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Epidemiology, the Impact of COVID-19, and the Mycological Approach in Invasive Mold Infection

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Invasive fungal infections are a significant health challenge worldwide. Although invasive yeast infections are more prevalent, invasive mold infections (IMIs), such as *Aspergillus* spp., *Mucorales*, and *Fusarium* spp., are rising. The increase in IMIs is attributed to various factors, such as transplantation, aggressive treatments, invasive medical devices, and the emergence of new at-risk populations, such as patients with influenza and SARS-CoV-2-associated aspergillosis. The European Organization for Research and Treatment of Cancer and the Mycosis Study Group initially provided definitions based on host and clinical factors, including radiological findings and mycological evidence. The recent 2020 updates of the definitions state that a case confirmed by microscopy, histopathology, and culture from a sterile site is "proven"; however, regarding mycological criteria, this guideline introduced revised thresholds for the galactomannan assay based on sample types, excluded the 1,3-β-D-glucan detection assay, and acknowledged positive *Aspergillus* polymerase chain reaction tests from serum, plasma, or bronchoalveolar lavage fluid. The current diagnostic approach improves the accuracy and timeliness of diagnosing fungal infections and facilitates more effective patient management.

Key Words: *Aspergillus* PCR, Conventional methods, COVID-19, Galactomannan antigen, Invasive mold infections

INTRODUCTION

Invasive fungal infections (IFIs) are systemic infections resulting from the establishment of yeasts or molds in deep-seated tissues, blood, and sterile sites. Nearly 70% of these infections worldwide are attributed to invasive candidiasis, followed by cryptococcosis (20%) and aspergillosis (10%)[1]. Endemic fungal strains, such as *Blastomyces*, *Histoplasma*, *ParaCoccidioides*, and *Coccidioides*, also contribute to severe systemic infections. IFIs affect approximately 1.9 million individuals annually, and 3 million people globally suffer from chronic severe fungal infections[1,2].

Although invasive mold infections (IMI) are relatively less common than invasive yeast infections, the incidence of mold-related diseases and hospitalizations is continuously increasing[1,3]. The disease burden of mold infections is increasing in Korea[4,5]. IMI can be caused by *Aspergillus* spp. and other septated hyaline molds such as *Penicillium*, *Paecilomyces*,

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Acremonium, *Scopulariopsis*, *Fusarium*, and *Scedosporium*, *Mucorales*, and dematiaceous fungi[3,4]. Mold infections are seldom controlled at the initial stage but can escalate in severity due to delayed diagnosis, complicating treatment and leading to higher mortality[1,3]. This increase in mold-related diseases and hospitalizations highlights the urgent need for timely and accurate fungal species detection and identification.

This article reviews the epidemiology and impact of COVID-19 on these infections and current nonculture-based diagnostic methods, such as serological and molecular methods, with the updated consensus definitions for Invasive Fungal Diseases (IFD) by the Cancer/Mycoses Special Interest Group (EORTC/MSG)[6].

EPIDEMIOLOGY AND THE IMPACT OF COVID-19

Invasive mold infection is exacerbated by aggressive medical treatments such as chemotherapy and transplants, invasive devices, such as central venous catheters, and the emergence of new at-risk populations, including individuals with influenza and SARS-CoV-2-associated Aspergillosis[7-9]. Invasive aspergillosis (IA) and invasive mucormycosis (IM) are the most prevalent IMIs. The mortality rate for candidemia exceeds 25%, while IA results in death in 40% to 90% of immunocompromised patients. The rate approaches 85% for untreated IA and nearly 100% for untreated IM, depending on the patient's underlying conditions[2,9].

Aspergillus fumigatus is the leading cause of culture-positive IA, followed by *A. flavus*, *A. niger*, and *A. terreus*. Infections from nonfumigatus *Aspergillus* species are more likely to become disseminated[1]. Mucormycosis is primarily caused by fungi from the order *Mucorales*, which frequently results in respiratory infections that can extend to the sinuses, eyes, or central nervous system. IM is commonly caused by *Rhizopus* spp., while other species, including *Mucor* spp., *Lichtheimia* spp., *Apophysomyces* spp., *Cunninghamella* spp., and *Saksenaea* spp., also cause IMI[2,4].

