]. In lung transplant recipients, solitary macronodules (typically without a perifocal halo) may represent IPA or other causes, such as lung cancer or post-transplant lymphoproliferative disease [59–61]. Centrilobular tree-in-bud nodular opacities may be present, indicating inflammatory disease in the small airways [60, 61]. The use of serial CT to monitor the evolution of nodular lesions has been shown to be useful in the follow-up of IPA and assessment of the outcome in patients with hematologic malignancy [18, 50, 65].

HS

The term “halo sign” refers to a pulmonary nodule or mass surrounded by a halo of ground-glass opacification on chest CT; it corresponds to an area of pulmonary infarction surrounded by alveolar hemorrhage [14, 66] (Figure 2A, 2B). A halo is not necessarily a complete 360° rim, it might be limited by pleura, fissures, or adjacent subsegmental atelectasis. In a large cohort of 235 probable IPA cases, most with hematologic malignancy, the prevalence was 61% [48]. The sensitivity of the HS for IPA is variable and related to the degree of the host’s immunosuppression and the stage of infection, but specificity is limited [67]. In general, in patients with hematologic malignancies and neutropenia, the HS is present on baseline CT (at the onset of symptoms) in >70% of IPA cases [49–51, 68]. The prevalence of the HS decreases rapidly over time in this population (<40% after 7 days and <20% after 14 days) [50, 51]. The sensitivity of the HS is reported to be lower in other groups with less profound neutropenia, such as hematopoietic stem cell transplant recipients with graft-versus-host disease, solid organ transplant recipients, or intensive care unit patients [53, 54, 56, 59, 62–64, 68, 69]. The HS is less frequently observed in the pediatric population [70, 71].

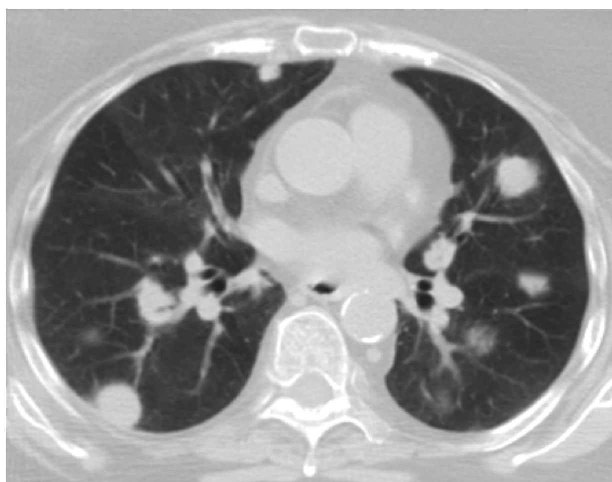

Figure 1. Computed tomography showing bilateral nodular opacities in an orthotopic liver transplant recipient with probable invasive aspergillosis.

The HS was reported to have good specificity (>90%) and positive predictive value (>60%) for the diagnosis of IPA in several studies [16, 56, 62, 68]. However, in other studies, including 2 with only biopsy-proven IPA, lower specificity and a lack of association with fungal pneumonia was reported [57, 72–74]. In a study of 61 patients, the HS indicated pneumonia caused by *Aspergillus* species or *Mucoraceae* verified in 49 (80%) [75]. The presence of nodules with accompanying hemorrhage resulting in a HS on CT has been reported in cryptococcosis, tuberculosis, and candidiasis [66, 73, 76–79]. The prognostic value of the HS has been assessed, and its presence is associated with improved survival, possibly related to early detection prompting early initiation of antifungal therapy [48, 49].

Reversed HS

The reversed HS (RHS) is an area of ground-glass opacification with a peripheral ring of consolidation originally described in patients with cryptogenic organizing pneumonia [80] (Figures 3, 4A). On histopathologic exam in the setting of angioinvasive fungal disease, the reversed HS corresponds to peripheral hemorrhage around infarcted lung tissue [66]. This CT sign is also called the “atoll sign” and can be seen in several diseases, including pulmonary infarction (secondary to thromboembolism), sarcoidosis, and tuberculosis [81–85]. A recent study suggested it occurs more frequently in patients with PM than IPA (54% vs 6%; $P < .001$) [86].

ACS

The ACS is a peripheral crescentic-shaped collection of air that separates the wall of a cavity from an inner mass (Figure 4B). In patients with IPA, this is seen on CT when there is retraction of necrotic tissue within a nodule [17]. In contrast to the HS, the ACS is observed at later stages of IPA, typically appearing on CT imaging about 2 weeks after the initial diagnosis of IPA and usually preceding the appearance of more complete cavitation [50–52]. It may be observed in as many as one-half to two-thirds of cases during follow-up imaging of IPA but is usually not present at initial presentation; thus, its diagnostic value is limited [50–52]. Moreover, the ACS is rarely observed in nonhematologic patients or in the pediatric population [54, 63, 69–71]. The ACS has been described in other fungal infections (notably PM), bacterial infections, and noninfectious conditions [77, 87–89]. The development of the ACS and of cavitation in general is typically associated with recovery of neutrophils [51, 54, 90].

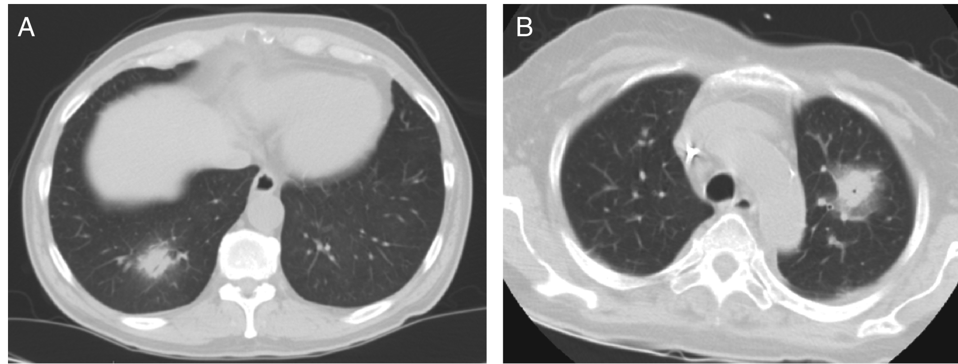

Figure 2. *A,* Computed tomography (CT) image showing a 2.5-cm right lower lobe nodule with ground-glass halo and air bronchogram in an acute leukemic patient with invasive pulmonary aspergillosis. *B,* CT of a febrile neutropenic patient with probable invasive pulmonary aspergillosis showing rounded consolidation in the left upper lobe with a halo of ground-glass opacification.

HDS

The HDS on nonenhanced CT is a discrete central area of lower attenuation that becomes detectable within a focus of consolidation or a mass [19] (Figure 5). This finding corresponds to a zone of infarction caused by an angioinvasive fungus, such as *Aspergillus* or *Mucor* and precedes cavitation [19]. The conspicuity of the HDS is optimized on soft reconstruction kernels and a window level that increases the visibility of small attenuation differences. In contrast to this is the central ground-glass component characteristic of the RHS, best seen in lung window settings. The central necrosis may be associated with a sudden stop of an air-filled bronchus (bronchus cutoff), indicating the underlying parenchymal distortion. This HDS was observed in 30% of IPA cases in one series, while it was absent in a comparative group of immunosuppressed patients with nonfungal pneumonia [19]. Another study reported the HDS in 17 (68%) of 25 IPA episodes among liver transplant recipients [61]. The frequency of this sign may be higher in non-neutropenic and less immunosuppressed populations [91]. The HDS, appearing about 1 week after the initial nodular presentation and 1 week before the development of the ACS, may represent an intermediate radiologic stage of IPA and an adjunct in the diagnosis of IPA [19].

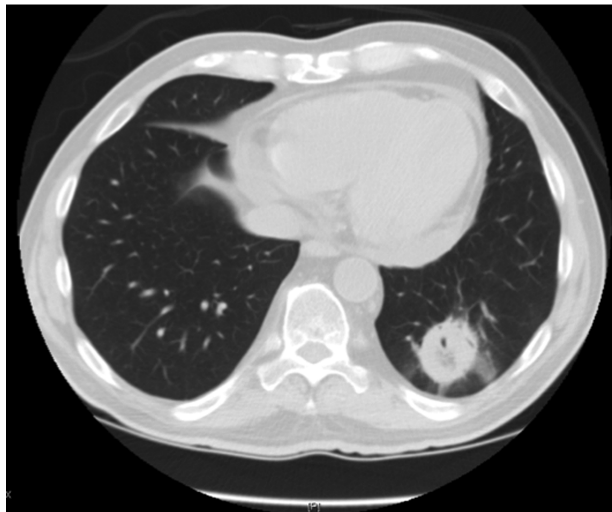

Figure 3. Computed tomography showing a 4.5 × 4 cm focus of consolidation with central ground-glass opacification (the reversed halo sign) in the left lower lobe, extending partially to the pleura, in an acute leukemic patient with mucormycosis (*Rhizopus* species).

Other Findings of IPA

Concomitant pulmonary infections and noninfectious lung diseases in patients with IPA are relatively common and may influence the radiographic findings. Masses, areas of consolidation, or ground-glass opacities have been documented in up to 20%–50% of cases of IPA and may represent a predominant pattern in solid organ transplant [48, 49, 59, 61, 63, 92] (Figure 6). Bronchial wall thickening associated with the tree-in-bud pattern on CT was a common finding among lung transplant recipients with airway-invasive aspergillosis but is less common in IPA [59, 60] (Figure 7). These findings may be the only radiologic signs of IPA in lung transplant recipients as the typical macronodular lesion may be absent [54, 59, 63]. Cavities are observed at a high frequency (50%–75% of cases) at later stages in patients recovering from bone marrow aplasia but are typically absent from early stages and are not predictive of IPA [52, 54, 90]. Empyema (infected pleural effusion) is uncommon and is associated with a poor prognosis [53, 93–95]. The recent observation of the increasing incidence of IPA among intensive care unit patients with severe influenza also poses a diagnostic challenge because of the lack of specificity of lung lesions, which may mimic bacterial pneumonia on chest CT [96].

RADIOLOGIC CT CHANGES ASSOCIATED WITH PM

Fewer studies have specifically investigated the radiologic features of PM [77, 97, 98]. In one study that compared CT findings

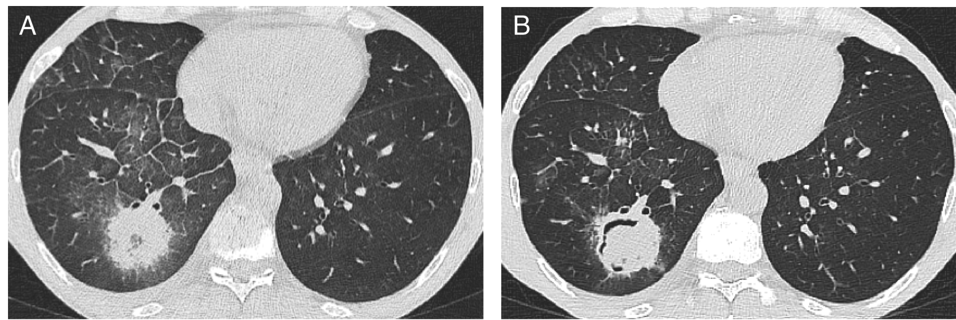

Figure 4. Computed tomography showing a 3.5-cm mass with the reversed halo sign in the right lower lobe in an acute myelocytic leukemic patient with invasive aspergillosis. *A,* After 9 days, the central necrosis was shrinking and developing into a smaller fungus ball while the peripheral thin wall became visible. *B,* From a feeding bronchus, air flows into the cavitation and an air crescent sign becomes visible by the shape of the air between the cavitation of peripheral inflamed viable lung tissue and the smaller central necrotic ball.

of PM and IPA in patients with hematologic malignancies, PM patients were more likely to have multiple nodules (≥10) and pleural effusions [77]. However, the frequency of the HS did not differ significantly between PM and IPA patients (25% vs 21%, respectively; $P = .93$). In a recent study, there was an increase in the frequency of the RHS in patients with PM compared with patients with IPA (54% vs 6%; $P < .001$) [86]. Indeed, another study demonstrated the presence of RHS in >90% of leukemic patients with PM within the first week of the disease [98]. Another study, including 37% of patients with hematologic malignancy and 63% with diabetes mellitus and/or Solid organ transplant (SOT), found that consolidation was the most frequent presentation of PM (65% of cases), followed by cavitation (40%) and masses (25%); nodules were observed in only 16% of cases [97]. Pleural effusions as well as extrapulmonary radiologic findings were observed frequently. The RHS appears to be more specific of the early phase of PM in patients with hematologic malignancies [98, 99].

INDICATION AND TIMING OF IMAGING

Radiographic findings and time course of IPA and PM are influenced by immune reconstitution and antifungal therapy. Thin-section thoracic CT should be conducted when there is clinical suspicion for IFD [1]. Because of low sensitivity, an unremarkable CXR should trigger a CT in the setting of neutropenic fever or signs of pulmonary infection. CT findings can be subtle and nonspecific. Table 3 summarizes the typical CT findings of IPA and PM. Two studies evaluated the prevalence and timing of radiographic signs of IPA in hematologic cancer patients and arrived at similar conclusions [50, 51]. On days 0, 3, 7 and 14 of the infection, the HS was present in decreasing prevalence of 88%–96%, 63%–68%, 22%–37%, and 18%–19% of patients, respectively. In contrast, the ACS showed an increasing prevalence from 0% (day 0) to 5%–8% (day 3), 10%–28% (day 7), and 25%–63% (day 14). The HDS is considered an intermediate stage and is seen at a median of 1 week from the start of IPA [19]. In patients with IPA, the extent of pulmonary infiltrates can increase during the first week despite effective antifungal therapy [18]. However, this finding alone does not indicate a treatment failure. Reduction of the halo and the development of an ACS typically indicate a favorable response [18].

The timing of radiologic findings in nonhematologic patients is less clear [54, 97]. Consolidations, masses, and

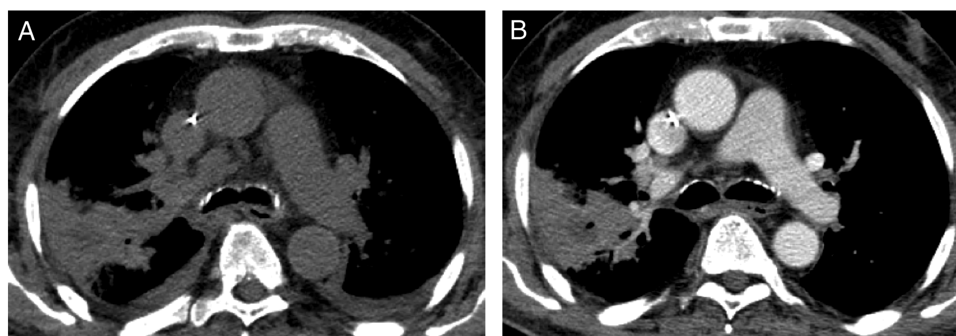

Figure 5. Computed tomography (CT) showing a triangular-shaped 7-cm mass in the right upper lobe with the 1.5-cm hypodense sign indicating central necrosis. *A,* For detection of possible vessel erosion, a contrast-enhanced CT was performed 5 days later. *B,* This illustrates the central necrosis even better than the nonenhanced initial CT.

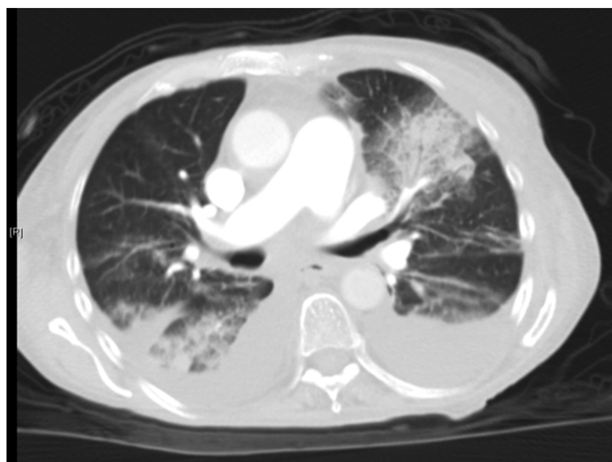

Figure 6. Computed tomography of a neutropenic patient with proven invasive mucormycosis secondary to *Myocladus* species. Note that extensive bilateral opacifications consisting of dense consolidations and ground glass in nondependent areas of the left upper lobe and right upper and lower lobe.

ground-glass opacities are more frequent in this subset of patients for both IPA and PM, while cavitation is present in an important proportion of patients being diagnosed at later stages of the disease [54, 97]. Without a significant clinical change, follow-up of thoracic CT scans should not be performed earlier than 7 days [100].

