Human Fungal Pathogens and Drug Resistance Against Azole Drugs

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Abstract Pathogenic fungi causing severe infections in humans with immunocompromised immune system have been the major reasons of deaths in the world. *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* are among the most prevalent human fungal pathogens. The most widely used therapy used for the invasive fungal infections is the treatment with azole antifungal drugs; however, drug resistance against azole drugs is a major limitation in treatment of fungal infections. High-throughput techniques such as genomics and proteomics have been applied to understand the molecular mechanisms involved in drug resistance against azole drugs in human pathogenic fungi. These studies could be useful to prevent the increase in drug resistance and better response to antifungals. Here, we focus on the incidences of drug resistance against azole antifungal drugs in human fungal pathogens, molecular mechanisms of drug resistance, and new strategies for combating drug resistance to improve clinical treatment of invasive fungal infections.

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1 Introduction

Opportunistic fungal infections are among the most difficult diseases to manage in patients. These infections frequently endanger the success of cancer treatments, transplant and surgery complications, autoimmune disease therapies, and also intensive care. In spite of several antifungal drugs and prophylaxis available, there is an increase in reported invasive fungal infections (IFIs). Worldwide mortality due to candidiasis, by *Candida albicans* (*C. albicans*) and related species, was 46–75 %; aspergillosis, by *Aspergillus fumigatus* (*A. fumigatus*) and related species, was 30–95 %; and mortality rate of cryptococcosis mainly caused by *Cryptococcus neoformans* (*C. neoformans*) worldwide was 20–70 % (d'Enfert 2009).

Fungal infections are established by fungi that are ubiquitous in nature and routinely inhaled by us in daily life. However, only individuals with compromised or diminished immunity are susceptible to invasive mycoses. Pathogenic fungi mainly *C. albicans*, *A. fumigatus*, and *C. neoformans* are leading pathogens among IFIs in humans. *C. albicans* is common microflora in human that may cause systemic infections in individuals with immunocompromised immune system leading to high mortality rates approaching up to 40 % (d'Enfert 2009). Other *Candida* species colonizes specific locations in the hosts, mainly in the gastrointestinal tract, genital tract, and the skin, whereas *C. glabrata* and *C. parapsilosis* also cause IFIs. Opportunistic pathogens, such as *A. fumigatus* and *C. neoformans*, are ubiquitously present in the soil. Normally host innate immunity, especially alveolar macrophages, manages these fungal spores, yeasts, or mycelial fragments. However, inhaled fungal spores can survive for extended period in macrophages and can establish infection when host immunity is weakened. The common causative agent of invasive aspergillosis is the filamentous mold *A. fumigatus*, with mortality rates up to 40–90 % (Dagenais and Keller 2009). The other opportunistic fungal pathogen known to cause infections in immunocompromised individuals is *C. neoformans* that leads to complications in central nervous system (CNS) such as cryptococcal meningitis among acquired immune deficiency syndrome (AIDS) patients (Sloan and Parris 2014). *Pneumocystis* spp. among other pathogenic fungi causes pneumonia in immunocompromised hosts.

These fungi have developed molecular mechanisms to combat the defense system in immunocompromised hosts. Detailed understanding of the complex interactions between genetic variations and its contributions to the disease phenotype is lacking and essential to study. Insight into host-pathogen interactions with respect to host genetic susceptibility can improve the identification of novel therapeutic targets and the design of better antifungal prophylaxis strategies.

It is a challenge to develop new techniques for early diagnosis of IFIs and effective treatment considering the increased burden of the disease and death in patients with their vulnerable immune status (Panackal et al. 2006). Diagnosis is extremely challenging, due to lack of sensitive or specific diagnostic methods, and results from currently used test are often available too late to be clinically useful.

Considering the population at risk for fungal infections and the presence of wide range of fungal pathogens in the environment niche, these opportunistic fungi present significant challenge for diagnostics and therapeutics. In comparison to the bacterial antibiotics, few antifungal drugs have been reported with minimum risk of side effects and the occurrence of resistance.

Resistance to first-line drugs in most of the pathogenic fungi causing invasive infections ranges from 0 to almost 100 %. Though the evaluation of the overall impact of drug resistance in health is difficult, it is quite clear that morbidity and mortality due to resistant pathogens have increased in view less effective treatment [WHO global strategy for containment of antimicrobial resistance, 2001]. Resistance to various antifungal drugs, azoles in particular, has been reported in pathogenic fungi (Warris et al. 2002). Rates of resistance to widely used azole and fluconazole (FLC) have been reported between 10 % and 25 % in invasive candidiasis. Cross-resistance to other azole agents like voriconazole and itraconazole has also been reported among some of these FLC-resistant isolates (Cuenca-Estrella et al. 2006; Arendrup et al. 2013). Resistance rate to triazoles in *Aspergillus* spp. is less common and assumed to be below 5 % in most of countries (Snelders et al. 2008; Alastruey-Izquierdo et al. 2013). However, resistance in *A. fumigatus* seems to increase majorly due to evolution of resistant isolates in response to azole fungicides that is being widely used in Europe for crop protection (Snelders et al. 2008). Several factors have been reported to be associated to drug resistance against azoles in pathogenic fungi including upregulation of functional genes controlling drug efflux, alterations in sterol synthesis, mitochondrial dysfunctioning, decreased affinity for the cellular target and chromosomal abnormalities, and high-osmolarity glycerol (HOG) pathway. The major factor for resistance to azoles involves alteration in target enzyme, 14α-demethylase, and specific drug efflux pumps in terms of the quantity or quality (Ghannoum and Rice 1999; Nascimento et al. 2003), mainly two classes of efflux transporters, class ATP-binding cassette (ABC) and major facilitator superfamily (MFS) (Slaven et al. 2002; da Silva Ferreira et al. 2006).