Other hyaline molds, *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp., and *Acremonium* spp., are also implicated in IMIs. *Fusarium* spp. typically disseminates in patients with a hematologic malignancy and hematopoietic stem cell transplant (HSCT) recipients but appears localized in solid organ transplant (SOT) recipients, leading to better outcomes than in neutropenic patients[10]. *Scedosporium* spp. can cause conditions ranging from mycetoma to lethal disseminated infections, especially in SOT recipients from nearly drowned donors[11]. Dermatiaceous fungi such as *Alternaria* spp., *Exophiala* spp., and *Curvularia* spp. can cause diseases ranging from skin lesions to disseminated infection in high-risk patients[10]. SOT recipients frequently exhibit skin conditions, while HSCT recipients are prone to lung complications, which are particularly problematic in these patients[9,11]. Indeed, *Aspergillus* spp., *Fusarium* spp., and *Mucorales* are known to cause healthcare-associated infections in chronic disease patients[12].

COVID-19 has impacted the incidence of fungal infections. COVID-19-associated pulmonary aspergillosis (CAPA) was first identified in China in early 2020, affecting about 10% of invasively ventilated COVID-19 patients and about 20.1% of those with acute respiratory failure requiring ventilation. *A. fumigatus* is the predominant pathogen causing CAPA linked to azole-resistant *Aspergillus* strains[8]. The patients most commonly affected are those with acute respiratory failure due to COVID-19, particularly patients treated with systemic corticosteroids or tocilizumab[13]. Unlike influenza-associated pulmonary aspergillosis, CAPA is usually diagnosed about 8 days after ICU admission and has a mortality that exceeds 40%. During India's second COVID-19 wave in early 2021, over 47,500 COVID-19-associated mucormycosis (CAM) cases were reported. The prevalence of CAM among hospitalized COVID-19 patients was 0.27% in India and about 1~2% in Europe[8,14]. CAM commonly leads to rhino-orbital-cerebral and pulmonary diseases in COVID-19 patients with high mortality and extensive necrosis, often necessitating disfiguring surgical debridement. Major risk factors include uncontrolled diabetes and systemic corticosteroid overuse[13].

DEFINITION OF INVASIVE MOLD INFECTION

Several criteria, such as those from EORTC/MSG, Bulpa, Blot, and the modified Aspergillosis in ICU (AspICU), have been recommended for the timely diagnosis and management of IFIs in various clinical settings[6,7,15]. Definitions by the EORTC/MSG for immunocompromised individuals were first proposed in 2002 and later updated in 2008 and 2020, incorporating host factors, clinical criteria, and mycological criteria[6,15,16]. However, the AspICU criteria proposed for ICU patients do not include microbial biomarkers such as the galactomannan (GM) antigen and *Aspergillus* polymerase chain reaction (PCR)[7].

According to the EORTC/MSG definition, a case confirmed by microscopy, histopathology, and culture from a sterile site is "proven". The diagnosis is "probable" if three criteria are met: the presence of at least 1 host factor, a clinical feature, and mycologic evidence. Conversely, a case that meets the

Table 1. Mycological approaches based on the updated 2020 European Organization for Research and Treatment of Cancer-Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycosis Study Group (EORTC/MSG) guideline for defining proven, and probable invasive mold disease