CONCLUSIONS

Radiologic assessment of the lung is an important component of the diagnostic work-up and management of IFD, and CT imaging is recommended [1]. One finding, the HS (in the appropriate clinical setting), is highly suggestive of IPA and associated with specific stages of the disease. The ACS is not specific for IFD and occurs in the later stages of infection. In contrast, while they occur less frequently, the RHS and the HDS seem to be more typical of PM. In other populations, both IPA and PM are more frequently associated with "atypical" nonnodular presentations, with consolidation, ground-glass opacities, or tree-in-bud patterns associated with bronchial wall thickening. As the spectrum of immunosuppressed patients is expanding, atypical patterns of IFD may be more frequently observed, in particular, among nonhematologic (nonneutropenic) patients. The group acknowledged this and broadened the radiologic criteria of IFD in the updated definitions, also including wedge-shaped and segmental or lobar consolidation as a fourth criterion of IPA and the RHS for PM, in addition to the 3 criteria of the previous definitions (dense well-circumscribed lesion with or without HS, ACS, and cavity) [1, 101]. While IFD criteria can be restricted to these radiologic features for defining IFD in clinical trials, the aforementioned CT lung abnormalities (eg, tree-in-bud pattern, diffuse area of ground-glass opacity, empyema) can be suggestive of IFD and should be interpreted in an appropriate context of host risk for guiding therapeutic decisions. Continuous monitoring of radiologic features of IFD in different patient populations at risk is warranted to inform future guidelines.

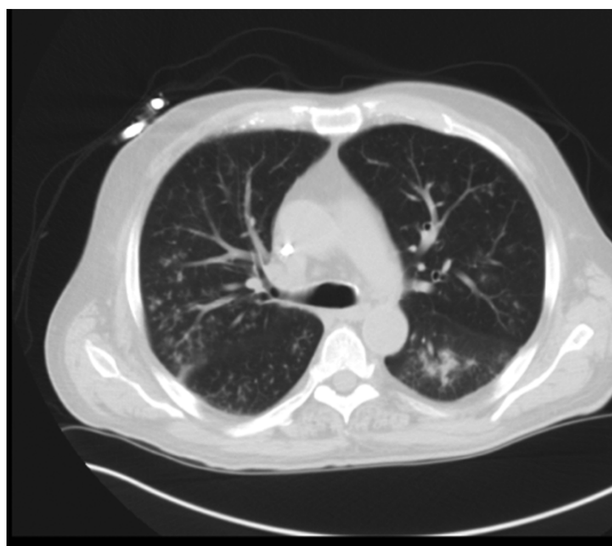

Figure 7. Diffuse tree-in-bud opacities with more focal consolidative opacity in the upper segment of the left lower lobe in a neutropenic patient with probable invasive pulmonary aspergillosis.

Notes

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Table 3. Computed Tomography Findings in the Course of Invasive Pulmonary Aspergillosis or Pulmonary Mucormycosis

Immunosuppression or Host Type	Invasive Fungal Disease	Timing of Computed Tomography Findings		
		At Diagnosis	At 1 Week	At ≥2 Weeks
Hematologic cancer (neutropenic)	Invasive pulmonary aspergillosis	Nodules with/without halo sign	Nodules	Air crescent sign, cavitation
	Pulmonary mucormycosis	Nodules with/without halo sign; reversed halo sign	Hypodense sign; multiple nodules	Pleural effusions; cavitation Cavitation
Other[a]	Invasive pulmonary aspergillosis and pulmonary mucormycosis	Consolidation Masses, nodules, bronchial wall thickening associated with tree-in-bud nodules	Hypodense sign	Cavitation

[a]Other includes solid organ transplant recipients, patients under intensive care, and patients with diabetes.

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Clinical Infectious Diseases

SUPPLEMENT ARTICLE

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Defining Galactomannan Positivity in the Updated EORTC/MSGERC Consensus Definitions of Invasive Fungal Diseases

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The consensus definitions of invasive fungal diseases from the EORTC/MSGERC were recently revised and updated. They now include consensus cutoff values for the galactomannan test that support the diagnosis of probable invasive aspergillosis. In this supplement article, we provide a rationale for these proposed thresholds based on the test's characteristics and performance in different patient populations and in different specimen types.

Keywords. invasive aspergillosis; galactomannan; consensus definitions; thresholds.

The EORTC/MSGERC consensus definitions of invasive fungal diseases were first published in 2002 [1] and have since been widely adopted in clinical research, including epidemiologic studies, validation of diagnostic tests, and trials on antifungal drugs. In addition, regulatory agencies such as the Food and Drug Administration (FDA) and the European Medicines Agency have accepted these diagnostic criteria (in particular, for proven and probable diseases) for defining the target population of clinical trials that evaluate novel antifungal agents. Of course, continuing advances in the diagnostic technology and the identification of new populations at risk have led to revisions of this document. By the end of 2019, the second revision of these consensus definitions, including new host factors, radiologic features, and microbiologic tests, was published [2]. As clearly stated in the original 2002 manuscript and re-emphasized in the 2008 and 2019 revision documents [1–3], these definitions are not intended to direct or guide patient care but should be used exclusively to increase the likelihood of having the fungal disease of interest in patients included into epidemiologic, diagnostic, or therapeutic research [4, 5].

Aspergillus antigen detection was a mycologic criterion to classify probable invasive aspergillosis (IA) cases in the 2002 consensus definition, although, at that time, a commercial assay was not widely available [1]. In the 2008 revised definitions, detection of *Aspergillus* galactomannan in plasma, serum, bronchoalveolar lavage fluid (BALF), or cerebrospinal fluid (CSF) was considered mycologic evidence that supported a probable diagnosis, but a cutoff value was not provided [3]. As a consensus could not be reached on the galactomannan cutoff, the optical density index (ODI) value that was recommended by the manufacturer (0.5 for serum and BALF) was used [6]. However, although clinical studies of IA generally classify patients by these EORTC/MSGERC definitions, different thresholds for a positive galactomannan ODI have been used in case definitions [6]. Therefore, there is a need for further standardization of the galactomannan detection criterion.

The *Aspergillus* galactomannan group (referred to as group 3 in the main document [2]) evaluated galactomannan detection for both adults and children and its utility and clinical validity for different specimens and proposed thresholds for positivity for different clinical specimens. The group fully acknowledges that antifungal therapy is often initiated based on lower levels of evidence (based on lower thresholds for galactomannan detection) than in research settings but also felt that it was crucial to increase the likelihood of having IA in research projects. With the newly proposed thresholds we aim for high specificity (ie, to minimize the rate of false positivity) while maintaining good sensitivities, and without dramatically limiting the number of patients who would be eligible for clinical trials. As such, these proposed consensus thresholds vary from the analytical threshold that is usually recommended by the manufacturer (ie, ODI of 0.5).

BACKGROUND

Galactomannan is a polysaccharide that consists of a mannose backbone and a variable number of galactofuran side chains

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and makes up a major part of the cell wall of *Aspergillus* spp. [7]. These galactofuranose-containing polysaccharides vary in size from 35 to 200 kDa and are secreted by the fungus during growth. It is therefore an interesting biomarker to detect the presence of growing *Aspergillus* inside the human body. In May 2003, the commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (Platelia *Aspergillus* EIA; Bio-Rad, Marnes-La-Coquette, France) was approved by the Center for Devices and Radiological Health, FDA. The assay was based on the EB-A2 rat monoclonal antibody and allowed the detection of serum galactomannan. The test was approved as an adjunct for the diagnosis of IA when used in conjunction with other diagnostic procedures such as microbiologic cultures, histologic examination of biopsy samples, and radiologic evidence of disease. The test was cleared for testing of BALF in 2011.

This Platelia *Aspergillus* enzyme immunoassay is a 1-stage immuno-enzymatic sandwich microplate assay that detects all sorts of galactofuranose-containing molecules, including but not restricted to galactomannan. However, in general, the term "galactomannan" is used collectively for all molecules containing cross-reactive galactofuranose polymers.

Galactomannan is not specific for *Aspergillus* spp. as cross-reactivity with polysaccharides from closely related fungi, such as *Histoplasma capsulatum*, *Fusarium* spp., *Cryptococcus* spp., *Talaromyces* spp., *Acremonium* spp., *Alternaria* spp., *Penicillium* spp., or *Geotrichum* spp., has been described [8–12]. As a consequence, identification to the species level or detection of specific traits, such as antifungal drug resistance, is required by additional tests. Other causes of clinically significant "false positivity" result from the presence of exogenously produced galactomannan that is introduced into the body as part of another product. For example, many organic molecules such as gluconate (used in plasma expanders such as Plasmalyte Baxter) or B-lactam antibiotics (such as piperacillin/tazobactam or amoxicillin/clavulanate) are produced on an industrial scale by fermentation through *Aspergillus niger* or *Aspergillus terreus* [13]. Even after filtration, galactomannan often contaminates the final solution of this process. After oral or parenteral administration of these products, galactomannan enters the bloodstream resulting in "false positive" test results [14, 15]. Fortunately, manufacturers of these products have succeeded in reducing the amount of galactomannan in their formulations sufficiently to effectively eliminate false-positive assays [16, 17]. Food stuffs containing galactomannan (either naturally or through the addition of organic molecules produced using *Aspergillus* fermentation) can cause positive results when the permeability of the gastrointestinal barrier is increased, as is the case in intestinal graft versus host disease or severe gut mucositis [18, 19].

On the other hand, the sensitivity of the test is significantly lower in patients receiving simultaneous mold-active antifungals, either prophylactically or therapeutically [20, 21]. Moreover, sensitivity depends on the study population and the specimen [20, 22–26].

GALACTOMANNAN IN SERUM OR PLASMA

A large body of evidence supports the use of serum or plasma galactomannan detection for the diagnosis of IA, including several meta-analyses. Although testing of galactomannan in plasma has never been evaluated by the manufacturer, a postmarketing head-to-head comparison showed that the performance in plasma was equal to or better than that in serum [27]. Overall, serum or plasma galactomannan testing has a moderate to good pooled sensitivity of 0.48–0.92 and a pooled specificity of 0.85–0.95 across the different meta-analyses [20, 22, 23]. However, the diagnostic characteristics are greatly influenced by the cutoff that is being used. The assay reports an index that compares the optical density of the patient's sample with that of 2 standardized comparator samples included with the test kit. As this value is a continuous variable, different cutoffs can be selected based on the clinical scenario. For example, a lower cutoff will increase the sensitivity by picking up cases with lower values, which can be useful in a screening setting, at the cost of decreasing the specificity by also including false positives. On the other hand, if a high degree of diagnostic certainty is required—for example, in the context of a clinical trial—a higher cutoff can be chosen to increase the specificity, at the expense of lower sensitivity (Table 1). For the updated definitions, a cutoff ODI of 1.0 was selected to increase the probability of having IA compared with a cutoff ODI of 0.5 (as currently recommended by the manufacturer), as these definitions are to be used for including patients in clinical trials, where a high diagnostic likelihood is required [6]. There are even sound arguments for returning to the original threshold of 1.5 [28]; this would further increase the specificity and positive-predictive value, although at the cost of a significant reduction in sensitivity. As the conduct of clinical trials on IA is already very challenging, too high a threshold could severely limit the number of patients found eligible for enrollment. In addition, increasing the cutoff potentially induces a bias by enrolling patients with a higher fungal disease burden [4]. A serum or plasma ODI cutoff of 1.0 was therefore chosen as the best compromise between diagnostic likelihood and patient eligibility for future studies.

A second cause of heterogeneity between different studies, besides the cutoff used, is the patient population being studied. Most studies were performed in hematology patients as they are at the highest risk of IA, especially those undergoing allogeneic stem cell transplantation or induction chemotherapy for acute myeloid leukemia [29]. In this population, the sensitivity of the assay is the highest, especially when these patients are neutropenic [23]. On the other hand, the sensitivity is significantly lower in other populations that are typically not neutropenic,

Table 1. Summary of Meta-analyses of the Performance of Galactomannan in Serum or Plasma in Different Subgroups

Subgroup	Sensitivity	Specificity	PLR	NLR	Informedness
Cutoff					
0.5 ODI	0.78–0.79	0.85–0.86	5.20–5.64	0.24–0.26	0.63–0.65
1.0 ODI	0.65–0.71	0.90–0.94	6.50–11.83	0.31–0.39	0.55–0.65
1.5 ODI	0.48–0.63	0.93–0.95	6.86–12.60	0.39–0.56	0.41–0.58
Population					
HM	0.58	0.95	11.60	0.44	0.53
HSCT	0.65	0.65	1.86	0.54	0.30
SOT	0.41	0.85	2.73	0.69	0.26

Data from references [20, 22, 23]. Informedness = sensitivity + specificity – 1, also known as Youden's index or the J-statistic. Abbreviations: HM, hematologic malignancy; HSCT, hematopoietic stem cell transplantation; NLR, negative likelihood ratio; ODI, optical density index; PLR, positive likelihood ratio; SOT, solid-organ transplantation.

such as solid-organ transplant recipients and patients in the intensive care unit [23].

The performance of serum galactomannan testing appears to be largely similar in pediatric patients and adults, with a pooled sensitivity of 0.81 and a pooled specificity of 0.88 in a meta-analysis of studies in pediatric patients with cancer and hematopoietic stem cell transplant recipients [30]. However, the sensitivity of serum galactomannan testing appears to be low in patients with chronic granulomatous disease or with hyperimmunoglobulin E (hyper-IgE) syndrome (formerly Job syndrome), despite their increased risk of IA [31, 32].

GALACTOMANNAN IN BRONCHOALVEOLAR LAVAGE FLUID

Although the Platelia assay was initially only approved for use in serum, the manufacturer later also added BALF as a validated sample type. As with serum/plasma, uncertainty remains around the appropriate cutoff to be used. Overall, BALF galactomannan testing has a pooled sensitivity of 0.61–0.92 and a pooled specificity of 0.89–0.98 across several meta-analyses [24–26]. As expected, the sensitivity is highest when using the lowest ODI cutoff of 0.5, at the cost of having the lowest specificity of 0.89–0.92 as well (Table 2). Increasing the cutoff to 1.0 increases the specificity to an excellent 0.94–0.95 while only slightly lowering the sensitivity [24–26]. Further increases in the cutoff (eg, ≥1.5) only marginally improves specificity but comes with a significant decrease in sensitivity (or false-negatives) [24–26]. A cutoff ODI of 1.0 was therefore selected for the updated consensus definitions.

As with serum/plasma, the sensitivity of galactomannan detection in BALF is lower in patients exposed to mold-active antifungals [25]. Moreover, the sensitivity is negatively affected by pretreatment of viscous BALF samples with mucolytic agents [33, 34]. Unlike with serum galactomannan, the sensitivity is similar in hematology versus nonhematology patients and in neutropenic versus nonneutropenic patients [24–26].

Bronchoalveolar lavage fluid galactomannan was consistently more sensitive than serum galactomannan, both in hematology patients [26] as well as in nonneutropenic patients [35, 36]. The addition of serum galactomannan to BALF galactomannan led to a small increase in sensitivity when a positive result was defined as a positive test in either serum or BALF [26]. Therefore, the group's consensus was that the combination of 2 low positive test results, 1 in BALF and 1 in serum/plasma (BALF ODI ≥0.8 and serum/plasma ODI ≥0.7), also suggests the presence of IA, although no study has ever looked into this combination specifically.

GALACTOMANNAN IN CEREBROSPINAL FLUID

The performance in CSF was studied in a total of 42 cases of central nervous system aspergillosis [37–40]. The pooled sensitivity across these studies was 0.88 at an ODI cutoff of 0.5, 0.86 at a cutoff of 1.0, and 0.84 at a cutoff of 2.0. The pooled specificity was 0.98 across the 2 studies that used a control group and reported galactomannan values for this group, independent of the cutoff used [37, 40]. The single "false positive" in this control group was caused by a patient with a brain abscess on magnetic resonance imaging, with a CSF galactomannan ODI of 8.2, but negative culture and biopsy and no other localizations of IA [40]. He was therefore classified as not having IA in accordance with the study protocol, although this could of course also be a misclassification by the gold standard used in this study. By comparing the CSF albumin/serum albumin gradient with the CSF galactomannan/serum galactomannan, Viscoli et al [38] showed that more than 99% of the galactomannan present in the CSF of the patients was produced intrathecally, indicating that high galactomannan levels in CSF are indeed indicative of localized infection and are not just the result of translocation from the circulation. It is unclear if the performance of galactomannan in CSF is different in children. In a small study of 9 pediatric cases of cerebral aspergillosis, an ODI cutoff of 0.5 showed a sensitivity of 0.66 and a specificity of 1.00 in 32 pediatric controls [41]. Based on the aggregated data, an ODI cutoff of 1.0 was agreed upon by the group for the updated consensus definitions.

Table 2. Summary of Meta-analyses of the Performance of Galactomannan in Bronchoalveolar Lavage Fluid in Different Subgroups

Subgroup	Sensitivity	Specificity	PLR	NLR	Informedness
Cutoff					
0.5 ODI	0.82–0.87	0.89–0.92	7.45–10.88	0.14–0.20	0.71–0.79
1.0 ODI	0.75–0.86	0.94–0.95	12.50–17.20	0.15–0.27	0.69–0.81
1.5 ODI	0.70–0.92	0.95–0.98	14.00–46.00	0.08–0.32	0.65–0.90
2.0 ODI	0.61–0.84	0.95–0.96	12.20–21.00	0.17–0.41	0.56–0.80
Hematologic malignancy					
Yes	0.85	0.91	9.44	0.16	0.76
No	0.87	0.89	7.91	0.15	0.76
Antifungal therapy or prophylaxis					
Yes	0.76–0.85	0.89	6.91–7.73	0.17–0.27	0.65–0.74
No	0.91	0.88	7.58	0.10	0.79

Data from references [24–26]. Informedness = sensitivity + specificity – 1, also known as Youden's index or the J-statistic. Abbreviations: NLR, negative likelihood ratio; ODI, optical density index; PLR, positive likelihood ratio.