Here, we have focused on incidences of drug resistance and molecular mechanism in three of the major human pathogenic fungi, *Candida*, *Aspergillus*, and *Cryptococcus*, against azole drugs.

2 Pathogenic Fungi and Invasive Fungal Infections

Pathogenic fungal infections are categorized as primary or opportunistic. Primary infections may develop in immunocompetent hosts, while opportunistic fungal infections are frequent in “high-risk” populations of immunocompromised individuals undergoing treatment for cancer, organ transplant cases, and autoimmune disease; in patients who are at risk of infection after prosthetic surgery or under the treatment of broad-spectrum antibiotics leading to changes in the normal flora; and in patients with HIV with immune deficiency (Nanjappa and Klein 2014). Opportunistic fungal infections could be superficial or systemic. IFIs have

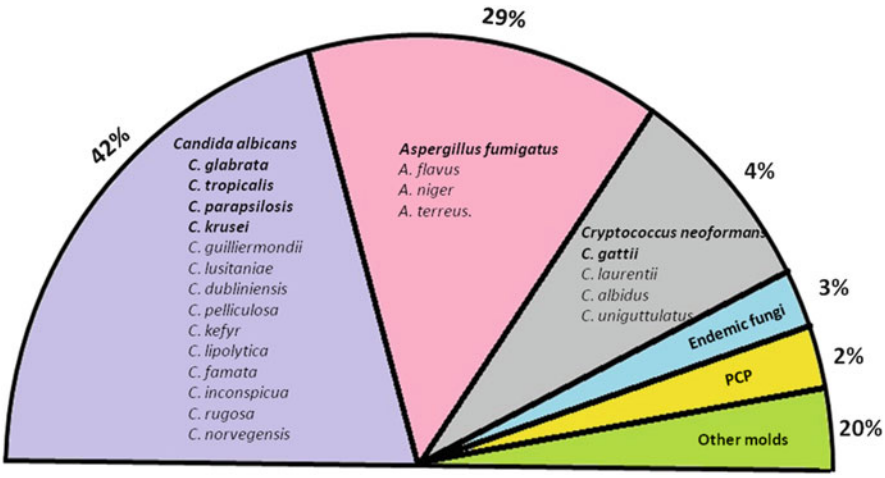

Fig. 1 Pathogenic fungi causing invasive fungal infections among transplant recipients. *Candida* spp. predominates among pathogenic fungi followed by *Aspergillus* spp. and *Cryptococcus* spp. as reported by Pfaller et al. (2009)

increased in the last two decades with increased use of steroids. Opportunistic fungal pathogens include *Candida*, *Aspergillus*, and *Cryptococcus* species. Figure 1 shows pathogenic fungi belonging to these genus and percentage of infection caused by these fungi.

2.1 Candida *spp.*

Candida species are *Ascomycota* group of fungi of the order Saccharomycotina, normally commensal organisms in mucous membranes, mainly gastrointestinal tract. There are a total of 150 *Candida* spp., and only 15 of them are commonly associated with humans as colonizers or opportunistic pathogens (Kam and Xu 2002). Of these, *Candida albicans* is the frequently isolated human commensal and pathogen (Krcmery and Barnes 2002). *C. albicans*, *Candida tropicalis*, *and Candida glabrata* are more virulent species, while *Candida parapsilosis* and *Candida krusei* are less virulent spp. Infections due to *C. parapsilosis* are more prevalent among children, and *C. glabrata* are frequently encountered among older adults (Yapar 2014; Kullberg and Arendrup 2015). These species have the differences in virulence as well as susceptibility to the azoles and the echinocandin drugs.

C. albicans is a dimorphic fungus and has ability to transform into different morphologies such as yeast, hyphae, and pseudohyphae upon perception of environmental signals (van der Meer et al. 2010). Most healthy individuals carry *C. albicans*, which harmlessly colonizes mucous membranes in different anatomical sites. *Candida* spp. are predominant constituents of the vaginal microflora

(Seed 2014). However, it is an efficient invasive pathogen for establishing infections in individuals with ineffective adaptive cellular immunity and in patients lacking neutrophils. *Candida* spp. cause invasive candidiasis which is mostly reported among immunocompromised patients. Candidemia, also known as blood-borne systemic candidiasis, generally develops in neutropenic patients or in patients exposed to contaminated indwelling catheters during surgery (Yapar 2014). For this, *Candida* is majorly the cause of Candidemia associated with healthcare in the USA (Magill et al. 2014). Despite of proper antifungal therapy being given to the patients, mortality rate has still been reported as high as 40 %. Non-albicans *Candida* species is also gaining attentions as it is emerging resistance to antifungal drugs (Kullberg and Arendrup 2015).