Criteria	Fungus/ disease	Mycologic feature		
		Conventional method	Serology*	PCR based assay
Proven invasive mold disease	Mold	Histopathologic, cytopathologic, or direct microscopy by needle aspiration or biopsy Recovery by culture of a sterile specimen (excluding BAL fluid, a paranasal or mastoid sinus cavity specimen, and urine)	Not applicable	Amplification of fungal DNA by PCR combined with DNA sequencing when molds are seen in formalin-fixed paraffin-embedded tissue
Probable invasive pulmonary mold disease	Mold	*Aspergillus*, *Fusarium*, *Scedosporium* species, or *Mucorales* recovered by culture from sputum, BAL, bronchial brush, or aspirate Microscopical detection of fungal elements in sputum, BAL, bronchial brush, or aspirate indicating a mold		
	Tracheobronchitis	*Aspergillus* recovered by culture of BAL or bronchial brush Microscopic detection of fungal elements in BAL or bronchial brush		
	Sinonasal diseases	Mold recovered by culture of sinus aspirate samples Microscopic detection of fungal elements in sinus aspirate samples		
	Aspergillus spp., only	Recovered by culture from sputum, BAL, bronchial brush, or aspirate	Galactomannan Ag: Any 1 of the following Single serum or plasma: ≥1.0, BAL ≥1.0 Single CSF: ≥1.0 Single serum or plasma: ≥0.7 and BAL ≥0.8	*Aspergillus* PCR : Any 1 of the following PCR positive as ≥2 consecutive plasma, serum, or whole blood ≥2 duplicated BAL fluid PCR positive ≥1 PCR positive in plasma, serum, or whole blood and 1 PCR test positive in BAL fluid

*1,3-β-D glucan was not considered to provide mycological evidence of any invasive mold disease
Abbreviations: BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; PCR, polymerase chain reaction

appropriate host factors and clinical evidence but lacks mycological support is considered "possible"[16]. Due to increasing data on *Aspergillus* GM thresholds and the new PCR-based diagnostic tests, the 2020 update to the EORTC/MSG criteria includes modifications in mycological criteria and additional host factors, such as SOT and hematologic malignancies. This document underscores the importance of laboratory diagnosis in identifying IFD, highlighting the roles of GM testing and PCR assays for *Aspergillus*[6] (Table 1). Adopting the 2020 EORTC/MSG criteria led to a significant 27.3% reduction in the diagnosis of probable IPA, contributing to a more uniform patient classification of treatment response, prognosis, and mortality. This consistency enhances the reliability of mortality predictions compared to the 2008 criteria[17].

MYCOLOGICAL APPROACH

1. The conventional method: microscopy, histopathology, and culture-based tests

Histopathologic demonstration of tissue invasion by hyphae or mold recovery from a culture of a specimen obtained from a clinically sterile site is a "proven" IMD[16]. This category can apply to any patient, regardless of their immunocompromised status. However, these conventional methods have inherent limitations, including a prolonged turnaround time, the invasive nature of the samples, and lower sensitivity. The proportion of proven cases was relatively low, from 1.8% to 27.0%[18]. Blood culture sensitivity for aspergillosis was markedly low at 1~5%, in contrast to 50~95% for candidemia[1,19]. Recovery of *Aspergillus* spp. from blood cultures rarely indicates endovascular disease and is almost always contamination[6]. A nationwide study in Korea found that only 8% of IPA cases were proven with these methods[20].

2. Galactomannan (GM) antigen assay

GM is a polysaccharide cell wall component released by *Aspergillus* spp. during growth. It can be detected in serum, bronchoalveolar lavage (BAL) fluid, cerebrospinal fluid (CSF), and others[15,21]. The 2002 EORTC/MSG definition included *Aspergillus* antigen detection, such as GM, as a criterion for probable IA[16]. However, the Platelia *Aspergillus* EIA (Bio-Rad, Marnes-la-Coquette, France), an immune-enzymatic sandwich microplate assay using a rat antigalactofuranose monoclonal antibody (EB-A2) was first approved in 2003 and remains the only FDA-approved GM assay, despite the availability of multiple commercial assays. Initially, the cut-off for GM was between 1.0 and 1.5 to minimize false positives, but in 2008, this threshold was lowered to 0.5 to improve performance in adult hematology patients[21,22]. The 2020 EORTC/MSG definition raised the cut-off back to 1.0 for individual plasma, serum, BAL fluid, or CSF samples, setting specific criteria for simultaneous positive results in serum and BAL fluid: ≥0.7 for serum and ≥0.8 for BAL[6].

