Chapter

Aspergillus-Human Interactions: From the Environment to Clinical Significance

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Abstract

Aspergillus species are ubiquitous fungi found in the environment worldwide. The most common *Aspergillus* species causing diseases in humans are *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus.* However, species causing human infections are also depending on human immune status. Host immune status and previous underlying diseases are important factors leading to different clinical manifestations and different disease spectra of *Aspergillus* infections. The most severe form of *Aspergillus* infections is invasive aspergillosis in human tissue, especially invasive pulmonary aspergillosis (IPA), which has high morbidity and mortality in immunocompromised patients. ICU patients with influenza infections and COVID-19 infections are recently risk factors of invasive pulmonary aspergillosis. New diagnostic criteria include galactomannan antigen assays, nucleic acid amplification assays, and lateral flow assays for early and accurate diagnosis. Voriconazole and the newest azole, isavuconazole, are antifungals of choice in IPA. Nevertheless, azole-resistant *Aspergillus* strains are increasing throughout the world. The etiology and spreading of azole-resistant *Aspergillus* strains may originate from the widespread use of fungicides in agriculture, leading to the selective pressure of azole-resistant strains. Therefore, there is a necessity to screen *Aspergillus* antifungal susceptibility patterns for choosing an appropriate antifungal agent to treat these invasive infections. In addition, mutations in an ergosterol-producing enzyme, i.e., lanosterol 14-α demethylase, could lead to azole-resistant strains. As a result, the detection of these mutations would predict the resistance to azole agents. Although many novel azole agents have been developed for invasive *Aspergillus* infections, the rate of novel antifungal discovery is still limited. Therefore, better diagnostic criteria and extensive antifungal resistant *Aspergillus* screening would guide us to better manage invasive *Aspergillus* infections with our existing limited resources.

Keywords: *Aspergillus*, *Aspergillus*-human interactions, invasive aspergillosis, antifungal susceptibility test, azole, voriconazole, amphotericin B, influenza-associated pulmonary aspergillosis, COVID-19-associated pulmonary aspergillosis

1. Introduction

Aspergillus species are saprophytic ubiquitous filamentous fungi [1]. They are in Phylum Ascomycota with both sexual and asexual forms [1]. In their sexual form, they produce asci and ascospores within the appropriate environment, while they produce conidia, or asexual spores, on phialides surrounding their

vesicles at the tip of conidiophores in their asexual form [1]. *Aspergillus* conidia are different in size and shape depending on *Aspergillus* species, which affects the dispersion and infectivity properties of *Aspergillus* [1]. Their conidia can be found in the soil, decomposed piles, air, animals, and humans. They cause diseases in immunocompromised hosts, e.g., patients with acquired immunodeficiency syndrome (AIDS), allogenic hematopoietic stem cell transplant or solid organ transplant candidates, patients with immunosuppressive drugs, patients with prolonged neutropenia, and patients with other underlying diseases [2]. The common pathogenic *Aspergillus* species are *A. fumigatus, A. flavus, A. niger,* and *A. terreus* [3]. There are a wide variety of disease spectra of *Aspergillus* infections, i.e., invasive aspergillosis, chronic aspergillosis, and allergic forms of aspergillosis [1, 2]. The most severe form causing high morbidity and mortality rate, especially in immunocompromised hosts, is invasive aspergillosis (IA) [2, 4]. An increase of immunocompromised hosts would also increase patients with IA with a high mortality rate [4–14].

Invasive aspergillosis (IA) is recently increasing in patients with allogenic hematopoietic stem cell transplantation (HSCT) and solid organ transplantation [5, 8, 13, 15–22]. Underlying conditions of patients with IA are hematological malignancies, e.g., leukemia or lymphoma, bone marrow transplant, and solid-organ transplant patients [5, 8, 13, 15–22]. Recently, not only neutropenic patients are at risk for IA, but non-neutropenic patients with immunosuppressive agents, e.g., biologics, small-molecule kinase inhibitors (SMKIs), Chimeric Antigen Receptor (CAR) T cells, are also at risk [23–28]. In developing countries, poor-controlled diabetes mellitus is one of the critical risk factors of IA [10, 12]. Therefore, risk factors of IA are now patients with malignancy, autoimmune, inflammatory diseases, complex immune-metabolic diseases from aging, immunosuppressive treatment, previous septic conditions, novel biologic treatment, including patients with hematological malignancies receiving SMKIs, patients in ICU, patients with a cytokine storm syndrome from CAR-T cells treated with high-dose corticosteroids, patients in ICU with severe influenza or other viral infections [23–36]. In an era of Coronavirus Disease 2019 (COVID-19) infections, IA was recognized as a severe complication of patients with COVID-19 infections in ICU [37–46].

2. Pathogenesis of *Aspergillus* and its virulence factors

Among thousands of *Aspergillus* species, only less than twenty species could cause diseases in humans [47]. The pathogenic species usually possess virulence factors that help them survive and cause infections inside hosts. *Aspergillus fumigatus* was utilized as a model to study virulence factors in many studies (**Table 1**) [1].

To survive inside the host environment, *Aspergillus* species need to adapt to heat and hypoxic conditions inside hosts. For the heat stress, the trehalose pathway was shown to have a role in heat tolerance and virulence of *A. fumigatus* [47]. Heat shock proteins (HSPs), especially Hsp90, are chaperone proteins associated with stress tolerance, not only for heat [48–50]. In mammalians, HIF1α, as a common transcription factor, controls cellular homeostasis in hypoxic conditions [51]. In fungi, a homolog of HIF1α, called the sterol regulatory element-binding protein (SREBP) or SrbA in *A. fumigatus,* is induced by hypoxia and iron starvation conditions [52–56]. SrbA protein is also associated with the virulence of *A. fumigatus in vivo* [52–54].