GALACTOMANNAN IN OTHER SPECIMEN TYPES

Besides BALF, serum/plasma, and CSF, galactomannan has also been detected in other human specimens, including urine. However, urinary antigens are expressed in different vehicles that require processing. Pilot studies have reported on the diagnostic performance of the current Platelia assay in urine using EB-A2 antibodies. These antibodies recognize long-chain galactofuranose molecules that are, however, not excreted robustly in urine [42, 43]. Using these antibodies, galactomannan could be detected in some patients, but its sensitivity was lower than in serum, despite using a lower cutoff (ODI of 0.3 or 0.1) [42, 43]. A follow-up study tried to circumvent this problem by normalizing the galactomannan ODI to urinary creatinine level [44]. In this study of only 6 cases of probable and proven IA, the urinary galactomannan to creatinine ratio had a sensitivity of 0.84 and specificity of 0.70 when using a cutoff ratio of 0.26. However, no information was provided on the performance of serum or BALF galactomannan in these same patients, making any comparison impossible.

Recently, investigators have used different antibodies that recognize shorter-chain galactofuranose epitopes, identifying fungal glycans that are expressed in animal and human urine both in free form and on the surface of extracellular vesicles [45–47]. Hence, detection of urine fungal antigen appears to be antibody dependent.

Finally, galactomannan detection in other fluids such as pus from abscesses or from suspected fungal rhinosinusitis has been described in case reports or case series [31, 48]. This appears to be useful in clinical cases where other tests were not helpful but has not been validated on a larger scale. As there are insufficient evidence and experience with all of these specimen types to date, they were not included in the latest revision of the consensus definitions of IA.

NOVEL *ASPERGILLUS* ANTIGEN TESTS

Following the most recent consensus meeting in 2015 (which resulted in the second revision and update of the EORTC/MSGERC definitions [3]), novel *Aspergillus* antigen detection tests have been investigated. Recently, the commercially available IMMY lateral flow assay (IMMY, Norman, OK, USA) and the OLM Diagnostics lateral flow device (OLM Diagnostics, Newcastle Upon Tyne, United Kingdom) have been approved for use as a diagnostic aid. These are fast and effective alternatives to galactomannan detection and prove to be especially useful for centers with low sample throughputs [49]. More recently, a lateral flow dipstick assay using the galactofuranose-specific monoclonal antibody (mAb476), which recognizes urine antigens after *Aspergillus* fumigatus pulmonary infection in animals, demonstrated good sensitivity and specificity, especially in patients with cancer [46]. This assay and an enzyme immunoassay are currently undergoing a multicenter clinical

validation. In addition, several new competing assays are under development by companies such as Dynamiker, Euroimmun, and IMMY, but large-scale data are lacking so far. Importantly, performance of the new tests in detecting fungal antigens in different body fluids will likely differ from the current Platelia galactomannan assay based on antibody epitope recognition (as discussed in urine) and test format. The group has decided not to incorporate these tools as a microbiologic criterion in the updated definitions as their performance as well as the corresponding cutoffs have not yet been fully assessed.

IMPACT OF NEW CUTOFFS

As discussed, we recommend these new cutoffs in order to increase the specificity of identifying cases, an important goal for enrollment in antifungal treatment trials. The group recognizes that our recommendation may impact clinical studies and clinical scenarios differently, depending on the focus. For instance, use of higher cutoffs to identify cases may not be feasible in the context of prophylaxis or prophylactic studies, where clinicians may want to treat patients with evidence of disease and antigen levels meeting the manufacturer's recommended lower cutoff. Adjustment of the cutoff has the potential secondary effect of changing the relevance of the "possible" IA category, with particular impact in prophylaxis and diagnostic studies, as a larger proportion of these patients can be considered to have real disease.

CONCLUSIONS

As the goal of the EORTC/MSGERC consensus definitions is to facilitate standardization and the selection of a more homogeneous population of patients with IA for clinical treatment trials, the proposed galactomannan cutoffs are higher than those typically used in clinical care. This results in a higher specificity and diagnostic likelihood, at the expense of a slightly lower sensitivity. In the end, the cutoffs that are being proposed are based on a consensus decision on the optimal tradeoff between diagnostic certainty and ensuring that a sufficient number of patients remain eligible for enrollment in treatment trials. It is important to note that all cutoffs mentioned in these consensus documents are based on the Platelia *Aspergillus* assay. We hope that the application of the new criteria in clinical, diagnostic, and epidemiologic research of IA will result in further standardization and improved comparability.

Notes

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SUPPLEMENT ARTICLE

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Aspergillus Polymerase Chain Reaction—An Update on Technical Recommendations, Clinical Applications, and Justification for Inclusion in the Second Revision of the EORTC/MSGERC Definitions of Invasive Fungal Disease

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Aspergillus polymerase chain reaction testing of blood and respiratory samples has recently been included in the second revision of the EORTC/MSGERC definitions for classifying invasive fungal disease. This is a result of considerable efforts to standardize methodology, the availability of commercial assays and external quality control programs, and additional clinical validation. This supporting article provides both clinical and technical justifications for its inclusion while also summarizing recent advances and likely future developments in the molecular diagnosis of invasive aspergillosis.

Keywords. *Aspergillus* PCR; EORTC/MSGERC definitions; technical aspects; clinical performance.

Aspergillus polymerase chain reaction (PCR) testing of blood and bronchoalveolar lavage fluid (BALF) has been recently accepted as a mycological criterion for probable invasive aspergillosis (IA) in consensus guidelines for research studies [1]. The basis for inclusion is the significant progress that has been made in the standardization of *Aspergillus* PCR methodology through the efforts of the European Aspergillus PCR Initiative (EAPCRI; now known as the Fungal PCR Initiative (FPCRI; [2] www.fpcri.eu), the availability of commercial assays, and increased confidence in performance as highlighted by a Cochrane review; various meta-analyses; and randomized, controlled trials that incorporate PCR technology [3–8].

When considering the suitability of any test for clinical use, the technical robustness and applicability, analytical and clinical performance, and clinical utility must be determined. All of these may be influenced by the reason for testing (screening vs diagnostic confirmation), which affects testing frequency, specimen choice, and subsequent result interpretation, where the emphasis will change dependent on the reason for testing [9]. While all parameters are important when considering a test for inclusion in the EORTC/MSGERC definitions, assay specificity is paramount, as accuracy in confirming a diagnosis is critical when enrolling patients into clinical trials of novel therapeutics or when assessing performance of new tests [1, 10, 11].

In this review, we summarize the evidence for inclusion of *Aspergillus* PCR into the recent EORTC/MSGERC definitions and describe recent advances, unmet clinical needs, and potential future developments.

TECHNICAL CONSIDERATIONS

Nucleic Acid Extraction

For years, the lack of commercial assays and limited methodological standardization prevented the incorporation of *Aspergillus* PCR into the EORTC/MSGERC definitions [10, 11]. The work of the EAPCRI/FPCRI demonstrated that the performance of molecular methods for the detection of *Aspergillus* was dependent on the nucleic acid (NA) extraction protocol to provide high-quality DNA of sufficient quantity with minimal inhibitory compounds [12]. The PCR amplification stage was not rate-limiting, providing consistent performance when testing comparable NA concentrations across methods. Following this, research commenced to develop optimal NA extraction protocols for whole blood (WB), serum, and plasma testing [12–14]. For all specimen types, sample volume (≥3 mL EDTA WB, ≥0.5 mL serum/plasma) and NA elution volume (<100 µL) were determined critical to success. The testing of WB to target organism-sourced DNA requires the processing of large volumes and manual procedures prior to automated extraction that increase processing time and limit the uptake of testing. The

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testing of serum/plasma for the detection of circulating DNA (DNAemia) was methodologically straightforward, using NA fully automated extraction platforms available in most molecular diagnostic laboratories [9]. NA extraction from serum likely provides enhanced specificity but compromises sensitivity compared with WB samples [3, 15]. However, the extraction from plasma was superior to that of serum, providing sensitivity comparable to testing WB but without the methodological complexity, which likely compromised specificity when testing WB [3, 14–16]. Given the documented presence of organisms in the respiratory tract and subsequent samples, mechanical disruption of the fungal cell is required to provide efficient NA extraction. However, the overall extraction process is less labor-intensive compared with WB extractions that require lysis of red and white blood cells prior to targeting the fungi [12]. Free fungal DNA will also be present in the respiratory tract through the actions of the host's immune response or antifungal therapy; this could be targeted exclusively using fully automated NA extraction platforms. By introducing BALF supernatant post-mechanical lysis of the BALF pellet, both organism-sourced and free DNA can be targeted in a single NA extraction procedure [9]. The FPCRI is currently finalizing NA extraction recommendations to standardize *Aspergillus* PCR testing of BALF and allow all potential DNA sources to be targeted, which will potentially improve performance. Given the viscous nature of some BALF, the sample may need to be liquefied before extraction to allow manipulation.

PCR Amplification

While PCR amplification has not been deemed critical to success when performing *Aspergillus* PCR, targeting a multicopy gene enhances the analytical sensitivity of PCR, and the ribosomal RNA gene cluster (18S/28S rRNA and the internal transcribed spacer (ITS) regions) has been frequently targeted [17]. As IA is most commonly caused by *Aspergillus fumigatus*, most assays are optimized to detect this organism. However, given that aspergillosis can occasionally be caused by other species, it is beneficial for assays to be pan-*Aspergillus.* However, a disadvantage is potential cross-detection of other genera (eg, *Penicillium* species) [9, 17]. Current *Aspergillus* PCR assays are better suited for the detection of *A. fumigatus*; however, the use of PCR assays that provide a genus level of detection and target the rRNA genes improves the detection of non-*A. fumigatus* species [17].

The use of real-time quantitative (qPCR) instruments minimizes the potential for contamination, provides rapid species-level identification, and generates a quantification cycle (Cq) that is proportional to the fungal burden, which is useful when interpreting the significance of a positive result. Typically, when testing blood samples, Cq values will be late (>35 cycles), entering the nonreproducible range of detection. Performing PCR amplification in duplicate to enhance detection of low NA concentrations and including an internal control are essential [8, 12]. Interpretation of PCR positives with late Cq values remains difficult. Determining the clinical significance of low burden is complicated, as it may be a consequence of testing specimens not directly associated with the infected site or a result of disease with little or no angioinvasion. Conversely, contamination can arise from both the clinical and laboratory settings and generate false-positive Cq values >35 cycles [9]. Negative controls should be used to monitor for procedural contamination. Commercial kits usually provide both positive and negative control material, and it is paramount that "in-house" methods follow suit.

While Cq is unique to each real-time PCR platform and the algorithm used for its determination, analysis across 29 protocols that tested blood samples spiked with varying burdens of *Aspergillus* genomic DNA identified a Cq threshold of 43 cycles as optimal, generating sensitivity and specificity of 86% and 95%, respectively, while lowering the thresholds to 34 cycles provided 100% specificity [13]. Setting a lower threshold of positivity (eg, an earlier Cq value) is a trade-off between sensitivity and specificity, and sensitivities <50% will compromise the positive likelihood ratio (<10), even if the specificity were 95%. Very high/late thresholds will optimize sensitivity but will produce more false positives, sacrificing specificity. This may be desirable in a clinical scenario where a clinician might prioritize avoidance of missing a true case over treating patients who may not be truly infected, since the consequence of not treating early IA can be devastating. However, the purpose of the EORTC/MSGERC guidelines is to restrict classification to cases that have a high degree of certainty for clinical trials, thus prioritizing high specificity. The recent Cochrane systematic review of the literature and meta-analysis on *Aspergillus* PCR testing of blood (29 studies, 34 datasets, 4718 patients, mean IA prevalence of 16.3%) that used the new EORTC/MSGERC criteria showed that pooled sensitivity/specificity for 2 consecutive positives was 60%/95%, corroborating the current inclusion criteria of requiring 2 positive PCR results [1, 3]. This trade-off between assay sensitivity and specificity emphasizes the limitations of trying to use the more restrictive criteria of the guidelines in the clinic.

BALF testing is invariably used to confirm suspected infection in a symptomatic high-risk patient. Thus, the pre-test probability is high and assay specificity is paramount. Meta-analyses have highlighted the high specificity (94%–95%) of *Aspergillus* PCR testing of BALF, and corresponding positive likelihood ratios (>12) confirm its suitability for confirming infection and its inclusion in the current definitions [5, 8, 18, 19]. Real-time PCR positivity is associated with a Cq value that is proportional to the fungal burden in the sample; this should allow thresholds that aid in the differentiation of infection from colonization/contamination in the respiratory sample to be implemented.

The availability of commercial *Aspergillus* PCR assays provides quality assurance and technical consistency, including the provision of control samples that facilitates adoption by more laboratories outside of specialty mycology reference facilities. Surprisingly, commercial assays have not demonstrated superior performance over laboratory-developed methods [3]. The commercial assays do not recommend specific NA extraction methods. Combining commercial assays with the FPCRI methodological recommendations for NA extraction provides a fully standardized method that can easily be replicated across centers. This methodological consistency coupled with the availability of external quality control methods for *Aspergillus* PCR testing (Quality Control for Molecular Diagnostics) [20] provide a process that is very robust. The availability of an international *Aspergillus* DNA calibrator, which is currently being used to develop an international standard for *Aspergillus* PCR, allows tests that use multiple platforms to be referenced to a single control material [21]. Given all of this information, ideally, *Aspergillus* PCR testing should only be performed using real-time PCR platforms.

CLINICAL APPLICATION AND PERFORMANCE

Screening vs Diagnosis

Aspergillus PCR testing is principally used by clinicians to either confirm diagnosis in patients suspected to have IA or to screen individuals at risk for developing IA in order to facilitate early diagnosis. Screening strategies are best applied in patients at moderate to high risk of IA (eg, acute leukemia or transplant recipients) since the pre-test probability governs how well the test performs.

When *Aspergillus* PCR was used to screen blood samples, meta-analytical reviews generated sensitivity and specificity values of 84%–88% and 75%–76%, respectively [4, 22]. The recent Cochrane review of *Aspergillus* PCR testing of blood generated similar statistics (sensitivity, 79%; specificity, 80%) [3].

Anti-*aspergillus* prophylaxis significantly reduces the pre-test probability of IA and was associated with a significant reduction in specificity (79%–64%) coupled with a nonsignificant increase in sensitivity (75%–82%) [3, 9, 23]. While this may seem counterintuitive and contradictory to the influence of antifungal therapy on galactomannan-enzyme immunoassay (GM-EIA), it may be explained by the possibility that prophylaxis will prevent an initial infection from progressing to overt disease while the presence of *Aspergillus* DNA is maintained or even enhanced due to release of NAs by antifungals that target the cell wall or membrane [3]. *Aspergillus* PCR testing of BALF to confirm a breakthrough diagnosis in a patient on prophylaxis is feasible [24].

When confirming IA in patients with suspected disease, specimens from the infection site are more advantageous than blood samples. In a retrospective, multicenter evaluation that compared *Aspergillus* PCR testing of BALF with concurrently taken blood samples, PCR sensitivity was significantly greater in BALF (63%) vs blood (8%). Also, although 75% of samples were taken during antifungal therapy, this did not have a major impact on performance in BALF [24]. Studies that directly compare the performance of screening and diagnostic confirmatory PCR approaches are currently lacking. However, in 73% of cases of IA regularly screened using both PCR and GM-EIA, a positive screening result in blood was recorded on average 11 days prior to bronchoscopy to confirm the diagnosis. This was due to the logistical delays inherent in getting bronchoscopy performed promptly [3, 25].

The meta-analytical performance of *Aspergillus* PCR for the testing of BALF is comparable to that for GM-EIA with comparable sensitivities and specificities that range from 76.8 to 79.65 and 93.7 to 94.5, respectively [5, 19, 20].

The optimal use of *Aspergillus* PCR is likely to be in combination with antigen detection [26]. In a study that tested BALF, the combination of PCR with GM (I>1.0) generated 100% sensitivity and 98% specificity [27]. This approach was confirmed using the commercial Pathonostics AsperGenius assay, where PCR combined with GM (I>1.0) generated 96% sensitivity and 100% specificity [28]. These findings provide some clinical validation of a combined strategy of using commercial PCR and antigen assays. A meta-analysis of antigen/PCR testing of BALF generated sensitivity and specificity of 84% and 94%. While combination testing of BALF increased sensitivity by 5%–9%, the specificity remained sufficient to confirm IA (positive likelihood ratio, 14) [29]. Various randomized, controlled trials and prospective cohort studies have highlighted the benefit of combined antigen/PCR testing of blood for the management of IA [6, 7, 30, 31]. A meta-analysis confirmed that if both were consistently negative, the sensitivity (99%) would be sufficient to exclude IA, whereas the specificity when both assays were positive was 98% [26]. The improved specificity achieved through combination testing of both blood and BALF may instill confidence regarding the certainty of a case of probable IA when both tests are positive. Conversely, if both tests are consistently negative, disease can be confidently excluded, which is critical if antifungal stewardship strategies are to be used successfully.

