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Review Article

Invasive fungal infection — laboratory diagnosis and antifungal treatment

Pei-Lan Shao[1,2], Li-Min Huang[2], Po-Ren Hsueh[1,3]

Departments of [1]Laboratory Medicine, [2]Pediatrics and [3]Internal Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan

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Invasive fungal infections (IFIs) have become increasingly prevalent in the recent decade along with the increasing populations of immunocompromised patients and widespread use of the broad-spectrum antibiotics. The morbidity and the mortality of IFIs remain high while the diagnosis and treatment of IFIs are highly challenging. Recent advances in diagnostic methods and antifungal agents provide the potential to improve the outcomes of these infections. Conventional diagnostic methods including microbiological cultures and histopathological diagnosis have the disadvantages of either insensitivity or requiring invasive procedures. The innovative techniques of detecting circulating fungal antigens and detecting fungal genomic DNA represent improvements in the diagnosis of invasive aspergillosis. Several antifungal agents have been developed in recent years, such as lipid formulations of amphotericin B, newer azoles, and echinocandins. These agents have either lower toxicities or greater activities against certain fungi compared with older treatments. With the availability of diverse antifungal agents, their use in combination has the potential to produce additive or synergistic effects, leading to better treatment outcomes. Large-scale randomized clinical trials are needed to confirm the efficacy of combination strategies.

Key words: Antifungal agents, diagnosis, laboratory techniques and procedures, mycoses, review

Introduction

Infection is a constant threat to human health. Remarkable progress has been made in the prevention and treatment of infectious diseases. At the same time, patterns of human infections have undergone significant changes during the past several decades. Bacterial infection was the predominant issue before the availability of antibiotics. The use of penicillins and cephalosporins led to an increase in Gram-negative bacterial infections in the 1960s and 1970s [1]. Thereafter, fungal infection started to emerge as a major clinical issue [2-4]. Two main factors contributed to the steady increase of fungal infection, namely, the widespread use of antibacterial agents and rapid increase in numbers of immunocompromised populations [3,4].

Clinically important fungi consist of yeasts, molds, and dimorphic species. Each poses a different challenge to clinicians. Overall, diagnosis of fungal infection is more difficult compared with bacterial infections by conventional culture, and treatment is also difficult because of the limited range of antifungal agents.

Modern therapeutic modalities such as cancer chemotherapy and organ transplantation have a greatly increased risk of invasive fungal infections (IFIs) [2,3]. Currently, morbidity and mortality from IFIs remain high in patients with hematologic malignancies who receive intensive myelosuppressive chemotherapy or undergo bone marrow transplantation [2,5-11]. Thus, there is considerable scope for improvement both in the diagnosis and management of fungal infection.

Advances in Laboratory Diagnosis

Early diagnosis of IFIs remains a great challenge. The symptoms and signs are often nonspecific and microbiological cultures are usually negative. Histopathological diagnosis, which requires invasive procedures to obtain the specimens, is often hindered by the grave conditions in these patients. The high

Corresponding author: Dr. Po-Ren Hsueh, Departments of Laboratory Medicine and Internal Medicine, National Taiwan University Hospital, No 7, Chung-Shan South Road, Taipei, Taiwan. E-mail: hsporen@ha.mc.ntu.edu.tw

mortality of IFI has contributed to the difficulties in establishing timely diagnosis and initiating prompt antifungal therapy. Recently, more rapid diagnosis has been achieved by use of detection of the circulating markers, including fungal cell wall components and fungal genomic DNA. These techniques have advanced the diagnosis of aspergillosis. Non-culture-based diagnosis for candidiasis and noninvasive diagnosis for molds other than *Aspergillus* are still mainly investigational [12].