A. fumigatus possesses enzymes to protect itself against host reactive oxygen species (ROS), e.g., catalase, superoxide dismutases, thioredoxin, glutathione, including mitochondrial electron transport chain [57–62]. In some animal

Virulence factors	Characteristics
Stress tolerance	• Thermotolerance • Hypoxic adaptation • pH/Reactive oxygen species (ROS) resistance • Secondary metabolites • Light response
Metabolism and nutrient uptake	• siderophores, Zinc Magnesium Copper transporter, calmodulin, calcineurin, phosphate permeases
Cell components	• Cell wall: β-glucan, chitin, rodlet • Galactosaminogalactan (GAG) • Melanin
Others	• Biofilm • Cellular heterogeneity

Table 1.
Essential virulence factors in Aspergillus fumigatus requiring for causing infections inside humans [1].

models, e.g., an eye infection model, demonstrated that these fungal enzymes were essential for fungal virulence [63]. Secondary metabolites are also playing a role in fungal virulence [64–66]. *A. fumigatus* secondary metabolites are gliotoxin, fumigaclavine, trypacidin, helvolic acid, fumitremorgin, fumagillin, and pseurotin, associated with host cellular toxicity [67–71]. However, the mechanisms behind this toxicity is still unclear and need to be further investigated *in vivo* [71]. *A. flavus* produces aflatoxins, which are important carcinogenic secondary metabolites, and other secondary metabolites, called Velvet complex, as environmental response mechanisms [72, 73]. Circadian rhythms or light response, which were studied thoroughly in the *Neurospora* model system, are essential to react with the environment [74]. Light-induced mycelial pigmentation and germination acted as a stress signaling pathway in *A. fumigatus* via transcription factor LreA and FphA, respectively [75–77].

For nutrient acquisition, exoenzymes or proteases are major enzymes produced by *A. fumigatus,* especially the alkaline protease Alp1 and the metalloprotease Mep1 [1, 78]. In *A. fumigatus,* a transcriptional repressor called CreA has a vital role in carbon catabolite repression. *Af*CreA regulates growth on different nitrogen, carbon, and lipid sources and has a role in amino acid transportation, nitrogen, and carbon assimilation, including glycogen and trehalose metabolism [79, 80]. Although CreA is not required for virulence, it is required for disease progression in invasive pulmonary aspergillosis (IPA) mouse models [79–81]. For nitrogen utilization, *Af*RhbA, a Ras-related protein in a nitrogen-regulated signaling pathway, and *Af*AreA, a GATA transcription factor requiring the expression of genes involving nitrogen utilization, are related to virulence in *A. fumigatus* [82–84]. *A. fumigatus* still needs divalent cations, i.e., iron, copper, magnesium, zinc, calcium, for its growth and virulence inside hosts via siderophores, calmodulin, calcineurin, specific importers, and exporters [85, 86].

Additionally, cell wall components of *Aspergillus fumigatus* are also essential virulence factors for fungal survival inside hosts and are important for host immune response [87–92]. Cell wall components consist of β-1,3-glucan, chitin, galactomannan, α-1,3-glucan, and melanin depending on different stages of *A. fumigatus*, i.e., conidial, or hyphal stage [91–95]. β-1,3-glucan, a central component of *Aspergillus* cell wall polysaccharide, is a pathogen-associated molecular

pattern (PAMP) recognized by host pattern recognition receptors (PRR), e.g., dectin-1 [88]. During its conidial stage, rodlet, or hydrophobins, and dihydroxynapthalene (DHN) melanin are present to protect fungal conidia against host immune response by evading host pathogen-associated molecular patterns (PAMPs) recognition, including protecting fungi from unfavorable stress conditions [93–97]. Furthermore, in its hyphal stage, galactosaminogalactan (GAG), which is a water-insoluble polymer consisting of a pyranose-form galactose, galactosamine, and N-acetylgalactosamine (GalNAc), is present as an extracellular matrix on an outer layer of the cell wall [98]. GAG is associated with biofilm formation and immunosuppression properties by masking PAMP exposure and resisting neutrophil killing via neutrophil extracellular traps (NETs) [99–102]. The linkage between cell wall components and metabolic pathways is still unclear. Nevertheless, these components share the same building blocks, e.g., UDP-glucose, glucose 6-phosphate, with specific metabolic pathways, e.g., glycolysis, trehalose biosynthesis pathway [81, 103–105]. It is possible that the homeostasis of cell wall biosynthesis is involved with some metabolic pathways, e.g., the trehalose biosynthesis pathway. Disruption of one of these trehalose enzymes or building blocks would result in decreased virulence due to changes in cell wall compositions [81, 103–105]. Understanding this homeostasis would lead to the discovery of novel antifungal targets in the future.

3. Diagnosis of invasive *Aspergillus* infections: challenge in the field

Aspergillus infections are associated closely with host immune status [106, 107]. Severe asthma with fungal sensitization and allergic bronchial pulmonary aspergillosis (ABPA) are found in immunocompetent hosts with hypersensitivity, while aspergilloma and chronic pulmonary aspergillosis are found in immunocompetent hosts with previous structural diseases, such as lung cavity from previous tuberculosis infections [108]. In immunocompromised hosts, invasive aspergillosis is common and severe, causing high morbidity and mortality in patients [108, 109].