The GM assay has high sensitivity and moderate accuracy in diagnosing IA in immunocompromised patients. In patients with hematologic malignancies, the GM assay has higher sensitivity and specificity in BAL fluid than serum[15,21]. Sensitivity is low in SOT recipients due to less frequent angioinvasion. False-positive results are usually reported in pediatric patients without clinical signs of IA. Galactofuranose in various fungi and certain substances has led to reports of false-positive results[21]. These false-positive results can arise from colonization by fungi, such as the genera *Fusarium*, *Histoplasma*, *Penicillium*, *Alternaria*, *Paecilomyces*, or *Geotrichum*, the administration of piperacillin-tazobactam or β-lactams, patients receiving total parenteral nutrition, such as Plasma-Lyte (Baxter, Deerfield, IL, USA), NP2 Enfant AP-HP parenteral nutrition (Fresenius, Kabi, Sevres, France), and intravenous gluconate solution[23,24].

Additionally, the cross-reactivity of BAL fluid samples with *Mycoplasma pneumoniae* or anesthetic drugs has been reported[24,25]. Patients with mucositis or compromised intestinal barriers have false-positive GM results due to dietary factors. When using BAL fluid for testing, lung transplant recipients have a significantly higher rate of false-positive results due to extensive airway colonization[25,26]. These inaccuracies lead to unnecessary diagnostic procedures, treatments, and radiation exposure from CT scans aimed at detecting IPA, adverse reactions from antifungal medications, and increased healthcare costs due to unnecessary tests and treatments. False negatives or reduced detection has also been reported in patients with chronic granulomatous disease or hyperimmunoglobulin E (hyper-IgE) syndrome (formerly Job syndrome) despite their increased risk of IA[27].

3. (1,3)-β-D-glucan (BDG) assay

(1,3)-β-D-glucan (BDG), a polysaccharide component of fungal cell walls, is a pan-fungal antigen detected in various species, including *Candida* spp., *Pneumocystis jivoveci*, *Aspergillus* spp., *Acremonium* spp., and *Fusarium* spp. with exceptions such as *Cryptococcus* spp., *Mucorales*, and the yeast phase of *Blastomyces dematitidis*[22]. The commercial BDG assay, the Fungitell Assay (Associates of Cape Cod Inc., East Falmouth, MA, USA) has received FDA approval and

numerous other BDG assays are available, each with various cut-off values: Fungitell assay, cut-off value 60~80 pg/mL; the Wako β-glucan test, cut-off value 11 pg/mL (Wako Pure Chemical, Osaka, Japan); and the Fungitec-G, cut-off value 20 pg/mL (Seikagaku, Tokyo, Japan)[28,29].

The revised 2020 EORTC/MSG criteria exclude BDG detection as a microbiologic criterion for IFD since BDG detection is not specific to a single IMD[6]. False positives can occur in concurrent bacteremia such as *Pseudomonas aeruginosa* and *Streptococcus* species, certain β-lactam antibiotics, specific antitumor chemotherapy agents, various fractionated blood components, and hemodialysis membranes. However, BDG detection is considered suitable for diagnosing probable IFD in specific clinical situations, such as patients with hematologic malignancies, post-hematopoietic stem cell transplantation neutropenia, and selection of high-risk ICU patients with complications from gastrointestinal surgery suspected of having an infection[29,30]. Additionally, due to its relatively high NPV, BDG testing is considered useful for excluding IFD in patients[28].

4. Polymerase chain reaction (PCR) assay

The PCR assay is recommended for *Aspergillus* spp. and *Pneumocystis jirovecii* infections, and serological testing will likely enhance the diagnosis. Molecular diagnostics represent the only nonclassical mycological approach for IM[4,31]. The International Fungal PCR Initiative (FPCRI) standardized DNA extraction protocols from serum, plasma, and whole blood to improve diagnostic accuracy[32]. BAL fluid is preferred for detecting respiratory pathogens, while debrided tissue is favored for skin and soft tissue infections. In formalin-fixed paraffin-embedded (FFPE) tissue, amplification of fungal DNA by PCR combined with DNA sequencing can be used when molds are seen. However, caution is needed with FFPE tissue due to a low DNA yield and potential amplification interference from colonizers[31].