While the kinetics of release of fungal biomarkers have been studied, data are limited, and the relationship between the release of the individual biomarkers and stages of disease is unclear [32–34]. Combination testing enhances the opportunity to detect the biomarkers that may vary differentially at various stages of the infection [35]. The recent The European Society for Clinical Microbiology and Infectious Diseases, the European Confederation of Medical Mycology and the European Respiratory Society (ESCMID/ECMM/ERS) guidelines for management of *Aspergillus* diseases moderately support the use of PCR to diagnose IA when testing blood, BALF, or cerebrospinal fluid (BII), and the strength of that recommendation is increased for combined GM-EIA and PCR

testing of BALF [36]. The 2016 Infectious Diseases Society of America Aspergillosis Guidelines advise that PCR be performed on an individual basis and in conjunction with other tests and clinical context [37]. The development of real-time *Aspergillus* PCR assays has raised the possibility of using the assay as a prognostic marker during therapy. Unfortunately, the late Cq values that are regularly encountered when testing blood samples only permit a qualitative interpretation, with patients usually becoming negative promptly after starting treatment. While PCR positivity in BALF is regularly associated with earlier Cq values that could be monitored for response to therapy, the invasive nature of obtaining the sample prohibits prognostic evaluations.

Nonneutropenic Patients

Most of the data regarding evaluation of *Aspergillus* biomarker assays have been generated in neutropenic and Hematopoietic stem cell transplant (HSCT) patients. Evaluations of the performance of biomarker assays in other nonneutropenic patients are limited, but the need is growing, with apparent greater frequency of IA occurring in the intensive care setting, including in coronavirus disease 2019 (COVID-19) patients [38, 39]. The utility of high-frequency screening of blood samples in nonneutropenic patients may be limited by less angioinvasion of IA. IA in nonneutropenic patients may be restricted to the respiratory tract (eg, *Aspergillus* tracheobronchitis post-influenza infection), and symptoms are attributed to the inflammatory response rather than tissue infarction, which is more characteristic of neutropenic infections. For these reasons, the use of PCR assays for diagnostic confirmation is preferred at present. However, given the increasing incidence of IA in certain intensive care unit (ICU) cohorts (19% in post-influenza and 33% in post–COVID-19 patients), screening strategies are a high priority [40, 41]. Using a commercial *Aspergillus* PCR, sensitivity was 100% and specificity was 99%–100% in BALF from ICU patients [42, 43]. Chong and colleagues showed that the performance of the Pathonostics AsperGenius assay when testing BALF was identical for hematologic and critical care populations, generating a sensitivity and specificity of 80% and 91%, respectively, for the ICU population [44].

Pediatrics

The number of studies that have evaluated the performance of *Aspergillus* PCR in children is limited, even more so for neonates. Most data are derived from studies that evaluated performance in high-risk pediatric populations (eg, leukemia, transplant, and chronic granulomatous disease). Overall clinical performance is variable, but data analysis is complicated because some studies included possible IA with proven/probable IA, incorporated a pan-fungal PCR technology, or failed to use the EORTC/MSGERC definitions for case classification [45–51]. For studies where the data were retrievable, the overall pooled sensitivity was 82.3% (95% confidence interval [CI], 75.8–87.3) and pooled specificity was 72.8% (95% CI, 68.8–76.4), which are comparable to the results included in the recent Cochrane review of *Aspergillus* PCR and suggest that the diagnostic yield of *Aspergillus* PCR does not differ significantly between adult and pediatric studies [3, 46–48, 51, 53–57].

Nevertheless, given the limited number of studies, there are no recent recommendations for the diagnosis and management of IA in pediatric patients as they relate to PCR testing [57]. While it is likely that the performance of *Aspergillus* PCR will be similar in adults and children, it is important to remember that radiological imaging, which is critical to attaining a diagnosis of probable IA in the EORTC/MSGERC, is typically nonspecific in pediatrics, making validation of IA using data from adult studies difficult [57].

FURTHER DEVELOPMENTS AND FUTURE REQUIREMENTS

Since different *Aspergillus* species may have different antifungal susceptibility profiles it is desirable for molecular methods to be able to differentiate between species. Analysis of the analytical specificity of mainly laboratory-developed PCR methods demonstrated that the detection of species other than *A. fumigatus* (eg, *Aspergillus terreus*) was reduced. While the detection of non-fumigatus species (eg, *Aspergillus lentulus*) within the *Aspergillus fumigati* complex was possible, most assays did not differentiate species within this complex [17]. Recently, several commercial assays (eg, Fungiplex Aspergillus, Bruker UK Limited, Glasgow, UK, and AsperGenius, Pathonostics, Maastricht, Netherlands) have been designed to identify *A. terreus* separately from other species. The AsperGenius assay has also been used "off-label" to distinguish *A. lentulus* and *Aspergillus felis* from other members of the *A. fumigati* complex via melt-curve analysis, although testing was limited to DNA extracted from cultures [58]. More work is needed to address the unmet need for species identification.

PCR technology has the potential to identify potentially resistant organisms, overcoming limitations of classic susceptibility testing which is time consuming and may be hindered by the poor growth in culture [28]. The identification of single nucleotide polymorphisms (SNP) or tandem repeats associated with triazole resistance in *A. fumigatus* is now well documented and commercial assays (Mycogenie, Ademtech, Pessac, France; Pathonostics AsperGenius) that target the most frequently encountered mutations (TR_{34}/L98H and TR_{46}/Y121F/T289A) are now available [59, 60]. An alternative approach is to use molecular tests to identify persistent organisms during therapy [61]. Unfortunately, the practicality is limited due to rapid disappearance of the NA signal in blood and persistent NA in BALF may not correlate with viable organisms [62].

Initially, PCR sequencing was required to identify mutations. However, this approach is time-consuming and the development

of real-time PCR that targets common mutations negates the need for gene sequencing and improves the time to result but limits the range of mutations that can be detected [62, 63]. Newer real-time PCR tests have been designed to detect multiple (n = 7, TR_{34}, TR_{46}, G54W, L98H, Y121F, and M220I) *cyp51A* mutations, but direct application to clinical samples has not yet been demonstrated [64]. A multicenter evaluation of the AsperGenius assay of BALF showed that the presence of mutations was significantly associated with treatment failure (75% vs 27%, $P = .01$) and increased 6-week mortality (50% vs 19%, $P = .07$) [65]. Nevertheless, the performance of these assays is variable [60, 66]. A comparison of the performance of the AsperGenius assay with direct PCR sequencing to identify mutations directly from samples, showed that PCR sequencing was only slightly better than real-time PCR [59]. Rapid pyrosequencing methods also have the capacity to detect an increasing number of mutations and have been applied directly to clinical specimens [67]. While direct sample testing to identify mutations has been applied to blood-based samples, the low circulating burden often limits successful amplification of the target genes [60, 68].

The application of next-generation sequencing (NGS) for the detection and identification of fungi directly from a clinical specimen likely represents the future of clinical mycological investigations, with the potential to identify to a species level (even within the mycobiome) and determine antifungal susceptibility and genotype organisms during outbreaks [69]. Currently, several limitations need to be overcome before it is suitable for routine use, including the identification of an optimal gene(s) to provide a sufficient degree of species differentiation (eg, ITS 1/2 regions only differentiate 75% of fungal species [70]), while maintaining the required analytical sensitivity (multicopy vs single-copy genes), optimization of the entire process from sampling through DNA extraction, PCR design, and overcoming the lack of required NGS bioinformatic tools and pipelines [69]. In a review of studies that used molecular approaches to study the complexity of the respiratory mycobiome, it was determined that *Candida* species were the dominant fungi, confirming the commensal nature of this yeast [71]. This highlights how NGS methods may need to be designed to avoid an overwhelming presence of a single commensal/colonizing species/genus of limited clinical importance, restricting the detection of less evident but clinically relevant fungi [72]. The application of digital droplet *Aspergillus* PCR represents an exciting development, potentially enhancing sensitivity and quantification [73].

CONCLUSIONS

Considerable evidence has been gathered about *Aspergillus* PCR assays to support both clinical utility and application to clinical research trials. With continued advances in molecular technology coupled with applications to address important unmet clinical needs, further developments in molecular technology will improve its use in clinical screening, diagnosis, and treatment selection.

Notes

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Clinical Infectious Diseases
SUPPLEMENT ARTICLE

IDSA Infectious Diseases Society of America
hivma hiv medicine association
OXFORD

Assessment of the Role of 1,3-β-d-Glucan Testing for the Diagnosis of Invasive Fungal Infections in Adults

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Detection of 1,3-β-d-glucan (BDG) in serum has been evaluated for its inclusion as a mycological criterion of invasive fungal infections (IFI) according to EORTC and Mycoses Study Group (MSG) definitions. BDG testing may be useful for the diagnosis of both invasive aspergillosis and invasive candidiasis, when interpreted in conjunction with other clinical/radiological signs and microbiological markers of IFI. However, its performance and utility vary according to patient population (hematologic cancer patients, solid-organ transplant recipients, intensive care unit patients) and pretest likelihood of IFI. The objectives of this article are to provide a systematic review of the performance of BDG testing and to assess recommendations for its use and interpretation in different clinical settings.

Keywords. beta-glucan; invasive aspergillosis; invasive candidiasis; hematologic cancer; intensive care.

1,3-β-d-glucan (BDG) is a polysaccharide that is a predominant and specific constituent of the cell wall in most fungi. BDG can be detected in serum during invasive fungal infections (IFI), serving as a biomarker for diseases like invasive aspergillosis (IA) and invasive candidiasis (IC) [1]. *Mucorales* and some basidiomycetous yeasts, such as *Cryptococcus* spp., are not usually detected by BDG testing because the polysaccharide is not a major cell wall component of these fungal species [1]. Growing evidence also supports the utility of BDG testing for the diagnosis of *Pneumocystis jirovecii* pneumonia [2, 3]; however, a review of its diagnostic role in this disease is beyond the scope of this paper.

Serum assays do not directly detect BDG. Rather, they measure BDG-mediated activation of the coagulation cascade in an amoebocyte lysate of the horseshoe crab [4]. Several BDG assays have been developed that differ in their threshold of detection, horseshoe crab species (*Tachypleus tridentatus* or *Limulus polyphemus*), and detection method (turbidimetric or colorimetric) [1]. Currently, 2 commercial kits are available: the Fungitell Assay (Associates of Cape Cod, Inc., Falmouth, Massachetts, USA) and the Wako β-Glucan test (Fujifilm Wako Pure Chemical Corp., Tokyo, Japan). The Fungitell assay, approved by the Food and Drug Administration (FDA) in 2004, is commonly used in the United States and Europe. The Wako test is used in Asia, and it was recently introduced into the European market. Other kits (eg, Fungitec-G, Seikagaku, Tokyo, Japan) have been used in the past.

The performance of the different BDG tests in serum for the diagnosis of IFI has been assessed in multiple studies, which have been pooled in several meta-analyses (Table 1) [3, 5–11]. BDG testing in serum has been included as a mycological criterion for diagnosing IFI in EORTC and Mycoses Study Group (MSG) definitions since 2008 [12].

BDG testing is currently not validated for use in samples other than serum. Some studies have evaluated BDG performance in bronchoalveolar lavage fluid, in particular among lung transplant recipients, with generally poor specificity [13]. BDG testing in cerebrospinal fluid has been evaluated for the diagnosis of fungal meningitis with encouraging results, but the test is uncommonly used for this indication [14, 15].

As part of the consortium for the updated EORTC-MSG definitions of IFI [16], our group was tasked with reassessing the role of BDG testing. We provide here evidence-based recommendations for the use and interpretation of BDG testing in serum for the diagnosis of IFI (in particular, IA and IC) in different subsets of adult patients.

HEMATOLOGIC CANCER PATIENTS

Evidence. BDG testing is used for IFI screening (mainly IA and IC) among high-risk hematologic cancer patients, such as allogeneic hematopoietic stem cell transplant (HSCT) recipients or patients with acute leukemia and prolonged

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Table 1. Performance of Serum BDG Testing for the Diagnosis of Invasive Fungal Infections: Results of Meta-Analyses

1st Author (year) [reference]	No. of Studies	Population	BDG Test	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	DOR	AUC-SROC
Karageorgopoulos (2011) [7]	16	All	All types	77 (67 – 84)	85 (80 –90)	19.2 (10.5–35.4)	0.89
Lu (2011) [9]	13	All	All types	73 (64 – 81)	91 (78– 96)	NA	0.83
Lamoth (2012) [8]	5	Hematologic cancer (cohorts)	All types (1× positive)	61 (48–73)	91 (83 – 95)	16.3 (6.5–40.8)	0.84
			All types (2× positive)	50 (34–65)	99 (97–99)	111.8 (38.6–324.1)	0.88
Onishi (2012) [3]	31	All	All types	80 (77 – 82)	82 (81 – 83)	25.7 (15.0 – 44.1)	0.88
He (2015) [5]	28	All	All types	78 (75 – 81)	81 (80 – 83)	21.9 (12.6 – 37.9)	0.89
Hou (2015) [6]	11	All (cohorts)	All types	75 (63 – 84)	87 (81 – 92)	19.5 (11.2 – 34.2)	0.89
Xiaoling (2018) [11]	37	All	All types	83 (38–61)	80 (81 – 82)	29.7 (18.9 – 46.5)	0.91
White (2019) [10]	10	Cancer	Fungitell	80 (74–89)	63 (64 – 88)	46	0.81

Abbreviations: AUC-SROC, area under the curve of the summary receiver operating characteristic curve; BDG, 1,3-β-d-glucan; CI, confidence interval; DOR, diagnostic odds ratio; NA, data not available; 1× positive, single positive test, 2× positive, 2 consecutive positive tests.

chemotherapy-induced neutropenia [17]. Its performance in this setting has been assessed in several studies that were included in meta-analyses along with other studies performed in nonhematologic cancer patients (Table 1) [3, 5–11]. Heterogeneity of the study designs was observed in types of analysis (case-control vs cohort or prospective versus retrospective studies), strategies of screening (serial BDG monitoring vs punctual BDG testing), criteria defining a positive BDG test (threshold for positivity, single vs 2 consecutive positive results), and definitions or types of IFI. One meta-analysis restricted to 5 cohort studies of patients with hematologic malignancies showed sensitivity of only 61% but specificity of 91%; specificity increased to 99% with the requirement of 2 consecutive positive tests [8]. Lower specificity (63%) was reported in another meta-analysis of patients with hematologic or solid cancers that had less restrictive inclusion criteria [10].

The rate of putatively false positive results varies considerably across studies [8, 10, 18], likely shaped by the types of patients tested, the approach to diagnosing IFI, or the risk of contamination associated with preanalytical/analytical conditions. Potential causes of false positive results include blood transfusion and blood-derived products, renal replacement therapy, or broad-spectrum antibiotics [1]. Immunoglobulin replacement therapy, which is used for the management of some hematologic cancers, was identified as a significant cause of false positive BDG results in this population [19].

Comparative analyses of BDG performance did not identify significant differences in diagnosing IA or IC [8]. Limited data suggest that BDG may be useful for detecting non-*Aspergillus* invasive mold infections (eg, infections due to *Fusarium* spp. or *Scedosporium* spp.); as mentioned above, mucormycosis is usually not detected [20–24]. Experience with yeasts other than *Candida* spp. is also limited. Some data suggest that BDG can be detected in about half of cases of *Trichosporon* fungemia, which is increasingly recognized among hematologic cancer patients [25, 26]. The impact of ongoing prophylaxis on BDG sensitivity is not well known, but current data suggest that performance is not affected [23, 24].

No significant differences in diagnostic performance between Fungitell and Wako assays have been observed. Cutoff values of these tests for optimal sensitivity/specificity may be lower in patients with hematologic malignancy than those recommended by the manufacturers (ie, 60 pg/mL instead of 80 pg/mL for Fungitell, and 5 pg/mL instead of 11 pg/mL for Wako) [8].

The performance of BDG testing was compared to the galactomannan assay for diagnosing IA in several studies, which found similar overall sensitivity and specificity [27–29]. The combination of these tests may improve the rate of detection. However, there are currently a limited set of studies addressing the combined performance of BDG and galactomannan in serum [30, 31]. Their results suggest that this approach may

improve the sensitivity, but to the detriment of decreased specificity.

The timing of BDG positivity in the course of IFI has been investigated in several studies [29, 32, 33]. Results are inconsistent when comparing time of BDG positivity to radiologic detection of IFI signs by computed tomography (CT) scan, mainly due to differences in diagnostic work-up strategies among studies (eg, timing of CT scan, frequency of BDG screening).