For invasive pulmonary aspergillosis, early diagnosis and prompt treatment are the keys to decrease the disease burden. Differentiation between *Aspergillus* colonization and invasive infections is still challenging [25, 92, 93]. Recently, the revised EORTC guideline for diagnosis of invasive fungal infections, including *Aspergillus* infections, recommended the diagnostic criteria including host factors, clinical, radiological, and microbiological criteria with new diagnostic methods (**Table 2**) [109]. Proven invasive aspergillosis is confirmed with histopathologic, cytopathologic, microscopic analysis, or nucleic acid analysis of sterile specimens or tissue or formalin-fixed paraffin-embedded tissue (FFPE), including culture recovered from sterile sites [109]. Species of common *Aspergillus* recovered from cultures are differentiated using macroscopic and microscopic morphology, but the nucleic acid analysis is necessary for the species complex (**Table 3**) [110]. For probable and possible invasive aspergillosis, host factors, clinical features, and mycological evidence are including for the diagnosis of invasive aspergillosis. Host factors include the history of neutropenia, which is less than 500 neutrophils/mm^3, for more than ten days, hematological malignancy, allogenic stem cell transplantation, solid organ transplantation, therapeutic-dose corticosteroids at not less than 0.3 mg/kg for not less than three weeks during the previous 60 days, treatment with T-cell or B-cell immunosuppressants, inherited immunodeficiency, or acute graft-versus-host disease grade III or IV [109]. For clinical evidence of pulmonary aspergillosis, a chest high-resolution CT scan is recommended to observe any halo

Diagnosis of invasive aspergillosis.	**Criteria**
Proven	• Microscopic analysis: from needle aspiration or biopsy OR • Culture: from sterile sites except for BAL fluid, paranasal sinuses, and urine OR • Tissue nucleic acid analysis from formalin-fixed paraffin-embedded tissue
Probable: 1 host factor + 1 clinical feature+1 mycological evidence	Host factors • Recent neutropenia • Hematological malignancy • Receipt of an allogenic stem cell transplant • Receipt of a solid organ transplant • Prolonged use of corticosteroids • Use of T-cell immunosuppressants • Use of B-cell immunosuppressants • Inherited severe immunodeficiency • Acute GVHD grade III or IV Clinical features: pulmonary aspergillosis • One of the following CT Chest patterns: ○ Dense well-circumscribed lesion with or without a halo sign ○ Air crescent sign ○ Cavity ○ Wedge-shaped and segmental or lobar consolidation Mycological evidence • Culture positive from sputum, BAL, bronchial brush, or aspirate • Direct examination positive from sputum, BAL, bronchial brush, or aspirate • Galactomannan antigen: plasma serum BAL CSF: any of: ○ Single serum or plasma >/= 1 ○ BAL fluid >/= 1 ○ Single serum or plasma >/= 0.7 and BAL fluid >/= 0.8 ○ CSF >/= 1 • *Aspergillus* PCR: any of: ○ Plasma, serum, or whole blood 2 or more consecutive PCR ○ BAL fluid 2 or more duplicate PCR ○ At least 1 PCR from plasma serum or whole blood & 1 PCR from BAL fluid
Possible: 1 host factor + 1 clinical feature	

Table 2.
Diagnosis of invasive aspergillus infections from revised EORTC/MSG criteria 2020 (BAL: bronchoalveolar lavage; CT: computed tomography; CSF: cerebrospinal fluid; GVHD: graft versus host disease; PCR: polymerase chain reaction) [109].

sign, air-crescent sign, cavity, or wedge-shaped and segmental or lobar consolidation [109, 111]. Probable invasive aspergillosis still needs at least one mycological evidence to support the diagnosis. Mycological evidence is including cultures recovered from sputum, bronchoalveolar lavage (BAL), bronchial brush, or

***Aspergillus* species**	**Macroscopic features**	**Microscopic features**
Aspergillus fumigatus	Typical blue-green colony with suede-like surface	Columnar uniseriate conidial heads with phialides limited to upper two-thirds of its vesicles; short and smooth conidiophores; basipetal green, rough-walled globose to subglobose conidia
Aspergillus flavus	Bright to dark yellow-green colony with a granular, flat surface	Radiate biseriate conidial heads with phialides over the surface of mature vesicles; coarsely rough conidiophores; pale green, globose to subglobose conidia
Aspergillus niger	Dark brown to the black colony with white to yellow color at the reverse side of the colony	Globose, large, dark brown, biseriate, radiate conidial head with long metulae; smooth, hyaline conidiophores; dark brown, rough conidia
Aspergillus terreus	Cinnamon-brown colony with suede-like surface and yellow to deep brown color at the reverse side of the colony	Compact, columnar, biseriate conidial heads; hyaline, smooth conidiophores; hyaline to yellow, smooth-walled conidia

Table 3.
Macroscopic and microscopic features of clinical-relevant Aspergillus species (colony on Czapek Dox agar at 30°C) [110].

aspirate [109]. *Aspergillus* galactomannan antigen assays with different thresholds depending on specimens, including serum, BAL fluid, plasma, and cerebrospinal fluid (CSF), support the diagnosis of invasive aspergillosis [112–115]. However, decreased sensitivity of galactomannan antigen assay is observed in patients with anti-mold therapy [115]. In addition, *Aspergillus* PCR from blood and BAL fluid is introduced to confirm the diagnosis and identify specific *Aspergillus* species with certain mutations related to triazole resistance [109, 116–124].

Nonetheless, revised EORTC/MSG criteria for diagnosing invasive fungal infections may be applied mainly for neutropenic patients or immunocompromised patients. Therefore, specific guidelines for the diagnosis of invasive aspergillosis in non-neutropenic patients in ICU (Invasive pulmonary aspergillosis in ICU, AspICU) or patients with influenza (Influenza-associated pulmonary aspergillosis, IAPA) or Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) (COVID-19 associated pulmonary aspergillosis, CAPA) co-infections were developed and published for early and accurate diagnosis (**Table 4**) [31, 125–127].