Detection of Fungal Antigens

Galactomannan detection

Galactomannan (GM) is a polysaccharide cell wall component that is released by the fungus into the serum during its growth in tissues [13]. A sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of GM antigen of *Aspergillus* was commercialized and has been applied in the diagnosis of invasive aspergillosis (IA). The ELISA test (Platelia *Aspergillus;* Biorad, Marnes-La-Coquette, France) uses the EB-A2 monoclonal antibody to recognize the galactofuran epitopes of the GM molecules [14]. The number of epitopes on the GM antigen released by the fungi may vary between strains, between species, and over time. Angioinvasion is assumed to be required for the GM antigens released from fungal hyphae to reach the circulation [14]. The degree of angioinvasion varies in relation to the underlying conditions and toxic damage caused by cytotoxic drugs or irradiation. In certain underlying diseases, such as chronic granulomatous disease, in which the formation of abscess predominates and may hamper the leakage of GM antigens into the circulation, IA may occur with the absence of the antigenemia [14]. In contrast, antigen detection has excellent sensitivity and specificity (up to 89.7% and 98.4%, respectively) in stem cell transplant recipients and in patients with prolonged neutropenia [15]. This result might indicate rapid progression of disease caused by the angioinvasive growth of *Aspergillus* in damaged lung epithelia [14].

Once the GM reaches the circulation, it might bind to substances present in the blood, including *Aspergillus* antibodies and other human proteins. Such binding could interfere with the performance of the ELISA test and cause false-negative results [14]. Clearance of the GM from the blood due to renal excretion and uptake by macrophage was demonstrated in an animal model [14]. Renal clearance depends on the renal function of the patient and the size of the GM antigen.

The performance of the GM ELISA test was reported to range from 50% to 100% in sensitivity and from 92% to 100% in specificity (Table 1) [13-20]. In these reports, sensitivity varied considerably, while specificity remained more consistent and was usually greater than 85%. When the test is applied to clinical diagnosis, double-checking of positive samples might be necessary because of the possible lack of reproducibility [17]. As mentioned by the manufacturer, IA

Table 1. Summary of studies on evaluation of the Platelia *Aspergillus* galactomannan (GM) enzyme-linked immunosorbent assay for diagnosis of invasive aspergillosis (IA)

No. of patients (episodes)	Underlying conditions	IA patients	GM cut-off index value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
135 (193)[a]	HeM with neutropenia SCT	17[b]	1.0	100	92	64	100	13
191 (362)	HeM with neutropenia SCT	39[c]	1.0	89.7	98.1	87.5	98.4	15
74	SCT	8[b]	1.5	75	100	100	97	16
807[a]	HeM or admitted to an intensive care units	34[c]	1.0	50	99.6	-	-	17
67	SCT	24[c]	1.0	54.2	100	-	-	18
797[a]	HeM, SCT	53[c]	1.5	90.6	94	-	-	19
71[a]	HeM with neutropenia and/or steroid treatment CGD, SCT	27[d]	1.0	92.6	95.4	93	95	20

Abbreviations: PPV = positive predictive value; NPV = negative predictive value; HeM = hematological malignancies; SCT = stem cell transplant; CGD = chronic granulomatous disease

[a]Includes pediatric patients.

[b]Includes proven, probable, and possible IA.

[c]Includes proven and probable IA.

[d]Includes proven IA.

should be considered when 2 consecutive positive samples from a patient have been obtained. In patients with only single positive samples, the sensitivity is low, perhaps around 40% or less [14]. The cut-off index value for a positive result suggested by the manufacturer was above 1.5. Some studies suggested that a cut-off value of 1.5 was too high and that lowering the cut-off to 1.0 could improve sensitivity without compromising specificity [13,20]. However, it also had been reported elsewhere that lowering the cut-off value to 1.0 did not improve sensitivity [19]. In patients with IA, the ELISA test usually gives positive results before the clinical symptoms and signs become detectable [18,19]. While serving as an early diagnostic tool, some study suggested decreasing the cut-off value further to 0.5 in order to increase the duration of test positivity before diagnosis could be established by clinical means [18]. Moreover, serial determination of serum GM index values is useful in order to evaluate the prognosis of IA. A 1.0 increase in GM level above baseline was a marker of disease progression and predictive of treatment failure in allogeneic stem cell transplant recipients [21]. Nevertheless, it is important to recognize the causes of false-positives and false-negatives when the ELISA test is utilized in the clinical setting.