The prognostic role of BDG testing in follow-up has also been evaluated [33–35]. Although BDG kinetics tended to correlate with clinical outcomes of both IA and IC, the assessment of the utility of BDG as a prognostic test is difficult due to its long half-life in serum. Persistently high BDG levels are frequently observed, and the test has limited value in assessing early responses to therapy.

The performance of BDG testing in patients with nonhematologic malignancies (eg, solid tumors) has been poorly investigated. Specificity may be lower in this population at lower risk of IFI and with lower pretest disease probability [10].

Recommendations. A robust set of data exists on the use of BDG testing for diagnosing IFI in hematologic oncological patients, such as allogeneic HSCT recipients or leukemic patients with prolonged chemotherapy-induced neutropenia. BDG can be used for the detection of IA, IC, or other IFI, with notable exceptions of mucormycosis and cryptococcosis. Monitoring patients during neutropenic and other high-risk periods with serial BDG measurements (once or twice per week) can be considered. Because of its limited sensitivity, BDG testing should not be used to rule out IFI in oncological patients considering the relatively high prevalence of IFI in this population. An intermediate or positive test (60–80 pg/mL for Fungitell, 5–11 pg/mL for Wako) should prompt further diagnostic work-up and be interpreted in conjunction with clinical, radiological and other mycological criteria of IFI according to the EORTC-MSG definitions [16]. A confirmatory BDG test after a first positive result is recommended to improve positive predictive value (PPV). BDG testing should be avoided in patients receiving immunoglobulin replacement therapy, which can cause false positive results.

SOLID-ORGAN TRANSPLANT RECIPIENTS

Evidence. Data about the performance of BDG testing for IFI diagnosis in other immunocompromised populations, such as solid-organ (SOT) transplant recipients, are scarce. Some studies have been performed among lung or liver transplant recipients [36–41]. Both IA and IC have been included in these analyses, often without clear distinction between the two diseases. Overall, sensitivity ranged from 60% to 80%, which resulted in negative predictive value (NPV) > 90% in this population with low incidence of IFI. However, specificity was low, with PPV that did not exceed 50%. Specific data for other SOT recipients or other immunocompromised populations (eg, patients with autoimmune disorders) are lacking.

Recommendations. The paucity of BDG data in immunocompromised hosts other than hematologic cancer patients (eg, SOT recipients) and the modest performance of the test in these settings do not currently support routine testing. However, BDG testing can be considered for excluding IFI among patients with low pretest probability, due to its NPV > 90%.

INTENSIVE CARE UNITS (ICU) PATIENTS

Evidence. Several studies have addressed the performance of BDG tests (mainly Fungitell) in detecting IFI (mainly IC) in ICU patients (Table 2) [42–61]. These analyses differ in design (cohort vs case-control), inclusion criteria, and the timing of BDG testing. Results demonstrate large heterogeneity in optimal BDG thresholds, and sensitivity and specificity. For the diagnosis of IC, most studies suggest a NPV > 90% and a PPV < 70%. False positive results in ICU patients may be due to many clinical variables and conditions (surgical gauzes, renal replacement therapy, albumin transfusion, broad-spectrum antibiotics) [1, 48, 53, 62]. Specificity and PPV can be increased by 2 consecutive BDG testing without significant impact on NPV [48, 55, 56, 61]. Use of higher cutoffs has also been suggested for improving sensitivity/specificity ratios [51, 58]. Some studies show better performance when BDG testing is restricted to patients at high risk of IC, such as those with complicated abdominal surgery (eg, recurrent gastrointestinal perforation or hepatobiliary anastomotic leakage; necrotizing pancreatitis) [49, 61]. Acceptable PPV (70 – 80%) can be achieved in this clinical subset but at the cost of lower NPV (around 80%).

The combination of BDG testing with other fungal biomarkers, mainly *Candida albicans* germ tub antibody (CAGTA), or clinical prediction rules (eg, *Candida* score) may improve performance, as suggested by some studies [51, 54, 55].

The role of BDG in guiding antifungal therapeutic decisions has been assessed in several studies [48, 49, 55, 63–65]. Because NPV is generally high, a negative BDG result can be used to withhold or discontinue empirical antifungal therapy. The role of positive BDG results in prompting preemptive antifungal strategies is less evident because of the relatively low PPV [48, 49, 65, 66]. BDG kinetics showed some correlation with response to therapy but with low potential utility for clinical application for reasons discussed above [48, 67, 68].

The performance of BDG in diagnosing IA in the ICU has been assessed in a few studies that included mixed populations of patients (hematologic and non-hematologic cancer patients) [42–46, 50]. Sensitivity was variable and specificity was relatively low (70–80%) with PPV not exceeding 50%. Higher

Table 2. **Performance of Serum BDG Testing for the Diagnosis of Invasive Fungal Infections in the Intensive Care Unit (ICU)**

1st Author (year) [reference]	Type of Study	BDG Cutoff[a]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Invasive candidiasis						
Leon (2009) [52]	Cohort (prospective)	75 pg/mL	78	53	12	97
Presterl (2009) [60]	Cohort (retrospective)[b]	40 pg/mL	52	76	46	80
Mohr (2011) [57]	Cohort (prospective)	80 pg/mL	87	73	NA	NA
Posteraro (2011) [59]	Cohort (retrospective)[b]	80 pg/mL	93	94	72	99
Acosta (2012) [43]	Cohort (prospective)	80 pg/mL	70	59	16	95
Hanson (2012) [48]	Cohort (prospective)	80 pg/mL	100	59	21	100
		80 pg/mL (2×)	100	75	30	100
Leon (2012) [51]	Cohort (prospective)	80 pg/mL	68	55	36	82
		259 pg/mL	52	87	59	83
Tissot (2013) [61]	Cohort (prospective)	80 pg/mL	83	40	49	77
		80 pg/mL (2×)	65	78	68	77
Poissy (2014) [58]	Case-control	80 pg/mL	97	31	NA	NA
		350 pg/mL	65	74	NA	NA
Lo Cascio (2015) [53]	Cohort (retrospective)	86 pg/mL	75	63	41	82
Martinez-Jimenez (2015) [54]	Case-control	80 pg/mL	84	92	87	90
Martinez-Jimenez (2015) [55]	Cohort (prospective)	80 pg/mL	87	53	44	90
		80 pg/mL (2×)	73	70	51	86
Martin-Mazuelos (2015) [56]	Cohort (prospective)	80 pg/mL	80	45	19	93
		80 pg/mL (2×)	80	76	35	96
Giacobbe (2017) [47]	Cohort (retrospective)	80 pg/mL	92	81	79	93
Kritikos (2020) [49]	Cohort (retrospective)	80 pg/mL	71	67	36	90
		80 pg/mL (2×)	70	68	41	88
Invasive aspergillosis						
Acosta (2011) [42]	Cohort (prospective)	80 pg/mL	80	75	44	94
De Vlieger (2011) [46]	Case-control	80 pg/mL	86	36	36	86
		140 pg/mL	86	70	54	92
Acosta (2012) [43]	Cohort (prospective)	80 pg/mL	92	63	26	98
		150 pg/mL	83	76	32	97
Cai (2014) [45]	Cohort (prospective)	80 pg/mL	48	79	48	79
Lahmer (2016) [50]	Cohort (retrospective)	80 pg/mL	88	82	NA	NA
Boch (2018) [44]	Cohort (prospective)	80 pg/mL	90	26	23	90

Abbreviations: ICU, intensive care unit, NA, data not available; NPV, negative predictive value; PPV, positive predictive value; 2×, 2 consecutive positive tests.

[a] Fungitell test was used in all studies. Data are presented for the cutoff recommended by the manufacturer (80 pg/mL) if available, and for the cutoff providing the best sensitivity/specificity ratio (if different).

[b] Include all type of invasive fungal infections with a majority of invasive candidiasis.

cutoffs (140–150 pg/mL) have been associated with improved specificity [43, 46].

Recommendations. Negative BDG results can be used to guide decisions to withhold or interrupt antifungal therapy among ICU patients at risk for IC, when interpreted in conjunction with other microbiological results and clinical signs/severity of infection. Use of BDG as a screening tool for preemptive antifungal strategies should be limited to groups of patients at particularly high risk of IC (eg, complicated abdominal surgery, particularly recurrent gastrointestinal perforation or hepatobiliary anastomotic leakage; necrotizing pancreatitis). In these settings, BDG testing can be combined with other markers of IC (eg, *Candida* score, CAGTA). Most data support the use of a positivity cutoff of 80 pg/mL for the Fungitell test, as recommended by the manufacturer. Data about the use of other BDG tests (eg, Wako) in ICU settings are currently insufficient to allow specific recommendations. The use of BDG for the diagnosis of IA in ICU cannot be recommended due to very limited datasets and concerns about low PPV.

CONCLUSIONS

Table 3 provides a summary of our recommendations for the use of BDG testing in serum for the diagnosis of IFI in adult patients. Because of limited sensitivity and specificity, the utility of serum BDG differs by patient population at risk of IFI. The prevalence of IFI in specific populations and the pre-test probability of IFI in individual patients should be taken into account when interpreting negative and positive results. The precise role of BDG testing in preemptive antifungal treatment strategies should be further investigated in prospective randomized interventional studies.

Table 3. Recommendations for the Use of BDG Testing in Different Patient Populations

Patient Population	Type of IFI	Negative BDG to Exclude IFI	Positive BDG to Start Antifungal Therapy for Probable IFI
Hematologic cancer patients[a]	IA, IC, other IFI[c]	Not recommended (low NPV)	If combined with other EORTC-MSG criteria of IFI; 2 consecutive positive tests recommended[d]
Solid-organ transplant recipients	IA, IC, other IFI[3]	If pretest probability of IFI estimated as low/moderate	Not recommended (low PPV)
Other type of immunosuppression[b]	IA, IC, other IFI[c]	If pretest probability of IFI estimated as low/moderate	Not recommended (low PPV)
ICU patients	IC	If pre-test probability of IC estimated as low/moderate	If testing is restricted to high risk patients[e]; 2 consecutive positive recommended[d]
	IA	Not recommended (low NPV)	Not recommended (low PPV)

Abbreviations: EORTC-MSG, European Organization for Research and Treatment of Cancer and Mycoses Study Group; IA, invasive aspergillosis; IC, invasive candidiasis; ICU, intensive care unit; IFI, invasive fungal infection; NPV, negative predictive value; PPV, positive predictive value.

[a]Allogeneic hematopoietic stem cell transplant recipients or patients with acute leukemia and prolonged chemotherapy-induced neutropenia (>10 days).

[b]Patients receiving long-term immunosuppressive therapy for autoimmune disorders or other diseases, patients with solid tumor and chemotherapy-induced neutropenia of short duration (<10 days).

[c] IFI due to molds other than *Aspergillus* spp. (eg, *Fusarium* spp., *Scedosporium* spp.) with the exception of mucormycosis (due to *Mucorales*). *Cryptococcus* is not detected by BDG.

[d]Higher specificity with two consecutive tests. However, antifungal treatment should not be delayed when clinical conditions justify prompt initiation.

[e]Patients with complicated abdominal surgery (ie, recurrent gastro-intestinal tract perforation or hepatobiliary anastomotic leakage), necrotizing pancreatitis or *Candida* score ≥ 3.

Notes

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Clinical Infectious Diseases

SUPPLEMENT ARTICLE

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Molecular Techniques for Genus and Species Determination of Fungi From Fresh and Paraffin-Embedded Formalin-Fixed Tissue in the Revised EORTC/MSGERC Definitions of Invasive Fungal Infection

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The EORTC/MSGERC have revised the definitions for proven, probable, and possible fungal diseases. The tissue diagnosis subcommittee was tasked with determining how and when species can be determined from tissue in the absence of culture. The subcommittee reached a consensus decision that polymerase chain reaction (PCR) from tissue, but not immunohistochemistry or in situ hybridization, can be used for genus or species determination under the new EORTC/MSGERC guidelines, but only when fungal elements are identified by histology. Fungal elements seen in tissue samples by histopathology and identified by PCR followed by sequencing should fulfill the definition of a proven fungal infection, identified to genus/species, even in the absence of culture. This summary discusses the issues that were deliberated by the subcommittee to reach the consensus decision and outlines the criteria a laboratory should follow in order to produce data that meet the EORTC/MSGERC definitions.

Keywords. formalin-fixed paraffin-embedded tissue; FFPE; tissue diagnosis; EORTC/MSG; immunohistochemistry.

Correct diagnosis of fungal genus and species from histopathology is a vanishing art, and the diagnostic accuracy of identification from traditional histopathology alone, even to the genus, is generally below 80% [1, 2]. This lack of diagnostic accuracy can have many consequences, ranging from inappropriate antifungal therapy to exclusion from a clinical trial. In addition, there are problems with fungal diagnosis from tissue; often, the histopathology detects a fungus but the culture is negative, or is simply not possible, for instance, because the specimen has been placed in formalin and not sent for culture. A study from the MD Anderson Cancer Center showed that culture was positive for only 30% of their histopathology positive fungal cases, and a study from 2 Spanish hospitals found that only 56% of their histopathology-positive fungal cases were also culture positive [3, 4]. In this scenario, it is difficult to determine the genus, let alone the species of the offending fungus.

The EORTC/MSGERC have revised the definitions for "proven," "probable," and "possible" fungal infection [5]. It has been 10 years since the definitions were last revised, during which time there has been substantial improvement in our methods and understanding of diagnosis of fungal infections from tissue, especially using molecular techniques. The tissue subcommittee of this group determined that the changes in technology that have come about since the publication of the definitions in 2008 warranted an update to the definitions with regard to identification of fungi from tissue [6]. The 2008 definitions stated the following regarding the use of molecular methods for detecting fungi in tissue: "By contrast, molecular methods of detecting fungi in clinical specimens, such as [polymerase chain reaction] PCR, were not included in the definitions because there is as yet no standard, and none of the techniques has been clinically validated"…"We had hoped that nucleic acid–detection tests, such as PCR, would have improved enough to incorporate the results of these tests into the definitions. However, standardization and validation have not yet been attained for these platforms" [5]. In the intervening period since these definitions were published, there has been substantial work on the development and application of PCR to amplify fungal DNA from both formalin-fixed paraffin-embedded (FFPE) and fresh tissue [4, 7-31]. However, the success rate of the various laboratory-developed protocols varies greatly due to many methodologic variables such as the method of DNA extraction, inoculum, sequencing targets, primer selection, specimen variables (open biopsy vs fine needle aspiration, fresh vs fixed tissue), providing mixed

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results in the literature. For example, Buitrago and colleagues found that PCR of fungal DNA from tissue shown by histopathology to contain fungi was 89% successful, although the species determined by DNA sequencing was discordant with the culture results in 13% of cases [4]. In contrast, a study from Japan showed that PCR of fungal DNA from FFPE tissue shown histopathologically to contain fungi was only 23% successful [15]. The apparent discrepancy in success between studies would indicate caution should be exercised because successful amplification of fungal DNA from tissue is dependent upon many factors, including the amount of fungi in the tissue, the amount of tissue available, the amount of time the tissue has been fixed in formalin, and whether the formalin was buffered or unbuffered.

One of the inherent difficulties in determining the success rate of fungal species identification from tissue is the suboptimal success rate of the "gold standard," which is the growth of a fungus from tissue [3]. If molecular detection and identification of fungi is more sensitive than the gold standard, as was seen with the recent fungal meningitis outbreak [32, 33], then the true sensitivity and specificity of the assay cannot be determined. A good example is the previously mentioned study from Spain, where histologic evidence of fungal infection was used as the standard, culture was only 56% sensitive [4]. Although tissue PCR was successful, there was no way to prove that the species identification was correct because the gold standard was less sensitive, leaving no isolate for comparison with many PCR positive cases.

The US outbreak of *Exserohilum* meningitis presented a good opportunity to directly compare culture with histologic evidence of infection and amplification of nucleic acids. In the study by Ritter and colleagues, patient FFPE tissues were stained using a panfungal polyclonal antibody directed to cell wall carbohydrates [33]. Tissue scrolls from both the histochemically positive and the histochemically negative cases were sent for PCR analysis. PCR from FFPE was not as sensitive as histopathological staining for the detection of fungi [33]. There were no cases where fungal nucleic acids were amplified in the absence of histological staining. By contrast, there were 16 cases where fungi could be detected by histochemical staining but no fungal DNA could be amplified. It is unclear if these results are fungus (*Exserohilum*) or tissue (meninges, brain)-specific or could be extrapolated to the plethora of other fungi causing invasive disease.

The 2008 definitions did allow for the designation of a proven infection based upon direct microscopic analysis of tissue. However, the guidelines did not allow for a species designation based on such analysis. Correct diagnosis of fungal species from histopathological specimens remains difficult [34]. Sangoi and colleagues compared histopathologic designation of genera with the cultured species identification [1]. Although the correct genera were described in 79% of cases, major errors were encountered, such as describing *Rhizopus* or *Scedosporium* as *Aspergillus* and describing *Histoplasma* as *Candida,* pointing to the limitations of any designation beyond "yeast or hyphae seen" [2, 7]. In a similar study, infections identified by histopathology as mucormycosis (MCR) were subsequently identified by PCR to be *Aspergillus* and *Scedosporium*, and conversely, cases identified as aspergillosis were subsequently identified as *Fusarium* or *Rhizopus* by PCR [29].