C. albicans infections are usually prevented by our defense system by mucosal tissues and the peripheral circulation. Polymorphic nuclear leukocytes (PMNL) are the first-line defense against blood-borne *Candida* infections. Neutrophil defects, decrease in neutrophil counts, and dysregulation in Th-cell reactivity are main risk factors which contribute to severe *Candida* infections (Romani 2004; Netea et al. 2004). *C. albicans* induces immunosuppression and leads to production of CD4+CD25+ T-regulatory cells. Further, the presence of specific antibodies also protects against fungal infections (Polonelli et al. 2000).

2.2 Aspergillus *spp.*

The *Aspergillus* species are *Ascomycota* group of fungi of the order *Eurotiales*, which grow on high osmotic concentrations and on carbon-rich sources. *Aspergillus* has 339 identified species (Samson et al. 2014); however, only few of them, namely, *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*, have been considered to be pathogenic to humans. *A. fumigatus* is the common etiological agent of human aspergillosis accounted for ∼90 % of cases; *A. flavus*, *A. niger*, and *A. terreus* are secondary agents. *Aspergillus* species causes extensive spectrum of diseases with clinical manifestations that ranges from colonization of fungi in the organs leading to asthma, allergic bronchopulmonary aspergillosis (ABPA), and invasive aspergillosis (IA). The major site of *Aspergillus* infection is the lung (Ellis et al. 2009). *A. fumigatus* is a ubiquitous, saprophytic mold that releases airborne conidia which are inhaled by humans everyday (Latge 1999). *A. fumigatus* causes IA which primarily occurs in transplant recipients and hematological malignancy patients. The smaller (∼2 μm) conidial size of *A. fumigatus* gives the fungus advantage to remain airborne for long periods and enter human alveoli. Conidia are coated with hydrophobic proteins and with the chemoprotectant melanin (Pihet et al. 2009) to withstand harsh environment in air and in vivo. On the onset of their germination, hyphae are recognized by innate immune cells in paranasal sinus or the lung. The airway mucus serves as a physical, chemical, and biological barrier secreting fluids that contain glycoproteins, proteoglycans, lipids, etc. and lead to clearance of fungal conidia (McCormack and Whitsett 2002).

A. fumigatus also produces various metabolites, e.g., gliotoxin, fumagillin, and helvolic acid which damages epithelium and can have inhibitory effects on ciliary movement (Amitani et al. 1995). Host proteins such as pattern recognition receptors (PRRs), lung surfactant proteins A and D (SP-A and SP-D), mannan-binding lectin (MBL), and toll-like receptors (TLRs) (Walsh et al. 2005; Johnson et al. 2014) are significant for the host defense against *Aspergillus*, and any alterations in these molecules may affect susceptibility to *Aspergillus* infections in the individuals (Johnson et al. 2014).

2.3 Cryptococcus *and Other Pathogenic Fungi*

Cryptococcus causes fatal infections in patients with weak immune status such as those associated with T-lymphocyte deficiency, common in AIDS patients. These infections are frequently reported in patients with stem cell recipients and malignancy, though 15–40 % of *Cryptococcus* cases are reported in HIV-negative patients (Speed and Dunt 1995). Glucuronoxylomannan (GXM) capsule of *Cryptococcus* is a well-known virulence factor that suppresses the host inflammatory response and prevents phagocytosis of the fungus (Speed and Dunt 1995).

The use of antifungal prophylaxis has become more prevalent for common and also uncommon fungal pathogens thus complicating clinical management. The other fungal pathogens include *Fusarium* spp., zygomycete, and the opportunistic yeastlike fungi such as *Histoplasma capsulatum* and *Pneumocystis* spp.; *Fusarium* species causes onychomycosis and fungal keratitis. *Fusarium* infections in the lungs majorly cause allergic bronchopulmonary fusariosis and hypersensitivity pneumonitis. Although radiological indications for fusariosis is similar to invasive aspergillosis, frequent occurrence of disseminated nodular skin lesions and blood culture positivity is the classical marker of *Fusarium* infection which is to differentiate it from aspergillosis (Nucci et al. 2015). Members of *Fusarium* genus may affect humans and cause mycotoxicosis by ingestion of toxin-contaminated food (Bennett and Klich 2003). Mucormycosis is another fungal infections usually found in spreading pneumonia due to invasion in blood vessels (Saxena et al. 2015).

3 Current Diagnosis and Clinical Treatment

3.1 Diagnosis

Early diagnosis of the infection along with species differentiation are of great importance for improved treatment of IFIs. Standard criteria for systemic fungal infections are histopathologic examination for the presence of fungus in the tissue or culture and isolation of etiologic agent from clinical sterile specimens (blood,

sputum, urine, cerebrospinal fluid, or tissue biopsy). Radiological tests are used for diagnosis of patients where invasive procedures for sample collection are very difficult and risky due to low immune status. Pulmonary lesions or nodules, infiltration, and halo signs are indicative of pulmonary fungal infections such as aspergillosis, fusariosis, scedosporiosis, or zygomycosis (Greene et al. 2007; Godoy et al. 2012). Among non-culture methods, serological tests are considered as standard practice for the diagnosis of fungal infections. Commercially available ELISA kits are available for *Candida* and *Aspergillus* antigens which detect mannan and galactomannan, respectively, and demonstrate good specificity but variable sensitivity (Musher et al. 2004). Detection of fungal DNA signatures in body fluids using polymerase chain reaction (PCR) assay in combination with serological test in high-risk patients is also viable diagnostic option. Advances in the qualitative methods, such as panfungal PCR, for fungal DNA in human blood samples, tissues, bronchoalveolar lavage, and other body fluids, are now in clinical practice as reliable test for fungal infections (Orsi et al. 2015).