The false-positive rate ranged from 5% [13] to 14% [15]. The occurrence of false positivity frequently coincided with mucositis, cytotoxic chemotherapy, and/or graft-versus-host disease [13,15]. GM was detected in various kinds of foods [14,17,19]. It is postulated that translocation of dietary GM via damaged or immature intestinal mucosa could result in false-positive results [15,17]. The false-positive rate was reported to be high in up to 83% of newborn babies [14]. Besides GM of food origin, lipoteichoic acid of *Bifidobacterium* spp., which heavily colonize the neonatal gut, might cause ELISA reactivity in infants after translocation through immature intestinal mucosa [22]. Cross-reactivity to other fungi or bacteria was not reported, with the exceptions of *Penicillium chrysogenum, Penicillium digitatum,* and *Paecilomyces variotii,* fungi that rarely cause human infections [23]. Some drugs of fungal origin, such as antibiotics, could also cause persistent or transient antigenemia [17]. False-positive results had been described in some patients receiving piperacillin-tazobactam [24,25] and in a patient receiving amoxicillin-clavulanic acid from a case report [26]. However, the nature of false-positives remains undetermined in many cases and the causes may be multifactorial.

False-negatives may result from low-level release of the GM of the growing fungi, the use of prophylactic antifungal agents, and limited angioinvasion. Exposure to antifungal agents such as amphotericin B (AmB) might reduce the mycelial growth and/or alter the hyphal release of GM [18], causing the false-negative results. Because of the risk of false-negative results, GM antigen detection does not replace other diagnostic tools, such as computed tomography imaging, in the exploration of IFIs in high-risk patients [16].

The utility of the GM antigen ELISA in specimens other than serum has been evaluated [27]. Detection of GM antigen in bronchoalveolar lavage (BAL) fluid had good sensitivity and was more sensitive than antigen detection in serum. Increased GM index in cerebrospinal fluid indicated IA of the central nervous system. The utility of the ELISA in urine remains controversial and needs further validation.

Noninvasive testing of GM ELISA has advanced the diagnosis of IA. In practice, routine follow-up of patients at high risk for aspergillosis should include serum GM determination twice per week during neutropenia and when patients have additional risk factors, such as graft-versus-host-disease, and/or prolonged corticosteroid therapy. GM detection remains one of the major criteria to establish the IA diagnosis even when mycological detection is negative, according to the consensus group of the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group [28]. Because of the overall good performance of the ELISA test, a positive result should trigger further evaluation for disease by radiography, and lead to prompt treatment if indicated, while a negative result should lead to the aggressive search for other etiologies.

Glucan detection

(1-3)-Beta (β)-D-glucan ([1-3]-BDG) is a component of the cell wall of a variety of fungi [29] and can be utilized as a nonspecific marker for IFIs. It can be detected by its ability to activate factor G of the horseshoe crab coagulation cascade [29]. Commercially available BDG assays (e.g., Fungitec G; Seikagaku, Tokyo, Japan) allow determination of serum BDG by colorimetric or kinetic assay [30]. The BDG assay had good performance in patients infected with *Candida, Aspergillus,* and *Fusarium* spp., but failed to detect patients infected with zygomycetes and *Cryptococcus,* which contain little or no BDG [31]. Sensitivity and

specificity were 69.9% and 87.1%, respectively, for diagnosis of IFIs in leukaemic patients in one study [31].

Detection of fungal DNA

The amplification of genomic sequences that are unique to fungi in clinical specimens theoretically allows a rapid and sensitive diagnostic method. Several polymerase chain reaction (PCR) protocols have been developed for the diagnosis of IA [32-34]. With the quantification PCR method (LightCycler), the fungal burden could be estimated and as little as 10-100 fg of genomic DNA, which correlates <10 to 100 conidia equivalents per mL, could be detected [30,33]. These assays have been evaluated in clinical specimens such as blood, serum and BAL [32,34,35]. The reported sensitivity of PCR assays varied between 50-70% [34].

The various noninvasive diagnostic methods for IA have different advantages and limitations in practice (Table 2) [32-37]. Kawazu et al prospectively evaluated the diagnostic potential for IA of GM ELISA, real-time PCR, and an assay for BDG in a weekly screening strategy [36]. The sandwich GM ELISA was reported to be the most sensitive test for the diagnosis of IA in high-risk patients with hematological disorders, using the cut-off index of 0.6. Combination use of these assays might further increase the diagnostic performance. Pazos et al reported that combination use of GM and BDG detection could identify false-positive reactions for each test and improve the specificity to 100% and the positive predictive value to 100% without affecting the sensitivity and negative predictive performance [37].