Immunohistochemical stains are commercially available for the Mucormycetes, *Aspergillus* species, and *Candida* species [35-37]. Although the specificity and sensitivity are high when used in culture-proven cases, the specificities of these stains have not yet been fully defined against a wide variety of other closely related species, especially among the other hyalohyphomycetes. Encouraging data with Mucormycetes, *Candida* species, and *Aspergillus* species are comprehensive and robust [35-39]. In a recent study, Jung et al investigated the accuracy of histomorphologic diagnosis of MCR and invasive aspergillosis (IA), using fungus-specific immunohistochemistry (IHC) in patients with proven/probable MCR or IA that had FFPE tissues available [38]. In 7 proven cases of MCR, the sensitivity and specificity of MCR IHC were 100% and 100%, respectively. In 8 proven cases of IA, the sensitivity and specificity of aspergillosis IHC staining were 87% and 100%, respectively. For probable cases, the sensitivity and specificity are much lower. In the absence of fungal culture results, the IHC tests seem helpful in differentiating between IA and MCR. However, such approaches remain problematic in the uncommon infections caused by organisms other than *Aspergillus*, Mucormycete spp, and *Candida*, because an abundance of negative control staining remains elusive. Until a comprehensive clinical study that provides more negative controls is published, specific immunohistochemical staining for genus identification cannot be fully endorsed for inclusion in the EORTC/MSGERC definitions. Similarly, difficulties are encountered with in situ hybridization techniques [30, 34, 40]. Some of the published probes have shown cross-reactivity and there are no validated protocols available. Although promising, more work needs to be done before it can be recommended.

Metagenomic next-generation sequence analysis is emerging as a powerful tool because it could be used to detect any fungal pathogen, even when intact fungal cells are not present in the tissue [41]. However, this tool is still under clinical validation and one of the few studies to look at fungi in tissue showed low sensitivity and specificity [42]. In addition, because of the high sensitivity of the assay, there is not yet a firm grasp on the clinical implications of the identification of

an organism, especially when histopathology and culture confirmation are absent [43]. As is the case with in situ hybridization and gene-targeted PCR techniques, rare fungi have few reference genomes and curated public databases, which complicates the analysis [44].

There are additional problems outside of the tissue-based molecular assays themselves that contribute to the difficulty of making a correct diagnosis. The first is that formalin treatment of tissue affects the integrity of the DNA and leads to shorter fragments, which may prevent amplification of the target sequence or may only allow the amplification of a fragment that is not long enough for species identification [45, 46]. Another is the paucity of sequences in publicly available validated and annotated fungal genome libraries [47, 48]. New fungal species are described on a regular basis but genome libraries are not consistently updated and annotated, often leaving sequences misidentified [49–51]. Although pan-fungal primers recognize most fungal taxa, they can also promote amplification of contaminating fungal DNA. Quantitative real-time PCR might obviate the problem of contamination, but its specificity limits the number of species that can be reasonably detected. Despite all of these difficulties, significant advances have been made. In a prospective, blinded study comparing histopathology, PCR and culture of aspergillosis and mucormycosis in FFPE tissue specimens, PCR identification fared quite well and even allowed the detection of mixed infection in 2 cases [52]. In a retrospective analysis to compare histopathology, culture, and PCR of MCR, PCR was positive for 10 of 12 culture confirmed cases and the sequence matched the cultured organism in nine of those cases [53]. In the 15 culture negative cases, the PCR was positive and a Mucormycete sequence was obtained for 12 cases.

A final problem related to assay performance is that many reagents used to process tissue, such as lyticase, proteinase K, and even the PCR master-mix, can be contaminated with fungal DNA during the manufacturing process [54–56]. In addition, fungal spores are ubiquitous in the indoor environment and the paraffin used for making blocks is not kept sterile in the pathology laboratory. The ubiquitous nature of fungal spores and fungal DNA greatly increases the chance of spurious amplification and false-positive PCR results.

Although the use of PCR for the detection of fungi from tissue has greatly advanced, the 2 issues outlined in the 2008 EORTC/MSG guidelines remain: there is no standardized technique for detection of fungal nucleic acid from tissue and there have only been a few validation studies to show that the obtained results are correct. The protocols used for this procedure are laboratory specific; there are no International Organization for Standards or Clinical and Laboratory Standards Institute guidelines to assure that protocols and interpretations are reproducible between laboratories [57, 58]. However, given that a validated consensus protocol is not forthcoming, we offer guidance on essential criteria while allowing some deviation in protocol, similar to those developed for quantitative real-time PCR [59]. For the purposes of the EORTC/MSGERC definitions for the identification of fungal infections, laboratory-validated protocols should rely on a common set of rules that may suffice until an international standard can be established. The subcommittee for tissue diagnosis recommended that PCR for species identification of fungi from tissue be adopted with the following rules and caveats:

1. PCR is appropriate for detection of invasive fungal infection from tissue samples only when fungal elements or structures have been detected by histopathology. PCR in tissue is not recommended in cases where fungal staining is negative.
2. Laboratories performing PCR-based identification of fungi from tissue must have a unidirectional workflow with strict separation of DNA-extraction, PCR preparation, amplification, and detection of PCR products. These processes should take place either in separate rooms or in separate isolation cabinets.
3. Quality control procedures are highly recommended to check for potential contaminating fungal DNA.
4. Primers should be panfungal, targeting the fungal barcoding sequences of either the ITS region or the D1/D2 region of ribosomal DNA [60]. Alternatively, other ribosomal or mitochondrial genes that target unique, species- or genus-specific proteins or antigens that have been fully validated must be used [52, 53, 61].
5. Every PCR reaction must include separate negative and positive amplification controls accompanied by a separate or tandem PCR reaction targeting a human housekeeping gene, like beta-globin, to indicate successful fungal DNA extraction and rule out inhibitors.
6. Every PCR product must be sequenced for identification—neither size of the PCR product nor hybridization such as in real-time PCRs, are regarded as sufficient for identification.
7. The length of sequence/PCR product should not be less than 150 base pairs, including primer-binding regions.
8. For species identification, a homology of the PCR product with the sequence in the database should be ≥98%. Use of a quality-controlled database, like the new database of the International Society for Human and Animal Mycology is strongly recommended [60].
9. When the species identification matches more than 1 species in the database to the same percentage, only the genus name should be used. Care should be given to periodic changes in fungal nomenclature [62].
10. The genus and species identification from PCR amplification should be consistent with the key histological features of the organism in tissue.

11. This test should be performed only in reference centers or high-volume centers that meet these requirements and not in small volume, community hospitals where volume might be low and expertise cannot be maintained or requirements met [63].

Although such tissue-based molecular assays may not provide a cost-effective tool for routine clinical diagnosis and management of fungal infections [64], in cases where there is histopathological evidence of a fungal infection without confirmation by culture, molecular tools may assist in identification of the etiological agent if the outlined criteria are met. Thus, fungal elements seen in tissue samples by histopathology and identified by PCR followed by sequencing should fulfill the definition of a proven fungal infection, even in the absence of culture. Again, because of the ubiquitous nature of fungal DNA and fungal spores, amplification of specific fungal DNA without histopathologic evidence of fungal infection is insufficient to confidently prove an invasive fungal infection. In cases of endemic mycoses where the only proof of infection is histopathology, molecular tools are recommended as proof of the diagnosis, especially in laboratories that have limited experience with these fungi [65, 66]. Laboratories that intend to perform fungal identification by PCR should first perform both a validation of their methodology as well as a validation of the DNA sequence database that they intend to use.

Because of the promise of advancement using newer molecular techniques, there is an urgent need for a multicenter study using prospective evaluation of a consensus protocol or set of guidelines [67]. Because this type of study is not immediately forthcoming, the criteria developed should be used as a framework for the correct use of PCR for the amplification of fungal DNA from tissue in individual laboratories.

Notes

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SUPPLEMENT ARTICLE

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Pneumocystis jirovecii Disease: Basis for the Revised EORTC/MSGERC Invasive Fungal Disease Definitions in Individuals Without Human Immunodeficiency Virus

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Background. *Pneumocystis jirovecii* pneumonia (PCP) causes substantive morbidity in immunocompromised patients. The EORTC/MSGERC convened an expert group to elaborate consensus definitions for *Pneumocystis* disease for the purpose of interventional clinical trials and epidemiological studies and evaluation of diagnostic tests.

Methods. Definitions were based on the triad of host factors, clinical-radiologic features, and mycologic tests with categorization into probable and proven *Pneumocystis* disease, and to be applicable to immunocompromised adults and children without human immunodeficiency virus (HIV). Definitions were formulated and their criteria debated and adjusted after public consultation. The definitions were published within the 2019 update of the EORTC/MSGERC Consensus Definitions of Invasive Fungal Disease. Here we detail the scientific rationale behind the disease definitions.

Results. The diagnosis of proven PCP is based on clinical and radiologic criteria plus demonstration of *P. jirovecii* by microscopy using conventional or immunofluorescence staining in tissue or respiratory tract specimens. Probable PCP is defined by the presence of appropriate host factors and clinical-radiologic criteria, plus amplification of *P. jirovecii* DNA by quantitative real-time polymerase chain reaction (PCR) in respiratory specimens and/or detection of β-D-glucan in serum provided that another invasive fungal disease and a false-positive result can be ruled out. Extrapulmonary *Pneumocystis* disease requires demonstration of the organism in affected tissue by microscopy and, preferably, PCR.

Conclusions. These updated definitions of *Pneumocystis* diseases should prove applicable in clinical, diagnostic, and epidemiologic research in a broad range of immunocompromised patients without HIV.

Keywords. cancer; transplantation; definitions; *Pneumocystis*; clinical trials; consensus; adults; children.

The EORTC/MSGERC consensus definitions of invasive fungal diseases (IFDs) published in 2002 [1] and updated in 2008 [2] have evolved into essential documents for research in clinical mycology. The definitions have fostered comparison of clinical research in patients with cancer and solid-organ and hematopoietic stem cell transplantation (HSCT) [3, 4]; they have been adopted by regulatory agencies for antifungal agents [4–7] and used to evaluate diagnostic tests [8] and for epidemiologic studies [9–12]. As such, they are specifically intended for research only, and not to direct patient care.

The 2008 revised definitions had their limitations, including poor applicability to patients treated in intensive care units (ICUs), lack of thresholds of positivity, and validation of, fungal biomarkers, and a focus on opportunistic mold infections [2]. Notably, no definitions were provided for diseases caused by *Pneumocystis jirovecii* including life-threatening pneumonia (PCP). *Pneumocystis jirovecii* pneumonia is particularly relevant in patients with profound impairment of T-cell–mediated immunity.

To overcome this limitation in a time of evolving anticancer immunotherapies, change in composition of immunocompromised patient populations, and new diagnostic tools, the EORTC/MSGERC established definitions for *P. jirovecii* disease in their second revision of IFD definitions [13]. The definitions were based on the established triad of host factors, clinical features, and mycologic tests with categorization into probable and proven disease, and were applicable to immunocompromised adults and children without human immunodeficiency virus

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(HIV). Here we present the scientific rationale behind these disease classifications.

METHODS

The *Pneumocystis* subcommittee of the EORTC/MSGERC was asked to restrict their purview to immunocompromised individuals without HIV [13]. This restriction was based on important differences in the biology and presentation of *Pneumocystis* disease between patients with advanced HIV infection and immunocompromised patients without HIV with attendant impact on applicability of diagnostic criteria [14–19].

Under the senior author (A. H. G.), host factors and diagnostic clinical and microbiologic criteria of *Pneumocystis* disease were evaluated through systematic literature review. Medical subject heading (MESH) terms were used as keywords to search articles published in English in PubMed. Host factors and clinical and microbiologic criteria were formulated and adapted after discussion within the group. The process for review and formulation of consensus is detailed in the 2019 revised IFD definitions [13].

DIAGNOSTIC CRITERIA

Host Factors

Pneumocystis jirovecii is a transient fungal colonizer of human pulmonary alveoli [20–22]. Although not completely elucidated, the mode of acquisition of infection likely occurs by the airborne route and person-to-person spread [23]. Seroepidemiologic studies suggest primary contact with the organism occurs in infancy [24, 25], with asymptomatic or mild upper respiratory tract infection [26]. There is no molecular evidence for a truly latent infection, and disease is believed to arise from prior colonization or by new infection [26, 27].

In immunocompromised patients, the organism may proliferate and cause lung disease through interaction with type I alveolar cells. Extrapulmonary manifestations are rare [28] and may be associated with atypical forms of *P. jirovecii* [29]. Pulmonary disease or PCP is typically diffuse with alveolar damage, an eosinophilic intra-alveolar foamy matrix, and interstitial inflammatory response, resulting in restrictive pulmonary disease, progressive hypoxemia, and death if untreated [26, 30]. Alveolar macrophages are the central effector cells in host defense against *P. jirovecii*, and PCP is exclusively associated with qualitative or quantitative impairment of T-cellular immunity [31–33].

Apart from institutionalized neonates with functional immaturity of cellular immunity, the principal populations at risk include children with primary T-cell immunodeficiencies, patients with very low CD4+ lymphocyte counts (eg, those undergoing intensive immunosuppressive therapy with glucocorticosteroids and other agents affecting CD4+ lymphocyte counts), and patients undergoing chemotherapy for cancer, or solid-organ transplantation or allogeneic HSCT prior to immune-reconstitution [26, 30, 34–36]. Historical incidence rates of PCP in immunocompromised patients without HIV not receiving prophylaxis are more than 20% for children with acute lymphoblastic leukemia [37], non-Hodgkin's lymphoma [38], or soft tissue sarcoma [39]; and for adults, rates are 20–30% in non-Hodgkin's lymphoma [40, 41], 5–15% in allogeneic HSCT recipients [42], 5–15% in solid-organ transplant recipients [43], and 6% in brain tumor patients with irradiation receiving glucocorticosteroids [44]. Compliance with trimethoprim/sulfamethoxazole renders *Pneumocystis* disease unlikely [39].

In case series, 80–90% of immunocompromised patients with PCP without HIV received corticosteroids [36, 45, 46] and, similar to patients with HIV [36, 47], a systematic review concluded that a CD4+ cell count of less than 200 was a sensitive biomarker of "high risk" in immunocompromised patients without HIV [48]. The strong association between *Pneumocystis* disease and immunosuppression suggests that risk should be focused on the net state of immunosuppression as opposed to underlying disease, including the use of glucocorticosteroids and therapies and conditions that specifically compromise T-cell–mediated immunity (Table 1).

Clinical Criteria

Symptoms and Signs

Unlike in patients with HIV where the onset of PCP is usually gradual and insidious, with few physical or radiologic findings, in immunocompromised patients without HIV, clinical presentation tends to be more acute with rapid onset of respiratory symptoms and faster progression to respiratory failure, higher ICU admission rates, and mortality exceeding 50% [16, 36, 49–51]. Clinical features include fever, progressive dyspnea, nonproductive cough, chest pain, circulatory failure, pneumothorax, and, very rarely, hemoptysis [36, 50–61]. The differences in clinical presentation between patients with and without HIV appear to be related to differences in severity of pneumonia and degree of lung inflammation. Patients with HIV have a higher organismal load and fewer granulocytes in the lung than do patients without HIV, with greater impairment of gas exchange [15] (Table 1). There is a paucity of findings at auscultation. Serum lactate dehydrogenase levels are not typically elevated in patients without HIV [49–51].

Very rarely, *Pneumocystis* disease may spread to other body sites and cause extrapulmonary manifestations whose signs and symptoms are nonspecific and will depend on the site involved [28, 29].

Table 1. Host Factors, Clinical Criteria, and Microbiologic Criteria Used for the Definition of *Pneumocystis jirovecii* Pneumonia

	Description
Host factors	• Use of therapeutic doses of ≥0.3 mg/kg prednisone equivalent for ≥2 weeks in the past 60 days • Low CD4+ lymphocyte counts (observed or expected; <200 cells/mm^3) induced by a medical condition, anticancer, anti-inflammatory, and immunosuppressive treatment, including but not limited to: - Primary immunodeficiencies with numeric/functional T-cell deficiency - Acute leukemia, non-Hodgkin's lymphoma, solid tumors, allogeneic HSCT - Solid-organ transplantation - Autoimmune- and hyperinflammatory disorders, including treatment with agents that lead to functional T-cell deficiencies
Clinical criteria	• Fever • Respiratory symptoms including cough, dyspnea, or hypoxemia • Bilateral or diffuse GGO on X-ray with interstitial infiltrates as the predominant feature; alveolar, alveolar-interstitial, and unilateral infiltrates are less frequent • Extensive, mostly diffuse GGO on CT scans, which typically has an upper lobe and perihilar predominance, sometimes with peripheral sparing or a mosaic pattern; consolidations, small nodules, and unilateral infiltrates are less frequent
Microbiologic criteria	• Visualization of *P. jirovecii* by microscopy using conventional staining methods (Gomori methenamine silver, Toluidine Blue O, Giemsa, Calcofluor White) or immunofluorescence staining in tissue, BAL fluid, induced sputum, expectorated sputum, or oral wash • Amplification of *P. jirovecii* DNA by quantitative real-time PCR in BAL fluid, induced sputum, or oral wash • Detection of β-D-glucan in serum if another invasive fungal infection and a false-positive result can be ruled out

Abbreviations: BAL, bronchoalveolar lavage; CT, computed tomography; GGO, ground-glass opacity; HSCT, hematopoietic stem cell transplantation; PCR, polymerase chain reaction.