3.2 Treatment

Standard antifungal drugs in use are polyenes, azoles, and echinocandins. Amphotericin B (AMB) deoxycholate, AMB-D and lipid formulations of AMB, and L-AMB are polyene drugs in use. Fluconazole and voriconazole are mainline azole compounds in use. Caspofungin, micafungin, and anidulafungin are recommended antifungal echinocandins. Fluconazole remains the choice of drug for invasive candidiasis, while for *Candida* species known to be susceptible to fluconazole, other compounds like echinocandins and AMB or AMB-D are recommended (Pappas et al. 2009a). Voriconazole is recommended for the primary treatment of invasive aspergillosis in most patients including infections resistant to AMB (also with *Aspergillus terreus* and *Aspergillus nidulans*) (Walsh et al. 2008). Recommended antifungal therapy for major fungal infections in adults is discussed in Table 1.

4 Azoles and Other Antifungal Drugs

The use of potassium iodide (KI) for treating sporotrichosis was the first successful chemotherapy in 1903. After this, nystatin, the polyene compound, was successfully used as antifungal followed by amphotericin B in 1956, which is still the standard antifungal drug to evaluate new systemic antifungals (Al-Mohsen and Hughes 1998). The antifungal drugs include the natural products (polyenes, griseofulvin, and echinocandins) and the synthetic chemicals (azoles, allylamines, flucytosine, and phenylmorpholines). The available antifungal drugs are broadly

Table 1 Summary of recommendations for the treatment of major IFIs in adult

Fungal infection	Primary antifungal therapy	Alternative antifungal therapy	References
Invasive pulmonary aspergillosis, extrapulmonary aspergillosis, *Aspergillus* infections of the heart, eyes, or cutaneous	Voriconazole IV or orally	L-AMB, ABLC, caspofungin, micafungin, posaconazole, itraconazole	Walsh et al. (2008)
Allergic bronchopulmonary aspergillosis	Itraconazole	Oral voriconazole or posaconazole	
Candidemia, nonneutropenic adults	Fluconazole or an echinocandin	L-AMB or AMB or voriconazole	Pappas et al. (2009a)
Candidemia, neutropenic patients	Echinocandin or L-AMB	Fluconazole or voriconazole	
CNS and disseminated cryptococcosis	Induction therapy, AMB-D and 5-FC (B); consolidation therapy, fluconazole; suppressive therapy, fluconazole	Induction therapy, AMB-D Third line, 5-FC plus fluconazole	Thursky et al. (2008)
Mucormycosis	L-AMB	Posaconazole	Chang et al. (2014)
Fusarium species infections	Voriconazole or L-AMB	Posaconazole	
Scedosporium	Commence voriconazole with terbinafine	–	
Rare and emerging fungal infections such as *Paecilomyces* spp., *Phaeohyphomycosis* spp.	Voriconazole, posaconazole, and itraconazole	–	

AMB amphotericin B, *L-AMB* liposomal amphotericin B, *AMB-D* amphotericin B deoxycholate, *5-FC* flucytosine

classified into azoles, polyenes, nucleoside analogues, and echinocandins based on their mechanism of action.

4.1 Azoles

Azole antifungal agents are now considered to be important for therapeutics of systemic fungal infections. Fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), posaconazole (POS), and ketoconazole (KTC) are some of the most widely used antifungal agents (Gallagher et al. 2003). Triazole drugs are more effective against many fungal pathogens with no severe nephrotoxic effects in comparison to AMB. Among azoles, FLC remains an effective and low-cost drug for the treatment of candidiasis and cryptococcosis. It is the first line of drug used for *Candida* (except *Candida krusei* and *glabrata*) and cryptococcal infections including

non-meningeal coccidioidal infections. This drug is safe; however, it is not effective against filamentous fungi. VRC has been effective for the treatment of aspergillosis; however, its use is limited by significant drug interactions. It is effective against most of the *Candida* and *Aspergillus* spp. ITC is effective in vitro and in vivo against *A. fumigatus*, *Candida* spp., *C. neoformans*, and the dimorphic fungi. Finally, POS is the latest addition to the azole drugs and is effective against the zygomycetes. POS is effective against most *Candida* and *Aspergillus* species in vitro. It is also active against *C. neoformans* and *Fusarium* spp. (Zonios and Bennett 2008). KTC was the azole that was available for oral usage and was found with constant levels in blood; however, its use is limited due to hepatotoxicity and resistance reported in patients with candidiasis or AIDS and esophageal and oropharyngeal candidiasis.