4. Treatment of *Aspergillus* infections

IA also includes the infections of the lower respiratory system, sinuses, and skin as entry routes. In addition, the cardiovascular system, central nervous system, and other tissues could be infected from hematogenous dissemination or direct extension from adjacent infected tissues [2]. Infectious Diseases Society of America (IDSA, 2016) and ESCMID-ECMM-ERS (2017) recommended voriconazole (6 mg/kg, intravenous route every 12 hours for one day, and then 4 mg/kg every 12 hours; 200–300 mg every 12-hour, oral route) as a first-line treatment for invasive pulmonary aspergillosis (IPA) [2, 128]. For alternative treatment, liposomal amphotericin B (3–5 mg/kg/day, intravenous route) and isavuconazole (200 mg every 8 hours for three days and then 200 mg daily) [2]. For other invasive aspergillosis syndromes, i.e., invasive sinus aspergillosis, tracheobronchial aspergillosis, invasive aspergillosis of the central nervous system or cardiovascular system, *Aspergillus* osteomyelitis,

Diagnostic criteria of IPA	**AspICU [125]**	**IPA with influenza (IAPA) [126]**	**IPA with SARS-CoV-2 (CAPA) [127]**
Host factors	One of the following: • Neutropenia (<500/mm^3) before or at ICU admission • Hematological or oncological malignancy with cytotoxic therapy • Glucocorticoid treatment with prednisolone equivalent >20 mg/day • Immunodeficiency	Entry criteria: influenza-like illness + positive influenza PCR or antigen + timing (7 days before and 96 hours after ICU admission)	Entry criteria: patients with COVID-19 infections (RT-PCR) in ICU with a temporal relationship to suspected IPA
Clinical features	One of the following: • Fever with appropriate antibiotic treatment for at least three days • Recurrent fever after a fever-free period for at least 48 hours with antibiotics and without other apparent cause • Dyspnea • Hemoptysis • Pleuritic chest pain or pleural friction rub • Worsening respiratory failure with appropriate antibiotics and ventilator support	None	None
Radiological evidence	• Any medical imaging by conventional chest X-ray or CT scan of lungs	• Pulmonary infiltrate OR • Cavitating infiltrate (not from other causes)	• Pulmonary infiltrate OR • Cavitating infiltrate (not from other causes)

Diagnostic criteria of IPA	**AspICU [125]**	**IPA with influenza (IAPA) [126]**	**IPA with SARS-CoV-2 (CAPA) [127]**
Microbiological evidence	• *Aspergillus* recovered from the lower respiratory tract (LRT) (entry criterion) • *Aspergillus*-positive culture of BAL fluid without bacterial growth together with a positive microscopic analysis showing branching hyphae (if no host factor)	• If pulmonary infiltrate presents, at least one of the following: ○ Galactomannan (GM) antigen assay: serum >0.5 or BAL ≥ 1.0 or ○ positive culture from BAL • If lung cavity presents, at least one of the following: positive sputum culture or tracheal aspirate culture	Probable CAPA: at least one of the following: • Microscopic detection of septate hyphae in BAL • Positive BAL culture • Serum GM >0·5 or serum LFA index >0·5 • BAL GM ≥1·0 or BAL LFA index ≥1·0 • Two or more positive *Aspergillus* PCR in plasma, serum, or whole blood or a single positive *Aspergillus* PCR in BAL (<36 cycles); or a single positive *Aspergillus* PCR in plasma, serum, or whole blood with a single positive in BAL fluid (any threshold cycle) Possible CAPA: at least one of the following: • Microscopic detection of septate hyphae in non-BAL • Positive non-BAL culture • Single non-BAL GM >4·5 • Non-BAL GM >1·2 twice or more • Non-BAL GM >1·2 plus another non-BAL PCR or LFA positive

Diagnostic criteria of IPA	**AspICU [125]**	**IPA with influenza (IAPA) [126]**	**IPA with SARS-CoV-2 (CAPA) [127]**
Categories	• Proven IPA: similar to EORTC/MSG 2020 criteria • Putative IPA: *Aspergillus*-positive from LRT + Clinical evidence + Radiological evidence + (Host factors or *Aspergillus* culture from BAL with positive microscopic analysis) • Colonization: ≥ 1 criterion for a diagnosis of putative IPA is not fulfilled	• Proven IAPA: entry criteria with tissue diagnosis similar to EORTC/MSG 2020 criteria • Putative IAPA: entry criteria + Radiological evidence + Microbiological evidence • Colonization: ≥ 1 criterion for a diagnosis of putative IPA is not fulfilled	• Proven CAPA: entry criteria with tissue diagnosis similar to EORTC/MSG 2020 criteria • Probable CAPA: entry criteria + radiological evidence + probable criteria of microbiological evidence • Possible CAPA: entry criteria + radiological evidence + possible criteria of microbiological evidence

Table 4.
Diagnostic criteria for invasive pulmonary aspergillosis (IPA) of patients in ICU (AspICU) or with influenza (IAPA) or SARS-CoV-2 (CAPA) coinfections (PCR: polymerase chain reaction; ICU: intensive care unit; RT-PCR: Real-time polymerase chain reaction; BAL: bronchoalveolar lavage; GM: galactomannan; LFA: lateral flow assay) [31, 125–127].

Aspergillus endophthalmitis and keratitis, cutaneous aspergillosis, and *Aspergillus* peritonitis, intravenous voriconazole is still the first-line therapy [2]. For IPA in ICU patients, patients with hematological malignancies, or solid organ transplants, IAPA, and CAPA, voriconazole and isavuconazole are still recommended as the first-line treatment (**Table 5**).