Radiographic Patterns

Between 10% and 15% of immunocompromised patients with PCP without HIV have normal chest radiographs and, among those with abnormalities, close to 30% have nonspecific findings. Typical findings are bilateral, diffuse ground-glass opacity (GGO) with interstitial infiltrates. Alveolar infiltrate patterns, unilateral involvement, lung nodules, or pleural effusions are less frequent. In mild or early presentations, opacities are usually perihilar. With advancing disease, opacities become diffuse and are in a butterfly pattern [19, 36].

Using high-resolution pulmonary computed tomography scans, extensive GGO is the main feature, representing exudate formation from alveolitis [15, 53]. Ground-glass opacity is usually symmetric, predominant in the perihilar regions and apices, with peripheral sparing (~20% of cases). A mosaic pattern has also been reported in 60% of cases, reflecting more severe disease [19, 62]. *Pneumocystis jirovecii* pneumonia treatment results in radiologic improvement, while ineffective therapy is associated with evolution to the mosaic pattern with architectural distortion and increasing pulmonary infiltrates [62] (Table 1). Because of host immune-mediated lung damage, GGO may be associated with rapid onset of lung consolidation [19]. Nodules and/or septal thickening are other findings [63]. Pulmonary cysts are rare and are attributed to longstanding, low-intensity inflammation, resulting in tissue destruction. Occasionally, pneumothorax or pneumomediastinum occurs [29, 64].

Microbiologic Criteria

Microbiologic diagnosis of PCP is hampered by the inability to cultivate the organism and little utility of serologic approaches using *P. jirovecii*–specific antibody tests. Although antibodies to *P. jirovecii* may be detected in up to 80% of individuals [25, 65], no commercial tests are available, results are variable, and the natural history of antibody persistence poorly understood. Many immunosuppressed patients are unable to produce antibodies.

The diagnosis of PCP hinges upon the visualization and/or detection of *P. jirovecii* in respiratory tract samples by (1) microscopy, (2) antigen detection, and (3) nucleic acid amplification tests (NAATs) (Table 1).

Microscopy

Definitive diagnosis of PCP has traditionally relied on microscopic visualization of *P. jirovecii* in respiratory specimens using optical brighteners, silver stains, and toluidine blue [66, 67]. Bronchoalveolar lavage (BAL) fluid or washings, with/without transbronchial biopsy, induced sputum (IS), and expectorated sputum are most often submitted for examination, but other upper tract specimens (eg, oral rinses, to avoid invasive sampling procedures) also have utility. Immunofluorescent staining for all the above specimen types exhibits superior sensitivity to conventional microscopy [66, 68, 69]. Today, conventional stains may be used (1) in laboratories that do not offer NAAT or immunofluorescent staining and (2) to visualize the cyst/trophic forms in histologic or cytologic specimens.

The use of mouse anti–*P. jirovecii* monoclonal antibodies to detect cysts and trophic forms in an immunofluorescent assay (IFA) format is the preferred method of microscopic diagnosis [70]. Direct antigen-detection formats identify both morphotypes while indirect IFAs detect only cysts. Although it may be an advantage to detect both forms, direct IFAs suffer from more artefact than those that detect cysts only. Rath and colleagues [66] recommend an IFA that detects only cysts as the most useful assay in contemporary routine diagnostics. The main limitations of IFAs are cost and need for a fluorescent microscope. Notably, detection of *Pneumocystis* microscopically in tissue, BAL fluid, or expectorated sputum remains the

criterion for proven PCP. Due to suboptimal sensitivity, negative microscopy does not rule out infection.

Nucleic Acid Amplification Test Approaches

Polymerase chain reaction (PCR) and other NAAT methods are more sensitive than microscopic examination for the detection of *P. jirovecii*; however, their high sensitivity does not allow for easy distinction between PCP and colonization with *P. jirovecii*. Hence, quantification of the fungal load is essential to interpret PCR results. Purely qualitative endpoint PCR tests (single round or nested) are not recommended for PCP diagnosis.

Instead, real-time PCR is now preferred, as this approach provides quantitative results, is rapid, and allows inclusion of a PCR inhibition control. The *Pneumocystis* multicopy mitochondrial large-subunit ribosomal RNA (*mtLSU*) gene is most commonly targeted, but assays targeting the mitochondrial small-subunit ribosomal RNA (*mtSSU*) gene, multi-copy major surface antigen (*MSG*) gene, 18S ribosomal RNA (rRNA), internal transcribed spacer (ITS), 5S rRNA, *DHPS, B*-tubulin, and *HSP70* genes have been developed. Overall, the performance of in-house PCR tests is similar to commercial tests [66].

Attempts have been made to define a quantitative PCR (qPCR) threshold. Based on literature data and including the results of a prospective multicenter laboratory evaluation over 4 years [71], consideration was given to defining 2 types of PCR "thresholds": a "high" threshold that would diagnose PCP with 100% specificity and a "low" threshold that would exclude PCR with a high degree of certainty (eg, where PCR is performed on BAL fluid). Inevitably, there will be patients with results in the gray zone in-between the 2 thresholds. Therefore, these results should be interpreted in the context of the patient's underlying disease, immunosuppressive therapies, and other treatments to inform decisions of whether or not to institute anti-*Pneumocystis* therapy. Indeed, all *Pneumocystis* qPCR assays (in-house or commercial) should be validated in the appropriate clinical context (eg, non–HIV-positive patients) to define the aforementioned thresholds.

Perret and colleagues [72] have suggested a single cutoff of 5×10^3 copies/mL to discriminate PCP from colonization while assessing an in-house qPCR on BAL samples from 71 patients with positive PCR results including 62 patients without HIV. However, test variability observed due to master mix and thermocycler parameters prevented the application of a consensual threshold and test standardization is essential [73]. The *Pneumocystis* Working Party of the Fungal PCR Initiative (www.fpcri.eu) has been working towards such a consensus method. A 16-laboratory international study confirmed the large (10 000-fold) variation between qPCR assays for a given sample. Assays targeting whole nucleic acid and the *mtSSU* gene were the most sensitive and have been put forward as a basis for standardizing *P. jirovecii* loads [74].

When using qPCR, IS and BAL fluid are equally appropriate as samples to diagnose PCP, and potentially the same interpretive cutoff values can be used; however, the number of patients with PCP in whom both IS and BAL fluid have been tested remains small [75]. Sensitivity of qPCR on upper respiratory tract samples is lower than on BAL fluid. Such positive results can be used as a microbiologic criterion to diagnose PCP, but negative results cannot exclude PCP.

Antigen Detection

Because of the invasiveness of BAL sampling and imperfect specificity of PCR, the utility of the fungal biomarker, 1,3-β-D-glucan (BDG), while not specific for *P. jirovecii*, has been studied in PCP diagnosis [66, 76–78].

BDG is concentrated in the cell wall of the cyst (but not the trophic) form of *P. jirovecii* [76] Several BDG commercial assays can be used—these differ in their cutoff value to call a "positive" test and hence affect study comparability. The most commonly studied are the Fungitell (Associates of Cape Cod, Inc, East Falmouth, MA) test and the Wako β-D-glucan Test (FUJIFILM Wako Chemicals, Osaka, Japan).

A systematic review and meta-analysis of the utility of serum BDG testing provided data studying 997 patients with PCP and 3062 controls [79]. Pooled sensitivity and specificity for PCP were 91% (95% confidence interval [CI], 87–94%) and 79% (95% CI, 72–84%), respectively. The sensitivity in patients with HIV was higher than in those without HIV (94% vs 86%; $P = .02$) with similar specificity. The authors concluded that a negative BDG test is only associated with a low post-test probability of PCP (≤5%) when the pre-test probability was low (≤20%) in patients without HIV. The moderate specificity can be explained by the positivity due to other fungal infections (eg, candidiasis) and the false positivity seen in patients with hemodialysis, receipt of immunoglobulins, and certain medications. A positive BDG result should hence trigger tests to exclude other IFDs (Table 1).

The BDG assay may also be able to distinguish *P. jirovecii* colonization from infection [80]. In 166 immunocompromised patients with pulmonary infiltrates, the results of BAL fluid PCR and serum BDG (Wako; FUJIFILM) were compared. BDG levels in patients with definite PCP were significantly higher than those in patients with probable infection, colonization, and patients without PCP (all $P < .001$). BDG levels in patients with definite/probable PCP (173.1 ± 18.8 pg/mL) were also higher than those in colonized patients who had PCR-positive results ($P < .002$). The cutoff level for discrimination was estimated at 33.5 pg/mL (positive-predictive value, 0.925).

The combination of qPCR and serum BDG testing may result in greater diagnostic performance. In 1 study, patients considered to have PCP (by qPCR on BAL fluid) had BDG levels of 100 pg/mL or higher (Fungitell; Associates of Cape Cod) compared with colonized patients (BDG <100 pg/mL), suggesting

that qPCR on BAL fluid plus serum BDG testing can differentiate between PCP and colonization [81]. In patients with unexplained lung infiltrates who underwent evaluation for suspected PCP with bronchoscopy, higher BDG values (>200 pg/mL; Fungitell; Associates of Cape Cod) were associated with clinical PCP among PCR-positive patients [82]. If BAL sampling is not feasible, combined BDG measurement with qPCR on nasopharyngeal aspirates has been an alternative [83]. It remains uncertain whether serum BDG can inform treatment response or prediction of the outcome [84]. There are no supporting data for BDG detection in BAL fluid.

BDG detection for PCP diagnosis has adequate sensitivity. Requiring 2 consecutive positive results improves specificity [85]. More experience is needed with commercial assays other than the Fungitell assay, with assignment of an optimal cutoff value. One study evaluating 116 PCP cases revealed the performance of the Wako β-glucan assay (FUJIFILM; cutoff 11 pg/mL) to be similar to the Fungitell (Associates of Cape Cod) assay with lower inter- and intra-run variability [86].

DISEASE DEFINITIONS

The criteria for the diagnosis of PCP by the 2019 Update of the EORTC/MSGERC Consensus Definitions of Invasive Fungal Disease are summarized in Table 1, and the disease definitions based on this triad of criteria are in Table 2.

The diagnosis of proven PCP is based on clinical and radiologic criteria plus demonstration of *P. jirovecii* by microscopy using conventional or immunofluorescence staining in tissue or respiratory tract specimens. The diagnosis of proven PCP does not require a host factor; however, in the absence of a host factor at the time of diagnosis, investigations for a predisposing host factor should be initiated. Quantitative PCR is not accepted as a microbiologic criterion for proven PCR because of the lack of standardized methodology and clear interpretation rules to distinguish colonization from infection.

Table 2. Diagnostic Criteria for Definition of Proven and Probable *Pneumocystis jirovecii* Pneumonia

	Description
Proven PCP	• Clinical and radiologic criteria, plus: - Demonstration of *P. jirovecii* by microscopy using conventional or immunofluorescence staining in tissue or - Demonstration of *P. jirovecii* by microscopy using conventional or immunofluorescence staining in respiratory specimens
Probable PCP	• Appropriate host factors and clinical and radiologic criteria, plus: - Amplification of *P. jirovecii* DNA by quantitative real-time PCR in respiratory specimen or - Detection of β-D-glucan in serum (alternative method; another IFD and a false-positive result should be ruled out)

Abbreviations: IFD, invasive fungal diseases; PCP, *Pneumocystis jirovecii* pneumonia; PCR, polymerase chain reaction.

Probable PCP is defined by the presence of appropriate host factors and clinical and radiologic criteria, plus detection of *P. jirovecii* by qPCR in respiratory tract specimens (BAL fluid, induced sputum, or oral wash) and/or detection of BDG in serum, provided that another IFD and a false-positive result can be ruled out. Two types of PCR "thresholds" for distinguishing colonization by *P. jirovecii* from disease have been proposed: a "high" threshold that would diagnose PCP with 100% specificity and a "low" threshold that would exclude PCR with a high degree of certainty, at least on BAL fluid; however, thresholds have not been defined by consensus. The inclusion of the serum BDG test is based on high sensitivity and excellent negative-predictive value; uniformly accepted thresholds, however, have not been defined.

Whereas the definitions of proven and probable IFDs are reliable for research purposes, a diagnosis of possible IFD per se is inconclusive due to lack of a microbiologic criterion but may be upgraded during the diagnostic workup if an appropriate microbiologic test result becomes positive. For PCP, possible disease is defined by appropriate host factors and clinical and radiologic criteria but absence of microbiologic confirmation by microscopy and PCR in tissue or respiratory specimens and BDG in serum, respectively (not done or negative result). *Pneumocystis jirovecii* pneumonia is highly unlikely in cases of failure to demonstrate *P. jirovecii* by microscopy in lung or to demonstrate *P. jirovecii* by PCR in BAL material and a negative BDG in serum immediately prior to or within 3–5 days after the start of appropriate treatment [82].

Finally, the diagnosis of extrapulmonary *Pneumocystis* disease requires demonstration of the organism in involved tissue by microscopy and, preferentially, by NAAT.

SUMMARY

These definitions represent consensus expert opinion based on current evidence. They will need regular review for relevance, particularly regarding the role of qPCR in supporting the definition of proven disease. However, this is more a consideration of scientific accuracy than of practical relevance, as probable and proven disease is usually grouped as 1 entity in clinical research.

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SUPPLEMENT ARTICLE

EORTC/MSGERC Definitions of Invasive Fungal Diseases: Summary of Activities of the Intensive Care Unit Working Group

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The EORTC/MSGERC recently revised and updated the consensus definitions of invasive fungal disease (IFD). These definitions primarily focus on patients with cancer and stem cell or solid-organ transplant patients. They may therefore not be suitable for intensive care unit (ICU) patients. More in detail, while the definition of proven IFD applies to a broad range of hosts, the categories of probable and possible IFD were primarily designed for classical immunocompromised hosts and may therefore not be ideal for other populations. Moreover, the scope of the possible category of IFD has been diminished in the recently revised definitions for classically immunocompromised hosts. Diagnosis of IFD in the ICU presents many challenges, which are different for invasive candidiasis and for invasive aspergillosis. The aim of this article is to review progresses made in recent years and difficulties remaining in the development of definitions applicable in the ICU setting.

Keywords. invasive aspergillosis (IA); invasive candidiasis (IC); biomarker; definition; histology.

Diagnosing invasive fungal diseases (IFD) in intensive care units (ICU) presents many challenges, which are different for the 2 most frequent IFD encountered in nonneutropenic critically ill patients: (1) invasive candidiasis (IC) and (2) invasive aspergillosis (IA). Especially for the latter, difficulties arise from the heterogeneity of the population admitted to the ICU, including a large proportion of immunocompetent hosts in whom classical host factors predisposing to IFD (eg, neutropenia, hematological malignancies, or organ transplantation) are not present. This heterogeneity implies variable and frequently unclear risk profiling, in turn affecting several key aspects (eg, difficulty in measuring the true prevalence of the disease and the performance of diagnostic tests) necessary for defining IFD in a standardized fashion from both clinical and research standpoints [1–6]. The objective of the EORTC/MSGERC ICU Working Group was to try to overcome these difficulties and provide definitions for IC and IA that are relevant for ICU patients.

Following the EORTC/MSGERC approach, definitions were developed according to 2 levels of probability of IFD—namely, "proven" and "probable" IFD [7, 8]. This approach establishes a formal framework for defining IFD with a variable certainty of diagnosis. "Proven" IFD requires that a fungus be detected by blood culture or histology/culture of a specimen of tissue taken from a normally sterile clinical site. This category of IFD can apply to any host whether or not immunocompromised. By contrast, "probable" IFD is dependent on the setting/population and hinges on 3 elements—namely, a host factor that identifies the patients at risk, clinical features consistent with the disease entity, and mycological evidence that includes culture and microscopy but also indirect tests, such as antigen detection and molecular tools (polymerase chain reaction [PCR]) [7, 8]. Progress and difficulties encountered by the EORTC/MSGERC ICU Working Group in developing definitions for IC and IA in ICU patients are briefly reviewed in the present work.

INVASIVE CANDIDIASIS

Background

Invasive candidiasis is the most common fungal disease among ICU patients [6, 9–11]. It occurs when *Candida* species, which are frequent colonizers of cutaneous and mucosal surfaces, gain access to deeper, normally sterile sites. Invasive candidiasis comprises candidemia and deep-seated tissue candidiasis [12]. Deep-seated candidiasis arises either from hematogenous dissemination or from procedures that lead to direct inoculation of *Candida* into a sterile site.