Azoles inhibit fungal cytochrome P_{450} demethylase (an enzyme encoded by *CYP51* or ERG11 gene) which converts lanosterol to ergosterol. This leads to reduced amount of ergosterol in the cell membrane of the fungus (Andriole and Bodey 1994; Georgopapadakou and Walsh 1996; Andriole 1998, 1999). In order to understand the molecular targets of azole drugs, various high-throughput studies have been carried out. Altered expression of *Saccharomyces cerevisiae* with different classes of antifungal compounds (KTC, amphotericin B, caspofungin, and 5-fluorocytosine) was carried out to identify altered gene expression specific to each drug. Exposure of KTC led to altered levels of genes involved in ergosterol biosynthesis pathway as well as sterol uptake, while exposure to caspofungin led to altered levels of genes belonging to cell wall integrity, and exposure to 5-FC led to altered levels of genes involved in DNA damage repair, DNA synthesis, protein synthesis, and regulation of cell cycle. On the other hand, exposure to AMB led to altered levels of genes involved in cell stress, membrane reorganization, cell wall integrity, and transport (Agarwal et al. 2003). In order to understand the molecular targets of antifungal azoles, VRC, and ITC, high-throughput techniques have been used. Using microarray hybridization, 2271 genes were found as differentially expressed in wild-type strain which describes decrease in biosynthesis of genes involved in ergosterol biosynthesis and increased mRNA level of gene-encoding transporters (da Silva Ferreira et al. 2006). Proteomic profiling of *A. fumigatus* on exposure to ITC (ITC) using 2-DE followed by mass spectrometric analysis led to identification of 54 differentially expressed proteins including proteins related to cell stress, carbohydrate metabolism, and amino acid metabolism.

4.2 Other Antifungal Drugs

The polyene antifungal agents are fungicidal with large spectrum of antifungal activity than any other antifungal agents. Amphotericin B, the polyene compound, has been proposed to interact with ergosterol that leads to the production of aqueous pores (Holz 1974). These pores result in dysregulated permeability and leakage of necessary cytoplasmic components leading to the killing of the organism (Kerridge

1980; Kerridge 1985). Intravenous amphotericin B has been recommended for severe and for invasive aspergillosis, *Candida* infections of CNS, blastomycosis, coccidioidomycosis, and mucormycosis (Andriole and Kravetz 1962; Andriole and Bodey 1994; Andriole 1998, 1999). Liposomal amphotericin B has reduced nephrotoxicity than conventional AMB (Hiemenz and Walsh 1996; Groll et al. 1998).

Flucytosine 5-FC is a fluorinated pyrimidine deregulating RNA and protein synthesis in the fungal cell mainly targeting pyrimidine metabolism (Andriole 1998, 1999; Groll et al. 1998). Flucytosine activity in vitro has been reported against *Aspergillus* spp., *Candida* spp., *C. glabrata*, and *C. neoformans*. However, if treated with it alone, it is less effective and has increased chances of developing fungal resistance in *Candida* and cryptococcal organisms. Echinocandins are cyclic lipopeptide agents with fungicidal activity through inhibition of cell wall enzyme, 1,3-β-D-glucan synthase, not expressed in mammalian cells, and hence form a potential antifungal target (Georgopapadakou and Walsh 1996; Groll et al. 1998). The investigational compound, LY 303366, an inhibitor of β-1, 3-beta-D-glucan synthase, is found to be active against *Candida* and *Aspergillus* organisms (Andriole 1998, 1999).

5 Drug Resistance to Azoles in Human Fungal Pathogens

Antifungal drug resistance is among the major causes of therapeutic failure in invasive fungal infections other than low bioavailability of the antifungal drug, weakened immune function, or a higher metabolism of the drug. Primary resistance occurs in organisms which are not at all exposed to the specific drug, while secondary or acquired resistance is due to exposure of an organism to the drug. Clinical resistance is reversion of infection due to therapeutic failure in an organism and is not linked to in vitro resistance (Rex et al. 1997).

For effective treatment of IFI, we need to be familiar with the susceptibility of the resistant fungal isolates to the antifungal drugs. The Clinical Laboratory Standard Institute (CLSI) in the USA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe have recommended protocols for antifungal susceptibility testing. The minimum inhibitory concentration (MIC) of the drug is calculated as the threshold levels of drugs leading to in vitro growth inhibition. The CLSI has recommended antifungal MIC breakpoints to separate susceptible and resistant population for azoles and echinocandins by analyzing the in vitro susceptibility data, in vitro outcome, and pharmacokinetics/pharmacodynamic studies, while EUCAST has defined the breakpoint derived from MIC as the epidemiological cutoff value (ECV) to avoid confusion with clinical breakpoints. ECVs are the MIC values that capture >95 % of the observed population. In this chapter, we discuss antifungal drug susceptibility for three major pathogenic fungal spp. against azole drugs (Fig. 2).