Voriconazole is metabolized at the liver via CYP2C19 and CYP3A4 [135]. Medications with CYP2C19 and CYP3A4-dependent metabolism, antacids, proton pump inhibitors may affect serum voriconazole levels [136]. Adverse reactions and toxicity of voriconazole are associated with higher serum voriconazole levels [137]. Common adverse reactions include reversible visual disturbances, hepatotoxicity, photosensitivity, reversible visual or auditory hallucinations, tachyarrhythmias, and QT interval prolongations [137, 138]. Isavuconazole is a second-generation broad-spectrum triazole requiring a loading dose with a five-day half-life [139]. Isavuconazole has fewer adverse reactions in photosensitivity, hepatotoxicity, visual abnormality, and less drug–drug interaction [140–142]. Isavuconazole is a CYP3A4 inhibitor and can decrease the metabolism of sirolimus, tacrolimus, cyclosporine, and digoxin, leading to increased levels of these agents [142]. Furthermore, isavuconazole can induce dose-dependent QTc shortening [143]. Isavuconazole was shown to be non-inferior to voriconazole to treat invasive mold disease from the SECURE trial [144]. Posaconazole is also a broad-spectrum triazole used mainly for prophylaxis and salvage treatment of invasive fungal infections [145]. A suspension form of posaconazole has unpredictable bioavailability and needs a high-fat meal for better absorption [146]. However, tablet and IV formulations overcome this limitation. Posaconazole strongly inhibits CYP3A4 and is metabolized through UGT1A4 [145]. Using CYP3A4 substrates with posaconazole should be cautious [145]. The common adverse effects of posaconazole are gastrointestinal disturbances, hepatotoxicity, rashes, fever, hypokalemia, hypomagnesemia, and QTc prolongation [145].

Amphotericin B, a polyene antifungal agent binding to ergosterol in the fungal cell membrane, has many forms, i.e., conventional with deoxycholate and lipid-based form [2, 147]. Conventional amphotericin B has common adverse effects, including acute reactions after infusion (fever, chills, nausea), phlebitis, hypokalemia, hypomagnesemia, and nephrotoxicity (usually from renal tubular acidosis). The lipid-based form has less nephrotoxicity than the conventional form [2]. Nevertheless, acute infusion reactions may still present in liposomal amphotericin B [148]. In addition, hypokalemia, hypomagnesemia, mild bilirubin, alkaline phosphatase elevations are also present occasionally in lipid-based amphotericin B [2]. Lipid-based amphotericin B is recommended for alternative treatment of invasive aspergillosis in case that azoles cannot be used [2].

Echinocandins, e.g., caspofungin, micafungin, is a non-competitive β-1,3 D-glucan synthase inhibitor leading to loss of fungal cell wall's strength and integrity [149, 150]. Echinocandins have fewer adverse reactions and fewer drug–drug interactions [149, 150]. Echinocandins are recommended for salvage therapy or in azole-resistant *Aspergillus* infections combined with azoles for invasive aspergillosis treatment (**Table 5**) [2, 151–153].

Therapeutic drug monitoring (TDM) of azoles, e.g., voriconazole, posaconazole, isavuconazole, is necessary, especially in elderly patients, obese patients, critically ill patients, and patients with potential azole drug–drug interactions [2]. For treatment of IA, IDSA recommended TDM of voriconazole at a trough level of >1–1.5 μg/mL but less than 5–6 μg/mL to prevent neurotoxicity [2]. American Society of Transplantation Infection Diseases Community of Practice (AST) recommended TDM of posaconazole (suspension and tablet form) and isavuconazole at a trough level of >1–1.25 μg/mL and 2–3 μg/mL, respectively [154]. Timing

Condition	First-line treatment	Prophylaxis
IPA in ICU patients [129, 130]	Voriconazole (6 mg/kg, intravenous route every 12 hours for one day, and then 4 mg/kg every 12 hours; 200–300 mg every 12 hours oral route) or Isavuconazole (200 mg every 8 hours for 3 days and then 200 mg daily) (Liposomal amphotericin B, 3–5 mg/kg/day, intravenous route, in ICU patients with severe liver insufficiency, cirrhosis Child-Pugh scores B, C)	In immunocompetent patients in ICU, prophylaxis is not recommended for IPA
IPA in patients with hematological malignancies [131–133]	Voriconazole or Isavuconazole (Liposomal amphotericin B as alternative treatment)	Posaconazole (oral solution 200 mg every eight hours or tablet/intravenous route 300 mg every 12 h on day one then 300 mg daily) (in AML and MDS undergoing intensive chemotherapy with the incidence of invasive mold diseases >8% or in graft-versus-host disease) Voriconazole (200 mg orally every 12 h) (in HSCT)
IPA in patients with solid organ transplantation [134]	Voriconazole or Isavuconazole (Liposomal amphotericin B in hepatotoxicity, drug–drug interaction, ≥10% environment azole-resistant isolates found)	Kidney and heart transplantation are not recommended Lung transplantation: voriconazole, nebulized liposomal amphotericin B
IAPA [31, 126]	Voriconazole or Isavuconazole	No current recommendation Need further studies
CAPA [127]	For azole sensitive: Voriconazole or Isavuconazole (for 6–12 weeks) For azole-resistant: - Suspected: voriconazole + echinocandin (Caspofungin 70 mg first day followed by 50 mg daily) or isavuconazole + echinocandin - Proven: Liposomal amphotericin B	No current recommendation Need further studies

Table 5.
Treatment of invasive pulmonary aspergillosis (IPA) in ICU patients, patients with hematological malignancies, or solid organ transplants, influenza-associated pulmonary aspergillosis (IAPA), and COVID-19 associated pulmonary aspergillosis (CAPA) (AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; HSCT: hematological stem cell transplantation).

for measuring serum trough concentration of voriconazole, posaconazole, and isavuconazole is at 5–7 days, after 5 days, and within 7 days, respectively [154]. For prophylaxis, International Society for Heart and Lung Transplantation (ISHLT) recommended TDM of voriconazole and posaconazole at a trough level of ≥1 μg/mL and > 0.7 μg/mL, respectively [155]. Additionally, in CAPA, ECMM/ISHAM

recommended weekly TDM of voriconazole and posaconazole at a trough level of 2–6 μg/mL and 1–3.75 μg/mL, respectively [127].