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Candidemia is generally viewed as the most common type of IC, and it accounts for the majority of cases included in clinical trials. Candidemia is defined by the isolation of *Candida* species from at least 1 blood culture and is unequivocal. These patients are more easily identified than patients with deep-seated candidiasis, which includes entities such as intra-abdominal candidiasis (IAC), osteomyelitis, septic arthritis, mediastinitis, endophthalmitis, endocarditis, urinary tract infections, and meningitis. Most of these foci arise from an earlier episode of candidemia that is often undiagnosed. Conversely, direct introduction of *Candida* at a sterile site may occur—for example, IAC (abscesses, peritonitis, pancreatitis) following abdominal surgery. Among patients in the ICU with IC, 2 patients will have isolated candidemia for every 3 patients with deep-seated candidiasis (>20–25% of which can lead to secondary candidemia) [13]. In the ICU, IAC constitutes the majority of cases with deep-seated candidiasis [13–15].

Diagnosis

Three entities must be considered: (1) candidemia in the absence of deep-seated candidiasis (including catheter-associated candidemia), (2) candidemia associated with deep-seated candidiasis, and (3) deep-seated candidiasis not associated with candidemia [15].

A proven diagnosis of candidemia (either primary or secondary to deep-seated candidemia) relies on the isolation of *Candida* spp. from blood cultures. Candidemia is the most frequent diagnosis of proven IFD in the ICU. Two pairs of blood culture bottles (10 mL each) should be obtained for aerobic and anaerobic culture when candidemia is suspected before the initiation of antifungal therapy [16]. To potentially increase the yield of blood cultures above 90%, up to 4 blood culture pairs should be obtained in 24 hours [17]. Although with the limitation of potential overestimation due to the possible inclusion of some cases of catheter colonization, up to 40–50% of all episodes of candidemia may be associated with intravenous catheters [18–20]. This is relevant in the ICU since intravenous catheters are typically present in this setting [21]. In patients with central venous lines and suspected candidemia, blood cultures should be obtained via the central line as well as from a peripheral site [22]. A distinction between catheter-associated and non–catheter-associated candidemia might be achieved by comparing the time to positivity or by comparing the number of colony-forming units from the blood drawn via the catheter and the peripheral blood [23, 24]. When cultures of only a catheter tip grow yeasts, while blood cultures remain sterile, systemic antifungals may not be indicated in every case, depending on the clinical condition of the patient and the level of contamination of the catheter tip [25]. Candidemia cases may nonetheless remain undetected because of false-negative blood cultures [15]. In such a case, presumptive diagnosis of candidemia in ICU patients with signs and symptoms of systemic infection is usually made by clinicians by the use of risk-prediction models or non–culture-based diagnostic tests, but is not standardized [26].

The use of risk-prediction models (in this case for diagnosis and not for prediction) may allow early diagnosis, but they have a low positive-predictive value and their use for universal administration of antifungals remains controversial [27, 28]. On the other hand, their very high negative-predictive value allows the diagnosis to be excluded [29]. Of note, some, but not all, models include colonization or colonization of more than 1 nonsterile site by *Candida* spp. among the factors increasing the risk of candidemia (or of IC in general for some of the scores) [30–39]. An alternative (or a completion) to risk-stratification scores is to stratify the risk based on non–culture-based tests, which, in a way similar to risk scores, is still not standardized. They include serological markers (1,3-β-D-glucan, mannan and anti-mannan, and *Candida albicans* germ tube antibody) and molecular methods (including the T2Candida test [T2 Biosystems, Lexington, Massachusetts] , which combines PCR and magnetic resonance–based detection of the agglomeration of supermagnetic particles induced by the amplicons; the T2Candida test is approved by the Food and Drug Administration for the detection of *C. albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* in blood [40, 41]). The main characteristics of non–culture-based tests for the diagnosis of candidemia in ICU patients are summarized in Table 1.

Diagnosis of proven deep-seated candidiasis is much less frequent than that of proven candidemia, since histopathology is rarely available and cultures are often obtained from nonsterile sites. For example, *Candida* spp. recovered in peritoneal fluid drawn from intra-abdominal drains may reflect colonization of drains from the skin rather than true intra-abdominal candidiasis [2]. In contrast, samples drawn under sterile conditions during surgery or radiology-guided drainage of abscesses are indicative of deep-seated *Candida* infections [72, 73]. Although potentially very useful given the frequent absence of proven diagnosis, identification of deep-seated candidiasis by means of non–culture-based tests is not standardized, and extrapolation of evidence regarding their diagnostic performance is sometimes hampered by the fact that they were usually explored in candidemia or IC in general and not for specific forms of deep-seated candidiasis. A brief summary of the characteristics of non–culture-based tests for the diagnosis of deep-seated candidiasis is also shown in Table 1.

Defining Proven and Probable Invasive Candidiasis in the Intensive Care Unit

After several rounds of review and discussion, the proposed definition for proven IC by the ICU Working Group required

Table 1. Characteristics of Non–Culture-Based Tests for the Diagnosis of Candidemia and Deep-Seated Candidiasis in Intensive Care Units

Test	Candidemia	Deep-Seated Candidemia
Serum BDG	• High NPV (frequently 90–95%) [42–45]	• Mostly studied in candidemia and IC in general
	• Low PPV (possibly 20–40%) [46]	• In a prospective study in 89 ICU patients with acute pancreatitis or who underwent abdominal surgery and at risk of IAC, BDG (2 consecutive measurements) showed 65% and 78% sensitivity and specificity, respectively [47]
	• Inconclusive evidence from RCT regarding the overall impact on mortality of candidemia of a BDG-based therapeutic strategy, although an improvement in rates of safe antifungal discontinuation has been described [48, 49]	…
	• Combination with other fungal antigen/antibody-based test of inflammatory markers (eg, serum PCT) has been proposed for improving diagnostic accuracy [50–53] and not for detecting specific types of IC	…
Serum Mn/A-Mn	• Variable diagnostic performance in different studies [54–57]	• Sensitivity of Mn and A-Mn was evaluated separately in 233 ICU patients with severe abdominal conditions; of them, 20 developed IAC and 11 candidemia; sensitivity and specificity of Mn were 43% and 67%, respectively; sensitivity and specificity of A-Mn were 26% and 89%, respectively [50]
	• Sensitivity and specificity of 59% and 65%, respectively, for candidemia reported in a study of 43 ICU patients with candidemia and 67 controls [58]	…
Serum CAGTA	• Limited experience compared with BDG and Mn/A-Mn	• Sensitivity of CAGTA was 5% and 69% for isolated candidemia and blood culture–positive deep-seated candidiasis, respectively, in a study of 50 patients with IC [59]
	• Important heterogeneity in specificity has been reported [60]	…
	• A possible improvement in diagnostic performance when used in combination with BDG has been suggested [52]	…
PCR-based methods	• Heterogeneous performance of in-house and commercial methods [61–65]	• The same considerations expressed for candidemia applied for deep-seated candidemia, with the additional note that most studies refer to candidemia or IC in general and not to specific forms of IC
	• Unable to detect all *Candida* species	…
	• Promising results reported for T2Candida panel, which is FDA-approved for the detection of *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis*, and *C. krusei* in blood; to be further evaluated through further real-life experiences [40, 66–71]	…

Abbreviations: A-Mn, anti-mannan antibodies; BDG, 1,3-β-D-glucan; CAGTA, *Candida albicans* germ tube antigen; FDA, Food and Drug Administration; IAC, intra-abdominal candidiasis; IC, invasive candidiasis; ICU, intensive care unit; Mn, mannan antigen; NPV, negative-predictive value; PCR, polymerase chain reaction; PCT, procalcitonin; PPV, positive-predictive value; RCT, randomized controlled trial.

definitive evidence of the organism in a normally sterile site. It should include at least 1 of the following:

1. Histopathologic, cytopathologic, or direct microscopic examination of material from a normally sterile site, obtained by needle aspiration or biopsy showing budding cells consistent with *Candida* species (presence of pseudo-hyphae and/or true hyphae is highly suggestive of *Candida* species, but these structures are not present in all *Candida* species and may also be seen in *Trichosporon* spp., *Geotrichum* spp., and *Magnusiomyces capitatus* [previously known as *Geotrichum capitatum*], thus confirmation by culture or PCR is necessary).
2. Recovery of *Candida* spp. by culture of a specimen obtained by a sterile procedure (including a freshly placed [<24 hours] drain) from a normally sterile site showing a clinical or radiologic abnormality consistent with an infectious-disease process.
3. Blood culture yielding *Candida* species.

The proposed definition of probable IC in the ICU was based on the presence of at least 1 clinical criterion (compatible ocular findings by fundoscopic examination, hepatosplenic lesions by computed tomography [CT], clinical or radiological [nonpulmonary] abnormalities consistent with an infectious-disease process that are otherwise unexplained) plus at least 1 mycological criterion (positive serum 1,3-β-D-glucan in 2 consecutive samples, recovery of *Candida* in an intra-abdominal specimen obtained surgically or within 24 hours from external drainage), plus at least 1 of the following host factors:

1. Glucocorticoid treatment with prednisone equivalent of 20 mg or more per day
2. Qualitative or quantitative neutrophil abnormality (inherited neutrophil deficiency, absolute neutrophil count ≤500 cells/mm^3)

3. Impaired gut wall integrity (eg, recent abdominal surgery, recent chemotherapy, biliary tree abnormality, recurrent intestinal perforations, ascites, mucositis, severe pancreatitis, parenteral nutrition)
4. Impaired cutaneous barriers to bloodstream infection (eg, presence of central vascular access device, hemodialysis)
5. *Candida* colonization, defined as recovery of *Candida* species in cultures obtained from 2 or more of the following: respiratory tract secretions, stool, skin, wound sites, urine, and drains that have been in place for 24 or more hours
6. Hematopoietic stem cell transplantation (HSCT)
7. Solid-organ transplant (SOT)

INVASIVE ASPERGILLOSIS

Background

Invasive aspergillosis is a severe IFD increasingly reported in patients beyond the traditional risk groups, especially among critically ill patients in the ICU, mostly in the form of invasive pulmonary aspergillosis (IPA) [74–76]. The prevalence of IA in ICU patients varies across hospitals, although important uncertainty surrounds its true value considering the frequent lack of proven diagnosis and the heterogeneity of risk profiles in different types of ICU patients [1, 76]. Risk factors for IA in the ICU population include high-dose corticosteroids, chronic obstructive pulmonary disease, liver disease, malnutrition, burns, and diabetes [74–76]. In addition, rapid development of IPA has been reported in ICU patients admitted with respiratory failure secondary to influenza [77, 78]. Recently, the possibility of a nonnegligible risk of IPA in ICU patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has also been suggested [79–81].

Diagnosis

The diagnosis of IA in ICU remains difficult for a number of reasons [1, 3, 76]. Tissue sampling may be difficult or contraindicated in patients with hemodynamic instability, thrombocytopenia, or coagulation disorders. In addition, the yield of cultures is frequently suboptimal in terms of sensitivity [82, 83]. Further complicating the picture are the following: (1) classic radiographic signs of IA (such as the halo or air crescent sign) are generally absent in nonclassical populations [84]; (2) there could be difficulties in obtaining CT scans instead of bedside chest radiography; (3) discrimination of *Aspergillus* colonization versus infection is problematic [5]; and (4) *Aspergillus* tracheobronchitis, which is rare overall, is rarely considered in the ICU.

Against this background, diagnosis of IA is frequently presumptive, with the performance of non–culture-based tests being of interest for improving accuracy as much as possible. However, a major problem is that proven diagnosis of IA is also infrequent in research studies in the ICU. Consequently, the performance of the different non–culture-based tests has been often evaluated using the IA definition developed for the immunocompromised population with the uncertainty that these results may not be safely extrapolated to traditional ICU patient populations [1]. Nonetheless, some general patterns can be recognized regarding the performance of non–culture-based tests for the diagnosis of IPA in the ICU: (1) the diagnostic performance of bronchoalveolar lavage fluid (BALF) galactomannan is superior to that of serum galactomannan and (2) the use of either BALF or serum 1,3-β-D-glucan presents suboptimal specificity [1]. The performance of other non–culture-based tests such as the BALF *Aspergillus* lateral flow device and BALF/blood *Aspergillus* PCR is promising, but comparative/combined experience with other tests and against reliable reference in ICU patients is still limited [1, 83, 85–90].

Over time, different definitions of IA have been proposed (original or obtained by modifying/adding host factors to the 2002 and 2008 versions of the EORTC/MSGERC definitions) and used in different studies evaluating various aspects of the disease (eg, epidemiology, performance of a diagnostic test) in ICU patients [3, 4, 7, 83, 85–87, 91–93]. Although some of them have certainly helped improving recognition of IA, the large number of these proposed definitions testifies to the need for a standard, shared definition in order to optimize reliability and comparability of research studies with the ultimate aim of improving diagnosis and management in clinical practice.

Defining Proven and Probable Invasive Aspergillosis in the Intensive Care Unit

After several rounds of review and discussion, the proposed definition for proven IA by the ICU Working Group includes definitive evidence of filamentous growth plus associated tissue damage, and should include at least 1 of the following:

1. Histopathologic, cytopathologic, or direct microscopic examination of a specimen obtained by needle aspiration or biopsy in which hyphae compatible with *Aspergillus* spp. are seen accompanied by evidence of associated tissue damage (with necessary confirmation by means of culture or PCR)
2. Recovery of *Aspergillus* spp. by culture of a specimen obtained by a sterile procedure from a normally sterile site and clinically or radiologically abnormal site consistent with an infectious-disease process

The proposed definition of probable IA was limited to probable IPA in the critical care setting and included mycological evidence of *Aspergillus* spp. [at least 1 of the following: (1) cytology, direct microscopy, and/or culture indicating presence of *Aspergillus* spp. in a lower respiratory tract specimen; (2) galactomannan antigen index >0.5 in plasma/serum and/or galactomannan antigen >0.8 in BALF], provided that clinical and host factor criteria were met. Specifically, there should be

at least 1 clinical/radiological abnormality consistent with an otherwise unexplained pulmonary infectious-disease process:

1. Dense, well-circumscribed lesions with or without a halo sign
2. Air crescent sign
3. Cavity
4. Wedge-shaped and segmental or lobar consolidation
5. Tracheobronchial ulceration, pseudomembrane, nodule, plaque, or eschar detected by bronchoscopy (for *Aspergillus* tracheobronchitis)

Plus at least 1 of the following host factors:

1. Glucocorticoid treatment with prednisone equivalent of 20 mg or more per day
2. Qualitative or quantitative neutrophil abnormality (inherited neutrophil deficiency, absolute neutrophil count of ≤500 cells/mm^3)
3. Chronic respiratory airway abnormality (chronic obstructive lung disease, bronchiectasis)
4. Decompensated cirrhosis
5. Treatment with recognized immunosuppressants (eg, calcineurin or mammalian target of rapamycin [mTOR] inhibitors, blockers of tumor necrosis factor [TNF] and similar antifungal immunity pathways, alemtuzumab, ibrutinib, nucleoside analogues) during the past 90 days
6. Hematological malignancies/HSCT
7. SOT
8. Human immunodeficiency virus infection
9. Severe influenza (or other severe viral pneumonia, such as coronavirus disease 2019 [COVID-19])

CONCLUSIONS

With the exception of proven IFD, the ICU Working Group did not reach a high level of certainty with regard to IFD definitions in ICU patients and the proposed definitions were thus not included in the latest version of the EORTC/MSGERC consensus [8]. Several factors hindered reaching a firm definition of probable disease, including the heterogeneity of predisposing factors, but also uncertainty about the true prevalence of IFD in the ICU especially for IA and the unreliability of other definitions as the reference standard for evaluating tests and radiology performance for diagnosing IA in ICU playing an important role. A different approach may be necessary to explore whether or not to define "probable IA" in ICU and, if so, how best to achieve this. For example, the weight assigned to different host factors could vary to reflect the impact on the pre-test and post-test probability of the different clinical and mycologic criteria. From this standpoint, a dedicated updated systematic revision of the diagnostic performance of existing definitions and tests for the diagnosis of IC and IA in nonneutropenic critically ill patients was deemed necessary as baseline information on which to base future discussions and the ultimate development of definitions. For this reason, another initiative (FUNDICU project) has been undertaken and is currently completing the first steps (the first systematic review, focused on the diagnosis of IA in critically ill patients, has been recently published) [1, 94]. Certainly, the systematic literature assessment is only the basis for informing expert discussions; we also need to consider and accurately weigh potential solutions from already used/developed definitions (either in specific categories of ICU patients or in non-ICU patients) [3, 14, 47, 78, 80]. Eventually, we hope this long process, involving the combination of the proactive discussions held during the EORTC/MSGERC ICU Working Group meetings and the subsequent ongoing work of the FUNDICU initiative, may ultimately result in providing a standardized and optimized approach to research and management of IFD in nonneutropenic, critically ill patients in the ICU.

Notes

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