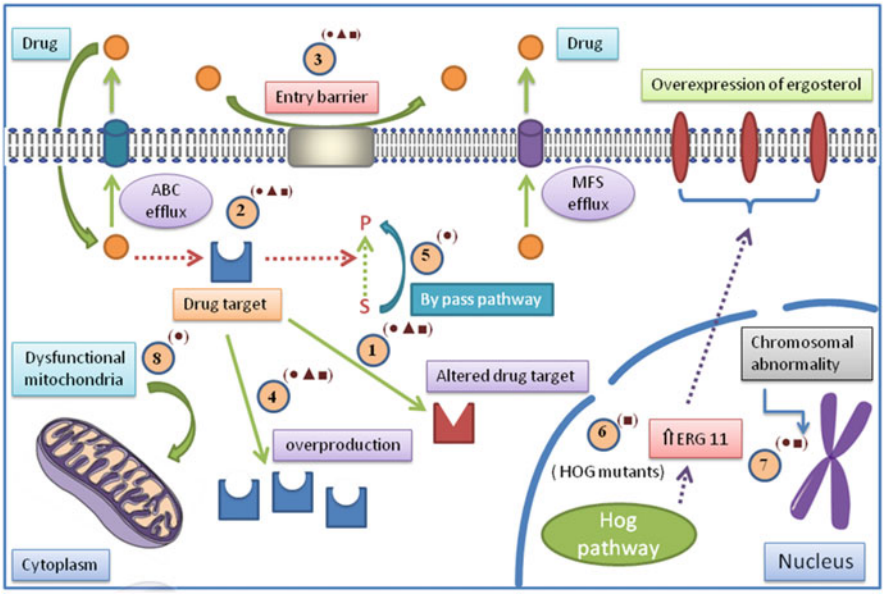

Fig. 2 Molecular mechanisms of drug resistance to azoles in human pathogenic fungi. Various mechanisms involved in fungal drug resistance are numbered from 1 to 8. Pathogenic fungi are shown with sign *closed circle* (*Candida* spp.), *closed triangle* (*Aspergillus* spp.), and closed square (*Cryptococcus* spp.). (1) Mutations in the target enzyme (lanosterol 14α-demethylase) result in the complete inhibition of the binding of the azole drug to its target. (2) The two different drug efflux systems in fungi, i.e., the ATP-binding cassette (ABC) superfamily and the major facilitator superfamily (MFS), contribute to azole drug resistance by pumping out the azole drugs out of the fungal cell leading to their less accumulation. (3) Modifications in the composition of plasma membrane affect the membrane asymmetry which alters the uptake of the drug. (4) Increased levels of target enzyme (lanosterol 14α-demethylase) may overwhelm the drug entering the cell, thereby resulting in increased level of resistance. Overproduction of the enzyme also results in cross-resistance between azoles. (5) The cell has a bypass pathway that balances for the loss-of-function inhibition due to the drug activity. (6) The HOG pathway negatively regulates the expression of ergosterol biosynthetic genes. Thus HOG mutants exhibit a decreased sensitivity toward azoles. (7) Chromosomal abnormalities have been linked with azole resistance in *Candida* and *Cryptococcus* species. *Candida* spp. acquire azole resistance by increasing the copy number of ERG11 (disomy) present on chromosome 5, whereas in *Cryptococcus* azole resistance is associated with chromosomes 1 and 4. (8) Loss of mitochondrial genome and changes in mitochondria membrane structures lead to the potential activation of drug resistance pathway in *Candida* spp.

5.1 Candida *spp.*

In vitro antifungal drug susceptibility of *Candida* spp. against different azole drugs (FLC, ITC, VRC, KTC) has been carried out each year for an update on the trends on resistant clinical isolates. Table 2 provides the ECV/MIC data compiled from various studies carried out in the last 2 years for five major *Candida* species. The comparative analysis of resistant isolates for four azole drugs ITC, FLC, VRC, and

Table 2 Antifungal drug susceptibility against azole drugs in *Candida* clinical isolates revealing drug resistant isolates

Source	Species/Total no. of isolates tested	Fluconazole resistant isolates (%)	Itraconazole resistant isolates (%)	Voriconazole resistant isolates (%)	Ketoconazole resistant isolates (%)	Method	References
NA	*C. albicans* *FLC (1196); VRC (593)*	**68 (5.7 %)**	–	27 (4.6%)	–	BM	Fothergill et al. (2014)
VVI	*C. albicans* *FLC (93; ITC (93); KTC (93)*	**83 (89.24 %)**	**8 (8.6 %)**	–	**23 (24.7 %)**	DD	Al-mamari et al. (2014)
BSI	*C. albicans* *FLC (183); VRC (183)*	0/183	–	0/183	–	BM	Won et al. (2015)
S,B,U	*C. albicans* *FLC (625); ITC (625)* *VRC (625)*	27/625 (4.3%)	8/625 (1.3%)	13/625 (2.1%)	–	BM	Zhang et al. (2015)
NA	*C. glabrata* *FLC (882); VRC (522)*	**70/882 (7.9 %)**	–	**96/522 (18.4 %)**	–	BM	Fothergill et al. (2014)
VVI	*C. glabrata* *FLC (18); ITC (18)* *KTC (18)*	**17/18 (94.4 %)**	0/18	–	**3/18 (16.6 %)**	DD	Al-mamari et al. (2014)
BSI	*C. glabrata* *FLC (58); VRC (58)*	6/58 (10.3%)	–	0/58	–	BM	Won et al. (2015)
S,B,U	*C. glabrata* *FLC (193); ITC (193)* *VRC (193)*	12/193 (6.2%)	4/193 (2.1%)	9/193 (4.7%)	–	BM	Zhang et al. (2015)
NA	*C. tropicalis* *FLC (327); VRC (205)*	32/327 (9.9%)	–	**36/205 (17.6 %)**	–	BM	Fothergill et al. (2014)