5. Azole-resistant *Aspergillus*

5.1 Etiology and clinical significance

Voriconazole and isavuconazole are the first-line therapy of invasive aspergillosis [2, 129, 130]. Furthermore, azoles, i.e., posaconazole and voriconazole, are also used as prophylaxis of invasive aspergillosis in patients with hematological malignancies and solid organ transplantation [131–134]. Therefore, azoles are important antifungal agents to combat invasive aspergillosis. Unfortunately, azole-resistant *Aspergillus fumigatus* strains are emerging and increasing, leading to increased treatment failure and mortality [156, 157]. The etiology of these emerging azole-resistant *A. fumigatus* (ARAF) may be from the environmental selective pressure associated with azole fungicides in the agricultural area, including Europe, Asia, Latin America, the Midwest, and Southeast states of the USA [158–161]. The supporting evidence of environment-derived ARAF is that ARAF strains were recovered from azole-naive patients [158, 162–165]. In addition, the most common mutations at the *cyp51A* gene (encoding lanosterol 14-α demethylase) causing azole resistance in ARAF strains, which are TR_{34}/L98H and TR_{46}/Y121F/T289A mutations, were also recovered from patients' homes and surroundings [166–171].

Azole fungicides, i.e., bromuconazole, difenoconazole, epoxiconazole, enilconazole, metconazole, prochloraz, propiconazole, prothioconazole-desthio, and tebuconazole, play an important role in the development of environment-derived azole-resistant *Aspergillus* isolates leading to cross resistance to medical azoles [169, 172, 173].

Antifungal susceptibility tests (AST) of *Aspergillus* species are essential for screening azole-resistant *Aspergillus* isolates. The indications to perform *Aspergillus* AST are that the fungus is recovered from sterile sites in regions with high azole-resistant rates, including long-term azole treatment in chronic bronchopulmonary aspergillosis and breakthrough *Aspergillus* infections or recurrent or persistent infections [2, 128, 174].

The standard antifungal susceptibility testing of filamentous fungi to observe the minimum inhibitory concentration (MIC) using broth microdilution assays was described by the Clinical and Laboratory Standards Institute (CLSI) and the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) [175, 176]. To determine antifungal resistance of *Aspergillus* species, e.g., *A. flavus, A. fumigatus, A. niger, A. terreus,* CLSI and EUCAST utilized two values, which are epidemiological cutoff values (ECVs or ECOFFs) and clinical breakpoints (BP) (**Table 6**). ECVs for CLSI and ECOFFs for EUCAST of each antifungal agent against each *Aspergillus* originate from MIC distribution of the wild-type *Aspergillus* population [175–178]. These values can divide *Aspergillus* strains into two groups, which are wild-type and non-wild-type strains. Non-wild-type strains may resist those antifungal agents [175, 176, 178]. Clinical breakpoints are based on antifungal pharmacodynamics, pharmacokinetics, data from clinical trials, and patient outcomes [175, 176, 178]. Resistance is determined by the MICs over R (resistant) (**Table 6**). For EUCAST, another value is the area of technical uncertainty (ATU), which is the value that needs to be addressed before reporting these results, i.e., repeating the test, using a genotypic test, changing the susceptibility category, or including ATU as a part of the report [176].

Aspergillus species	Antifungal agents	CLSI M59 & M61, 2020 (μg/mL)				EUCAST BP_ECOFF v2.0, 2020 (μg/mL)			
		ECV	S	I	R	ECV	S≤	R>	ATU
A. flavus	Amphotericin B	4	—	—	—	4	—	—	—
	Caspofungin	0.5	—	—	—	—	—	—	—
	Isavuconazole	1	—	—	—	2	1	2	2
	Itraconazole	1	—	—	—	1	1	1	2
	Posaconazole	0.5	—	—	—	0.5	—	—	—
	Voriconazole	2	—	—	—	2	—	—	—
A. fumigatus	Amphotericin B	2	—	—	—	1	1	1	—
	Caspofungin	0.5	—	—	—	—	—	—	—
	Isavuconazole	1	—	—	—	2	1	2	2
	Itraconazole	1	—	—	—	1	1	1	2
	Posaconazole	—	—	—	—	0.25	0.125	0.25	0.25
	Voriconazole	1	≤0.5	1	≥2	1	1	1	2
A. niger	Amphotericin B	2	—	—	—	0.5	1	1	—
	Caspofungin	0.25	—	—	—	—	—	—	—
	Isavuconazole	4	—	—	—	4	—	—	—
	Itraconazole	4	—	—	—	4	—	—	—
	Posaconazole	2	—	—	—	0.5	—	—	—
	Voriconazole	2	—	—	—	2	—	—	—
A. terreus	Amphotericin B	4	—	—	—	8	—	—	—
	Caspofungin	0.12	—	—	—	—	—	—	—
	Isavuconazole	1	—	—	—	1	1	1	—
	Itraconazole	2	—	—	—	0.5	1	1	2
	Posaconazole	1	—	—	—	0.25	0.125	0.25	0.25
	Voriconazole	2	—	—	—	2	—	—	—

Table 6.
Interpretation of antifungal susceptibility tests and epidemiological cutoff values (ECVs) of Aspergillus species according to CLSI M59 and M61, 2020 and EUCAST BP ECOFF version 2, 2020 (S: susceptible, I: intermediate, R: resistant, ATU: Area of Technical Uncertainty) [175–177].

Molecular methods to detect *CYP51A* mutations, e.g., TR_{34}/L98H, TR_{46}/Y121F, are established by using classic PCRs with sequencing, real-time PCRs, loop-mediated isothermal amplification (LAMP), or whole-genome sequencing (WGS) [179]. These molecular methods have a high negative predictive value to rule out these resistant strains' infections [179]. However, they had narrow coverage and mutations at this point depending on association data between mutations and antifungal resistance property. Furthermore, commercial tools are still not approved by the US FDA [179].