Advances in Antifungal Treatment

Agents targeting fungal cell membrane

Polyenes

AmB deoxycholate has long been the standard for the treatment of IFIs [38]. The major limits of its usage are the adverse effects such as fever, chills, nausea and vomiting, electrolyte abnormalities, and most importantly, nephrotoxicity [39]. Continuous infusion of AmB deoxycholate has been shown to reduce the incidence of side effects, compared with the traditional administration of the same amount over 2-6 h [40]. Continuous infusion also facilitates dose escalation, thus enabling the dose-dependent efficacy of AmB to be exploited. Currently, 3 lipid-based formulations of AmB are licensed: AmB lipid complex (Abelcet), AmB colloidal dispersion (Amphotec), and liposomal AmB (AmBisome). They were considered to be as effective as conventional AmB but less toxic [41-43]. In severe or refractory infections, lipid formulations of AmB have potential advantages over conventional AmB because they can be delivered at higher doses [44].

Routes of administration other than parenteral have also been studied. Intranasal AmB was reported to reduce inflammatory mucosal thickening and decrease the levels of intranasal markers for eosinophilic inflammation in patients with chronic paranasal sinusitis [45]. Inhaled aerosolized AmB has been evaluated for prophylaxis [46] even for the treatment of pulmonary fungal infections in an animal model [47]. The safety and efficacy of the non-systemic use of AmB needs further evaluation.

Table 2. Comparison of the performances of the Platelia *Aspergillus* galactomannan (GM) enzyme-linked immunosorbent assay (ELISA), beta-D-glucan (BDG) assay, and polymerase chain reaction (PCR) in the diagnosis of invasive aspergillosis

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Samples	No. of case/ total patients	Study design	Reference
GM ELISA (cut-off >0.7)	33.3	98.9			Serum	11/115	Prospective	32
Nested PCR	63.6	63.5			Blood, BAL, others			
GM ELISA (cut-off >1.5)	52	-			Serum	14/14	Retrospective	34
Real-time PCR	45	-			Serum			
GM ELISA (cut-off >0.5)	76	94			BAL	49/99	Retrospective	35
Real-time PCR	67	100			BAL			
GM ELISA (cut-off >0.6)	100	93	55	100	Serum	11/96	Prospective	36
BDG test	55	93	40	96	Plasma			
Real-time PCR	55	93	40	96	Plasma			
GM ELISA (cut-off >1.5)	87.5	89.6	70	96.3	Serum	8/40	Retrospective	37
BDG assay	87.5	89.6	70	96.3	Serum			

Abbreviations: PPV = positive predictive value; NPV = negative predictive value; BAL = bronchoalveolar lavage

Extended-spectrum triazoles

The azole antifungal agents fluconazole and itraconazole became available in the 1990s and proved to be advances in antifungal therapy. Fluconazole has good toxicity profiles but has a narrow spectrum of antifungal activity. Itraconazole has a broader spectrum of activity, including *Candida* spp., *Aspergillus* spp., and dermatophytes [48]. However, the use of itraconazole is limited by wide-ranging drug interactions and unpredictable oral bioavailability, which requires measurement of drug levels [48]. Intravenous itraconazole was reported to be equivalent to AmB deoxycholate as empirical antifungal treatment in neutropenic patients [49]. In a study evaluating prophylactic antifungal treament in allogeneic stem cell transplant recipients, itraconazole provided better protection against invasive mold infections, but similar protection against candidiasis compared with fluconazole [50]. However, greater hepatotoxicity and poor gastrointestinal tolerability were observed in patients receiving itraconazole. The place of intravenous itraconazole in antifungal therapy requires further clarification.