VVI	*C. tropicalis* *FLC (24); ITC (24)* *KTC (24)*	4/24 (17%)	0/24	–	1/24 (4.2%)	DD	Al-mamari et al. (2014)
BSI	*C. tropicalis* *FLC (82); VRC (82)*	2/82 (2.44%)	–	0/82	–	BM	Won et al. (2015)
S,B,U	*C. tropicalis* *FLC (84); ITC (84)* *VRC (84)*	**9/84 (10.7%)**	**4/84 (4.8%)**	6/84 (7.1%)	–	BM	Zhang et al. (2015)
NA	*C. krusei* *FLC (NA); VRC (98)*	NA	–	7/98 (12.2%)	–	BM	Fothergill et al. (2014)
VVI	*C. krusei* *FLC (6); ITC (6)* *KTC (6)*	**5/6 (83.3%)**	0/6	–	0/6	DD	Al-mamari et al. (2014)
BSI	*C. krusei* *FLC (4); VRC (4)*	**4/4 (100%)**	–	0/4	–	BM	Won et al. (2015)
S,B,U	*C. krusei* *FLC (29); ITC (29)* *VRC (29)*	22/29 (75.9%)	1/29 (3.4%)	2/29 (6.9%)	–	BM	Zhang et al. (2015)
NA	*C. parapsilosis* *FLC (497); VRC (298)*	11/497 (2.2%)	–	6/298 (2%)	–	BM	Fothergill et al. (2014)
BSI	*C. parapsilosis* *FLC (101); VRC (101)*	1/101 (0.99%)	–	0/101	–	BM	Won et al. (2015)
S,B,U	*C. parapsilosis* *FLC (13); ITC (13)* *VRC (13)*	**2/13 (15.4%)**	0/13	0/13	–	BM	Zhang et al. (2015)

BM broth microdilution; *DD* disk diffusion; *ECV* epidemiologic cutoff value for susceptible, susceptible dose-dependent and resistant isolates; *MIC* minimum inhibitory concentration at which more than 50% of the total isolates are inhibited in growth; *VVI* vulvovaginal isolates; *BSI* bloodstream infection isolates; *S*, *B*, *U* sputum, blood, urine; *NA* not available; (–) experiment was not performed for the corresponding azole and ECV/MIC values were not available

KTC showed maximum number of resistant isolates to FLC in *C. albicans*, i.e., 68 isolates (5.7 %) and 83 isolates (89.24 %); *C. glabrata*, 70 isolates (7.9 %) and 17 isolates (94.4 %); to VRC in *C. glabrata*, i.e., 96 isolates (18.4 %); *C. tropicalis*, 36 isolates (17.6 %); to ITC in *C. albicans* i.e. 8 isolates (8.6 %); *C. tropicalis* i.e. 4 isolates (4.8 %); to KTC in *C. albicans* i.e. 23 isolates (24.7 %); *C. glabrata i.e.* 3 isolates (16.6 %) (Fothergill et al. 2014; Al-mamari et al. 2014; Zhang et al. 2015; Won et al. 2015; 83 isolates (Al-mamari et al. 2014); *C. glabrata*, 111 isolates (Fothergill et al. 2014); to VRC in *C. albicans*, i.e., 82 isolates (Fothergill et al. 2014); *C. glabrata*, 258 isolates (Fothergill et al. 2014); and *C. tropicalis*, 94 isolates (Fothergill et al. 2014) (refer Table 2). The trend of increasing resistance of FLC leads to therapeutic failure and raises the need of new effective azoles for treatment. Reports of low resistance rates in VRC suggest that VRC may be used with confidence for the treatment of candidiasis (98 % success rates of VRC and 95 % in case of FLC) against FLC-resistant isolates (Ally et al. 2001).

There are several reports suggesting correlation of resistant isolates against different azoles and mutations in ERG11 gene in *Candida* spp. ERG11 (CnCYP51) gene encodes lanosterol 14α-demethylase enzyme which appears to be important target for azole antifungal drug. In a detailed study, ERG11 genes from 17 clinical isolates of *C. albicans* were analyzed for FLC resistance. These strains were observed to have 27 point mutations in ERG11 gene. Out of these, five mutations mainly Y132H, A114S, Y257H, G464S, and F72S substitutions were most prevalent in resistance spp., while two novel substitutions, T285A and S457P, in hotspot regions were relevant (Wang et al. 2015a). In the clinical isolates of *C. albicans*, the correlation between naturally occurring mutations in a gene for multidrug resistance regulator 2 (MRR2) and FLC resistance was analyzed. In a group of 20 FLC-resistant *C. albicans* isolates, 12 isolates showed overexpression of *Candida* drug resistance 1 (CDR1) gene. Of these, only three FLC-resistant isolates showed 11 identical missense mutations in MRR2 gene, 6 of which were among azole-resistant isolates. In addition, the role of MRR2 mutations in CDR1 overexpression and thus to FLC resistance was verified using recombinant strains with mutated MRR2 gene (Wang et al. 2015b). Hence, increasing rates of azole resistance in these species emphasize on the development of antifungal strategies with greater efficacy toward azole class of antifungals.

5.2 Aspergillus *spp.*

Appearance of azole resistance in strains of *A. fumigatus* has become a serious public health problem. Antifungal drug susceptibility in *Aspergillus* against different azoles has been performed by several groups. We looked for the data on antifungal drug susceptibility of *Aspergillus* spp. against different azole drugs from major studies in the last 10 years in literature. The comparative analysis of resistant isolates for three azole drugs in *A. fumigatus*, *A. flavus*, and *A. niger*

showed maximum number of resistant isolates to VRC in *A. fumigatus* [3.1 % (Espinel-Ingroff et al. 2010), 0.8 % (Pfaller et al. 2009), and 2.2 % (Rodriguez-Tudela et al. 2008)], to ITC in *A. flavus* (5.6 %), and to POS in *A. niger* (8.8 %) (Espinel-Ingroff et al. 2010) (see Table 3). The data suggest increased resistance to VRC, first line of therapy for invasive aspergillosis, in *A. fumigatus* (Mikulska et al. 2012). This is in line with the observation that VRC treatment is failing due to resistant isolates in the patients as mentioned earlier (Verweij et al. 2007).