5.2 Management of azole-resistant *Aspergillus* and novel antifungal candidates

Overexpression with a tandem repeat in the promoter area (TR_{34} or TR_{46}) and point mutations (L98H or Y121F/T289A) in the *cyp51A* gene, encoding azole's target called lanosterol 14-α demethylase, would lead to azole resistance in *Aspergillus*

fumigatus including voriconazole and isavuconazole [156, 178]. To treat these azole-resistant *Aspergillus* infections, monotherapy of each azole should be avoided, especially in areas with more than 10% of azole resistance prevalence [180]. In areas with high rates of azole resistance, liposomal amphotericin B and a combination of voriconazole and echinocandin should be considered [2, 127, 128, 156, 180]. Therefore, the prevalence of azole-resistant *Aspergillus* strains using conventional culturing methods together with broth microdilution assays or using molecular biology (RT-PCR) is essential to decide the optimal treatment and to choose suitable antifungal agents to get rid of these infections [156, 179].

From the increased speed of azole-resistant *Aspergillus* strains, novel antifungal agents with high efficacy and fewer side effects are crucial to combat these infections with very high mortality [156]. However, discovering these novel antifungal agents has many steps and methods to evaluate both *in vitro* and *in vivo* analyses for both antifungal activity and toxicity [181, 182]. The first step for screening antifungal activity has many methods depending on the screening purpose [181]. To observe the antifungal activity of novel antifungal candidates, the broth microdilution method is the standard method to provide the MICs [183]. This method is perfect for various compounds requiring high throughput assays [181]. Furthermore, this method requires a small number of compounds and can apply to different *Aspergillus* species simultaneously [181]. To observe combinatorial effects between novel antifungal candidates and current antifungal agents, checkerboard assays are used to determine the fractional inhibitory concentration index (FICI) [184, 185]. The FICI is calculated using the sum of the fractional inhibitory concentration (FIC_1) of the first compound, which is MIC_{1+2} of the combination of the first and the second compounds divided by MIC_1 of the first compound alone, and the FIC_2 of the second compound [184, 185]. Synergistic, additive, indifferent, and antagonistic effects are defined by FICI $\leq$0.5; >0.5–1; >1–4; and > 4, respectively [184–186]. For the cytotoxicity effects on human epithelial cells, many *in vitro* colorimetric assays, including mammalian tissue culture systems and vital dyes, are used, such as Alamar blue, MTT, XTT (tetrazolium) assays [181]. Next steps after *in vitro* studies to prove the antifungal activity and toxicity, *in vivo* animal models are used to study pharmacodynamics and pharmacokinetics, including *in vivo* antifungal activity and *in vivo* toxicity [181]. Then, these antifungal candidates would follow through the clinical trial phase I (safety), phase II (checking effectiveness), phase III (confirming effectiveness, side effects), and get approved [181, 182, 187].

Many novel antifungal compounds against both classical targets and novel targets are in clinical trials (**Table 7**) [262]. Novel targets against *Aspergillus* species include glycosylphosphatidylinositol (GPI) anchor protein, dihydroorotate dehydrogenase in pyrimidine synthesis, fungal mitochondrial respiration chain, siderophore iron transporter, Heat shock protein 90 (Hsp90), calcineurin, histone deacetylase (HDAC), inositol phosphorylceramide (IPC) synthase, chitin synthase, and sphingolipid pathway (**Table 7**). Nevertheless, more clinical trials are on the way for these agents before using them in the clinical practice against antifungal-resistant *Aspergillus*/fungal strains.

In addition, enzymes in the *Aspergillus* trehalose biosynthesis pathway, i.e., trehalose-6-phosphate synthase, trehalose-6-phosphate phosphatase, trehalase enzymes, were identified as important virulence factors, including proteins related to the trehalose pathway, i.e., *Af*SsdA, *Af*TslA [103, 105, 263, 264]. The trehalose pathway in *A. fumigatus* is associated with cell wall integrity and fungal virulence *in vivo* [103, 264, 265]. However, inhibitors of this pathway are still lacking and under-investigated. Validamycin A is one of the inhibitors of trehalase enzymes and was first demonstrated its strong antifungal activity against a plant fungal

Name.	Target	Mechanism	Advantage	Administration	Clinical trial
Classical targets					
Encochleated amphotericin B (CAmB) [188–192]	Ergosterol	Renovated structure of amphotericin B with cochleated lipid-crystal nanoparticles	Oral administration, broad-spectrum, less toxicity	Oral	Phase I
Rezafungin (CD101) [192–203]	1,3-β-D-glucan synthase (FKS)	1,3-β-D-glucan synthase inhibitor	Improved stability, long half-life (once a week), activity against *A. fumigatus, A. terreus, A. flavus,* and *A. niger*	Intravenous	Phase III
Ibrexafungerp (SCY-078) [204–210]	1,3-β-D-glucan synthase (FKS)	1,3-β-D-glucan synthase inhibitor	activity against *A. fumigatus, A. terreus, A. flavus,* and *A. niger,* including itraconazole-resistant *Aspergillus*	Oral and intravenous	Phase III
VT-1598 [211, 212]	Lanosterol demethylase (CYP51)	Tetrazole, inhibiting lanosterol demethylase	Less drug–drug interactions, long half-life, broad-spectrum: *Candida, Aspergillus*	Oral	Phase I
VT-1161 (oteseconazole) [213, 214]	Lanosterol demethylase (CYP51)	Tetrazole, inhibiting lanosterol demethylase	Less drug–drug interactions, long half-life: activity against azole-resistant *Candida,* onychomycosis	Oral	Phase III
VT-1129 (quilseconazole) [215–220]	Lanosterol demethylase (CYP51)	Tetrazole, inhibiting lanosterol demethylase	Less drug–drug interactions, long half-life, brain penetration, activity against *Cryptococcus, Candida*	Oral	Phase I
PC945 [221–227]	Lanosterol demethylase (CYP51)	Triazole, inhibiting lanosterol demethylase	Fungicidal, high lung exposure, activity against *A. fumigatus*	Inhalation	Phase II
Novel targets					
Fosmanogepix (APX001) [228–236]	Glycosylphosphatidylinositol (GPI) anchor protein synthesis (GWT1)	Inhibiting GPI	Fungal-specific target, broad-spectrum, activity against *A. fumigatus, A. terreus, A. flavus,* and *A. niger*	Intravenous and oral	Phase II
APX2096 [236]	Glycosylphosphatidylinositol (GPI) anchor protein synthesis (GWT1)	Inhibiting GPI	Strong activity against *Cryptococcus,* effective blood–brain barrier penetration	Intraperitoneal and oral	—
Olorofim (F901318) [237–239]	Dihydroorotate dehydrogenase in pyrimidine synthesis	Inhibiting pyrimidine synthesis	Activity against *A. fumigatus, A. terreus, A. flavus,* and *A. nidulans,* including azole-resistant *A. fumigatus*	Intravenous and oral	Phase III