Voriconazole is the first available second-generation triazole. A fluconazole derivative, voriconazole has improved antifungal activity and enhanced potency against fungal 14-alpha-demethylase [51,52]. Voriconazole has good activity against yeasts, molds, common dermatophytes, and dimorphic fungi in vitro [51-53]. The drug appears to be fungistatic for yeasts, as are other azoles, but fungicidal against filamentous molds, in common with other second-generation triazoles [54]. Voriconazole is available in both intravenous and oral formulations, with the latter having 96% bioavailability [55,56]. Two major studies established voriconazole as a potent antifungal agent [57,58]. A randomized, unblinded trial indicated that voriconazole is superior to AmB deoxycholate for the primary therapy of IA [57]. A successful outcome was achieved in 52.8% of the voriconazole group versus 31.6% of the AmB group. An improved survival rate was observed in the voriconazole group (70.8% vs 57.9%). Voriconazole was the better tolerated agent in this study. In another randomized trial of empirical antifungal treatment in cases of febrile neutropenia, voriconazole was shown to be equally effective as liposomal AmB [58].

In the treatment of invasive candidiasis, voriconazole performed as well as fluconazole for the treatment of esophageal candidiasis in immunocompromised patients [59]. Ostrosky-Zeichner et al evaluated voriconazole as salvage treatment for invasive candidiasis in 52 patients who were intolerant of or refractory to other antifungal agents [60]. The overall rate of response was 56%, with response rates of 44% for *Candida albicans*, 38% for *Candida glabrata*, 70% for *Candida krusei*, and 67% for *Candida tropicalis*. Hence, it appears that voriconazole is a useful agent as a salvage therapy of invasive candidiasis, even in the settings of previous azole treatment and infections due to *C. krusei*.

Common adverse effects of voriconazole include visual disturbance, skin rashes, and elevated hepatic enzymes, as well as a number of drug interactions [54]. Voriconazole is currently approved by the United States Food and Drug Administration for the primary therapy of IA and for the treatment of serious *Fusarium* infection. In Europe and other countries, voriconazole is also approved for the treatment of fluconazole-resistant *Candida* infections, including *C. krusei* infections.

However, with the increasing use of voriconazole, recent reports noted the potential for breakthrough zygomycosis during prophylactic and therapeutic use of voriconazole in patients with febrile neutropenia or stem cell transplant recipients [61-63]. The correlation between the use of voriconazole and zygomycosis needs further prospective evaluation. Zygomycetes are intrinsically resistant to voriconazole in vitro [63]. Since diagnosis of zygomycosis is very difficult and the infection has a high risk of mortality [5,8], clinicians should be aware of this emerging opportunistic infection.

Posaconazole is a second-generation extended-spectrum triazole antifungal agent, which is only available in oral form. In an open-label, multicenter, phase III study, it showed activities against various IFIs in patients who were refractory to, or intolerant of, other antifungal therapy [64]. In contrast to voriconazole, it also showed some efficacy in zygomycosis. Invasive zygomycetes infections are emerging in immunocompromised populations in the era of new antifungal agents such as voriconazole and caspofungin [30]. AmB formulations are the only marketed agents currently effective against zygomycetes. While the activity of posaconazole against these molds is clinically useful, clinicians may hesitate to use the oral formulation in critically ill patients. Furthermore, its usage as a primary therapy needs more validation.

Agents targeting fungal cell wall

Echinocandins

The echinocandins differ from other antifungal agents in acting on the cell membrane of fungi. Although fungi are eukaryotes, they possess cell walls that are absent in

mammalian cells. Fungal cell walls consist of large polysaccharides, including: (1-3)-BDG, (1-4)-BDG, (1-6)-BDG, chitin, mannan or GM, and alpha-glucans and various glycoproteins [65,66]. Antifungal agents acting on fungal cell walls provide 2 benefits. First, because cell walls are absent in humans, the adverse effects would be relatively few. Second, they provide a novel antifungal mechanism and theoretically can be usefully combined with agents acting on the cell membrane as combination therapy for difficult fungi.

All echinocandin agents (caspofungin, micafungin, and anidulafungin) are insufficiently bioavailable for oral use (<0.2% for caspofungin) [67], and hence must be given intravenously. The antifungal spectrum of the echinocandins is best suited to (and limited to) *Candida* spp. and *Aspergillus* spp.; the drugs are fungicidal in vitro and in vivo against most isolates of *Candida* spp., and fungistatic against *Aspergillus* spp. Echinocandins appear to be more active than AmB and fluconazole against all common *Candida* spp., with the exceptions of *Candida parapsilosis* and *Candida guilliermondii*, for which echinocandins, AmB and fluconazole may have similar activities [68]. Echinocandins can be used against *Aspergillus* infections, but they are probably fungistatic against *Aspergillus* spp. The susceptibility of *Aspergillus* spp. to echinocandins is variable, with *Aspergillus flavus*, for example, being less susceptible to anidulafungin [69]. Nevertheless, specific methods for susceptibility testing of the echinocandins have not yet been developed.