The 2020 updates to the EORTC/MSG definitions now include *Aspergillus*-specific plasma, serum, whole blood, and BAL fluid PCR assays as a mycological criterion for probable IA[6]. The European *Aspergillus* PCR Initiative (EAPCRI) Working Group of the International Society of Human and Animal Mycology has validated *Aspergillus* PCR assays in plasma, serum, and whole blood, except BAL fluid[19,32]. *Aspergillus* PCR assays have moderate accuracy for diagnosing IPA in immunocompromised patients. They require two positive results for confirmation to achieve higher specificity. In high-risk hematology patients, these assays in blood show a high NPV of 94% and a PPV of 70%, reducing the need for empirical antifungal therapy[33]. *Aspergillus* PCR assays in patients not previously treated with antifungals show a sensitivity of 71.4% and a specificity of 92.3%, with a PPV of 62.5% and an NPV of 98.3%; however, in patients with *Aspergillus*-active prophylaxis, the PPV drops to 5.4% while the NPV increases to 100%, indicating that PCR screening is more effective in patients not on primary antimold prophylaxis[34].

PCR assays for *Aspergillus* DNA in specimens such as BAL fluid, CSF, and tissue are useful for diagnosing IA in SOT recipients or patients under long-term immunosuppressive therapy[6]. However, BAL fluid assays may not differentiate between colonization and active infection, with a PPV of about 72% in nonhematology patients[14,35]. While *Candida* PCR assays lack standardization, *Aspergillus* PCR assays are well-standardized, aiding in result comparison and detecting azole resistance mutations. Still, the absence of an FDA-endorsed *Aspergillus* PCR assay limits its global use[31]. Despite the lack of significant differences in mortality between the redefined possible and probable IA groups, elevated DNA levels (greater than 150 copies/mL) in quantitative PCR have been associated with higher 90-day mortality rates[6,33,36]. The false-positive results in *Aspergillus* PCR assays are often due to environmental contamination, cross-reactions with other fungi, such as *Penicillium* spp., or respiratory tract colonization[37].

The absence of serological tests to confirm the diagnosis of IM highlights the need for a molecular assay. The increasing use of *Mucorales*-specific PCR assays enhances accuracy in detecting DNA in blood and BAL fluid, using dual loci (ITS and TEF1a) for DNA barcoding[31,38]. *Mucorales* DNA has been detected in blood 3 to 68 days before conventional methods, eight days earlier than histology or culture, and three days before imaging[31]. Quantitative PCR in serum samples has been proven to be of high prognostic value. Patients with decreasing fungal loads have better survival rates. However, more comprehensive studies are needed to clarify its sensitivity and role in patients treated with antifungals and those with rhino-orbital-cerebral mucormycosis[31,39].

CONCLUSION

IMIs are exacerbated by medical interventions, such as chemotherapy and transplants, invasive devices, and the emergence of new at-risk populations, including those with influenza and SARS-CoV-2-associated aspergillosis. Diagnostic mycological approaches have evolved beyond traditional microscopy and culture to include serology-based and molecular approaches, offering rapid, sensitive, and specific

detection. This fact is particularly vital for timely and accurate diagnosis, allowing the initiation of appropriate treatment. The 2020 EORTC/MSG criteria have integrated these newer diagnostic tools, reflecting advancements in nonculture-based methods that improve diagnostic accuracy and patient management in fungal infections. These molecular diagnostics are pivotal in differentiating invasive infections from colonization, especially in immunocompromised patients, and have significantly refined the approach to invasive mold infection detection and management.

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CONFLICT OF INTEREST

In relation to this article, we declare that there is no conflict of interest.

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