Name.	Target	Mechanism	Advantage	Administration	Clinical trial
T-2307 [240–242]	Intracellular mitochondrial membrane respiration potential	Inhibiting mitochondrial respiration chain (arylamidine)	Uptaking more by fungal cells, fungicidal activity against *A. fumigatus, A. terreus, A. flavus, A. nidulans,* and *A. niger*	Subcutaneous	Phase I
VL-2397 (ASP2397) [243–245]	Unknown	Uptaking by siderophore iron transporter (Sit1)	Fungicidal, activity against *A. fumigatus, A. terreus, A. flavus,* and *A. niger*	Intravenous	Phase II
Geldanamycin [246–248]	Heat shock protein 90 (Hsp90)	Inhibiting Hsp90	Synergy to caspofungin	Intravenous	—
Tacrolimus (FK506) [249–251]	Calcineurin	Inhibiting calcineurin	Synergy to caspofungin, activity against *A. fumigatus*	Intravenous and oral	—
Cyclosporin A [249, 252]	Calcineurin	Inhibiting calcineurin	Activity against *A. fumigatus*	Intravenous, oral, and topical	—
FK506 analogs (9D31OD-FK506) [251]	Calcineurin	Inhibiting calcineurin	Synergy to azoles, decrease T-cell toxicity and host immunosuppression	Intravenous	—
Trichostatin A [253]	Histone deacetylase (HDAC)	Inhibiting HDAC	Synergy to caspofungin, activity against *A. fumigatus*	Intravenous	—
MGCD290 [254]	Histone deacetylase (HDAC)	Inhibiting HDAC	Synergy to caspofungin, azole, broad spectrum	Oral	Phase II
Aureobasidin A [255–258]	Inositol phosphorylceramide (IPC) synthase	Inhibiting IPC synthase	Synergy to caspofungin	Intravenous and oral	—
Nikkomycin [259]	Chitin synthase	Inhibiting chitin synthase	Broad-spectrum	Intravenous	—
BHBM D13 [260, 261]	Sphingolipid pathway	Acylhydrazone, inhibiting fungal sphingolipid glucosylceramide (GlcCer) synthesis	Broad-spectrum, specific to fungi, fungicidal, blood–brain barrier penetration, less toxicity	Intraperitoneal and oral	—

Table 7.
Summary of novel antifungal agents against classical targets and novel targets for Aspergillus infections.

pathogen, *Rhizoctonia solani* [266–269]. Furthermore, validamycin A has antifungal activity against *Candida albicans* and *Aspergillus flavus* [186, 270]. Validamycin A also possesses combinatorial effects with conventional amphotericin B against *A. flavus* [186]. Nevertheless, *in vivo* experiments are still necessary to verify an antifungal activity of validamycin A. Additionally, the high-osmolarity glycerol (HOG)-mitogen-activated protein kinase (MAPK) signaling pathway is associated with trehalose production and stress response in *A. fumigatus* [271–274]. This signaling pathway may be another good antifungal target to be developed in the future. Therefore, there are many more pathways involved with *Aspergillus* virulence, and there are so many unexplored areas in *Aspergillus* pathogenesis to develop novel antifungal candidates. With this knowledge, we could overcome the shortage of antifungal agents against many more antifungal-resistant *Aspergillus* strains to emerge very soon.

6. Conclusion

Aspergillus species are common fungi found everywhere around humans. They adapt and express many virulence factors to survive inside hosts and cause infections in immunocompromised hosts. Recently, new risk factors that cause severe invasive pulmonary aspergillosis are ICU patients with influenza infections or COVID-19 infections. The diagnosis of invasive aspergillosis, especially without proven tissue or culture evidence, is still challenging. New molecular methods, i.e., nucleic acid assays, lateral flow assays, are introduced for supporting the diagnosis of probable and possible invasive aspergillosis. Nevertheless, voriconazole and isavuconazole are the first-line therapy in IPA in ICU patients, patients with hematological malignancies, patients with IAPA, and CAPA. Furthermore, posaconazole is the principal antifungal agent for the prophylactic treatment of IPA in patients with hematological malignancies. Additionally, emerging azole-resistant *Aspergillus* strains are increasing, and the management against these azole-resistant *Aspergillus* strains is the combination therapy between azoles and echinocandins, including liposomal amphotericin B. Although novel antifungal agents against *Aspergillus* species are on their way, antimicrobial stewardship of existing antifungal agents is also crucial to prevent further breakthrough antifungal-resistant strains in the future. With our better understanding of *Aspergillus* pathogenesis, the shortage of antifungal agents against *Aspergillus* and its resistant strains would no longer be for the better lives of patients suffering from *Aspergillus* infections.

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Conflict of interest

The author declares no conflict of interest.

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