At present, caspofungin is the only marketed drug in this class. It is as effective as and generally better tolerated than liposomal AmB when given as empirical antifungal therapy in patients with persistent fever and neutropenia [70]. Caspofungin is at least as effective as AmB for the treatment of invasive candidiasis and, more specifically, candidemia [71]. In the Infectious Diseases Society of America guidelines for treatment of candidiasis published in 2004, caspofungin is recommended as the primary therapy for candidemia in both neutropenic and non-neutropenic patients, as alternative therapy for candidal endocarditis and chronic disseminated candidiasis, and as alternative therapy for oropharyngeal and esopharyngeal candidiasis [72]. Maertens et al investigated the use of caspofungin as salvage therapy in IA [73]. A favorable response to caspofungin therapy was observed in 37 of 83 patients (45%), including 32 of 64 (50%) with pulmonary aspergillosis, and 3 (23%) of 13 with disseminated aspergillosis.

The echinocandins are highly active against *Pneumocystis carinii* [74-76]. Results of animal experiments showed that echinocandins have a good prophylactic effect at very low doses, but only a modest treatment effect [75]. Hence, echinocandins are not expected to be useful in treating very ill patients with *P. carinii* infection.

Other fungi that might be susceptibe to echinocandins include *Coccidioides immitis, Scedosporium* spp., *Alternaria* spp., *Bipolaris* spp., *Cladophialophora bantiana, Phialophora* spp., *Exophiala* spp., *Fonsecaea pedrosoi, P. variotii, Acremonium strictum*, and *Blastomyces dermatitidis*. Although echinocandins showed some in vitro activity against *Histoplasma* spp. [77], no significant therapeutic effect could be seen in a murine histoplasmosis model [78]. The echinocandins are not active against *Cryptococcus neoformans*, *Trichosporon* spp., *Fusarium* spp., or any zygomycetes.

Few adverse events and toxic effects of the echinocandins have been noted. Being basic polypeptide compounds, echinocandins are expected to induce histamine release. However, histamine-like reactions were not seen in patients after administration of caspofungin or micafungin, but could arise with anidulafungin if given too fast. Liver function impairment was not uncommon in patients receiving caspofungin, although it is difficult to evaluate the role of drug treatment versus pre-existing disease in this regard [67,71].

Clinically significant haemolytic anaemia seems to be rare in clinical studies although haemolysis has been observed in some animals treated with echinocandins. Fever is a frequent side effect of caspofungin treatment (occurring in about 35% of patients) [67,79-81], but not micafungin (about 1%) [68]. There is very limited experience with use of caspofungin in children [67]. One study suggested that a higher dose might be needed compared with adults [82].

Combination therapy

With the increased number of antifungal agents in recent years, there has been growing interest in combination antifungal therapy for IFIs. This has been stimulated by the opportunity to combine agents with different mechanisms of action, in the hope of producing additive or even syngergistic therapeutic effects. Most clinical experience of combination antifungal therapy has been obtained in the treatment of cryptococcus [83]. The use of AmB plus flucytosine results in a higher rate of improvement than AmB alone.

With regard to combination regimens for candidiasis, most in vitro studies demonstrated antagonism with AmB and azole (fluconazole, itraconazole, and other early azoles) combinations [83]. Prior exposure to an azole may reduce AmB activity through depletion or alteration of the ergosterol target for AmB [83]. However, conflicting results were achieved in different animal studies investigating the combination of fluconazole and AmB [83]. In a randomized clinical trial in non-neutropenic patients with candidemia comparing AmB deoxycholate plus fluconazole with fluconazole alone, there was a trend towards better overall success with the combination, and no evidence of antagonism [84]. In vitro evaluation of the efficacy of dual combinations of fluconazole, AmB, and caspofungin against *C. albicans* biofilms showed indifferent effects with all combinations [85]. Voriconazole combined with AmB or fluconazole in vitro showed additive or indifferent effects only [86]. Clinical evaluation of the combination of voriconazole or caspofungin is lacking.

Due to the increasing incidence and the high mortality of IA, there is a need for better therapeutic approaches to this condition. Many studies have evaluated combinations of different antifungal agents against *Aspergillus* spp. When evaluated in vitro against *Aspergillus* spp., AmB combined with itraconazole frequently showed antagonistic to indifferent effects [83, 86,87], and the effect of combinations of AmB with echinocandins ranged from indifference to synergy [83,86]. Caspofungin in combination with voriconazole was frequently reported to be additive to synergistic in vitro [83,86,88], but antagonism has been reported [86]. In animal models of IA, the combination of a second-generation triazole plus an echinocandin, or AmB plus an echinocandin, had better efficacy than monotherapy [83,89]. Kontoyiannis et al described 48 patients with hematologic malignancies who received liposomal AmB plus caspofungin for IA [90]. The majority (65%) received the combination as salvage therapy for diseases refractory to liposomal AmB, while the remainder received the combination as primary therapy. The overall response rate was 42%. The combination was more successful as a primary therapy (53%) than as a salvage therapy (35%) although the response rates were not statistically different. Marr et al compared the combination of voriconazole and caspofungin with voriconazole alone in patients with aspergillosis refractory to AmB [91].

The combination therapy was associated with higher 3-month survival rate (p=0.048). The probability of death due to aspergillosis was lower in patients receiving combination therapy (p=0.024). The combination of voriconazole plus AmB also demonstrated favorable responses in refractory aspergillosis [86]. However, although combination therapies seem promising, studies to date have mostly involved limited numbers of cases and have been limited to use as salvage therapy in refractory aspergillosis. Randomized trials with larger case numbers are warranted in order to further confirm these findings and to determine whether these combinations could have a role as primary therapy for aspergillosis (Table 3) [83-91].

Table 3. Studies on combinations of antifungal agents for treatment of invasive fungal infections

Disease	Combinations	Study type	Comments	Reference(s)
Candidiasis				
	AmB + Flu or Itra	In vitro	Mostly antagonism	83
	AmB + Flu	Animal model	Conflicting reports	83
	AmB + Flu	Clinical trial	Not antagonistic in non-neutropenic patients with candidemia	84
	Any 2 of AmB, Flu, or Cas	In vitro	Indifference	85
	Vori + AmB or Flu	In vitro	Indifference or additivity	86
Aspergillosis				
	AmB + Itra	In vitro	Antagonism or indifference	83,86,87
	AmB + Cas	In vitro	Indifference or additivity or synergy	83,86
	Liposomal AmB + Cas	Clinical trial	Success rate of 53% in primary therapy and 35% in salvage therapy	90
	Vori + Cas	In vitro	Additivity or synergy. Antagonism has been reported	83,86,88
	Vori + Cas	Animal model	Reduced numbers of positive cultures	89
	Vori + Cas	Clinical trial	Higher 3-month survival and lower death rate from aspergillosis as salvage therapy	91

Abbreviations: AmB = amphotericin B; Flu = Fluconazole; Itra = itraconazole; Cas = caspofungin; Vori = voriconazole

Conclusions

Laboratory diagnosis of IFIs has progressed in recent years, with advances mainly occurring in the area of diagnosis of IA. In the evaluation of the diagnostic methods for IA, the determination of sensitivity and specificity has been confounded by the uncertainty of the disease status; inclusion of probable cases of IA in the evaluation would affect performances of diagnostic tests. Non-culture-based diagnosis for candidiasis and noninvasive diagnosis for molds other than *Aspergillus* mostly remain investigational. New methods or better strategies are needed to improve diagnostic methods for IFIs.

There have been many advances in antifungal treatment in the last decade. The availability of more potent and less toxic antifungal agents, such as second-generation triazoles and echinocandins, has greatly improved the treatment of IFIs. However, the mortality of IFIs remains high. Combination therapy is promising conceptually as a means to increase the success rate of treatment, but more controlled clinical trials are needed to verify the efficacy of this approach.

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