There are several reports suggesting correlation of resistant isolates against different azoles and mutations in CYP51A1 gene in *A. fumigatus* (Denning et al. 1997). In a study (Bader et al. 2013), 527 clinical isolates of *A. fumigatus* were analyzed and found that 17 (3.2 %) strains showed increased MIC_0 values. Out of these 17 isolates, 14 were found to have resistance to ITC ($MIC_0 > 32$ mg/l) and 1 was highly resistant to *POS* [$MIC_0 > 32$ mg/l]. All the resistant isolates showed mutations in CYP51A1, and most common mutation was TR/L98H. Recently, ITC resistance also has been found to be increased due to TR/L98H mutations in CYP51A1 (Snelders et al. 2008). In another study, 38 clinical isolates were analyzed, where 3 isolates showed multi-azole resistance to ITC, VRC, and *POS*. It was found that all three isolates also showed exclusively TR_{34}/L98H mutations in *CYP51A1* gene, not present in the remaining 35 azole-susceptible isolates (Wu et al. 2015).

5.3 Cryptococcus *spp.*

The antifungal drug susceptibility for azoles in *C. neoformans* and *C. gatti* is provided in Table 4. Molecular typing for *Cryptococcus* spp. done using molecular methodologies like AFLP and PCR fingerprinting identified eight molecular types of *Cryptococcus*. An in vitro antifungal susceptibility test was done for the abovementioned molecular types of *Cryptococcus* species using the CLSI broth dilution method (Espinel-Ingroff et al. 2012). In *C. neoformans* strains with high FLC MICs ($\geq$32 μg/ml), it was observed that a point mutation (involving the glycine to serine substitution at 484th position) in the ERG11 (CnCYP51) gene resulted in an alteration in the binding site of the target enzyme (Rodero et al. 2003). Earlier work done by Sanguinetti et al. showed a reduction in concentration of FLC in *C. neoformans* due to overexpression of the gene *C. neoformans* Antifungal Resistance 1 (Cn*AFR1*) which codes for an ABC transporter, a membrane efflux pump (Sanguinetti et al. 2006).

Cross-resistance among pathogenic fungi against azoles is expected as their target of action is similar, but azole cross-resistance is rarely seen among the *Cryptococcal* strains. In *C. neoformans*, cross-resistance between ITC and FLC is not observed due to the dual targets of ITC (the lanosterol 14α-demethylase and the 3-ketosteroid reductase). In *C. neoformans* isolates, the cross-resistance is also reported between FLC (MICs >64 μg/ml) and VRC (MICs $\geq$ 2 μg/ml), but no cross-resistance was seen between FLC and ITC. The fluconazole-voriconazole

Table 3 Antifungal drug susceptibility against azole drugs in *Aspergillus* clinical isolates revealing resistant isolates

Aspergillus spp./Total number of isolates tested	Itraconazole		Posaconazole		Voriconazole		Method	References
	WT (%)	NWT (%)	WT (%)	NWT (%)	WT (%)	NWT (%)		
A. fumigatus *ITC (2554)* *POS (1647)* *VRC (2778)*	2488 (98.8)	68 (2.6)	1611 (97.8)	37 (2.2)	2692 (96.9)	**88 (3.1)**	CLSI M38-A2 microdilution method	Espinel-Ingroff et al. (2010)
A. fumigatus *ITC (637)* *POS (637)* *VRC (637)*	636 (99.8)	1 (0.2)	636 (99.8)	1 (0.2)	632 (99.2)	**5 (0.8)**	EUCAST broth microdilution (BMD) method	Pfaller et al. (2009)
A. fumigatus *ITC (393)* *POS (393)* *VRC (393)*	635 (99.7)	2 (0.3)	387 (98.6)	6 (1.4)	384 (97.8)	**9 (2.2)**	CLSI M38-A2 microdilution method	Rodriguez-Tudela et al. (2008)
A. flavus *ITC (536)* *POS (321)* *VRC (590)*	532 (99.3)	41 (0.7)	303 (94.7)	**18 (5.6)**	578 (98)	12 (2)	CLSI M38-A2 microdilution method	Espinel-Ingroff et al. (2010)
A. niger *ITC (427)* *POS (325)* *VRC (479)*	389 (91.2)	**41 (8.8)**	308 (94.8)	19 (5.2)	474 (99)	5 (1)	CLSI M38-A2 microdilution method	Espinel-Ingroff et al. (2010)

Maximum percentage (%) of non-wild-type isolates of *Aspergillus* spp. against specific azole drug is shown in bold
WT wild type, *NWT* non-wild type

Table 4 Antifungal drug susceptibility against azoles for *Cryptococcus* species revealing resistant isolates

Species/total no of isolates used in the study	Genotype	Antifungal agents				References
		Fluconazole	Itraconazole	Posaconazole	Voriconazole	
		WT/Non-WT [% of isolates with MICs > ECV]	WT/Non-WT [% of isolates with MICs > ECV]	WT/Non-WT [% of isolates with MICs > ECV]	WT/Non-WT [% of isolates with MICs > ECV]	
C. neoformans FLC (5637) ITC (3875) PSC (2879) VRC (4665)	Non genotyped	4370/76 [1.7]	2700/30 [1.1]	1999/121 [5.7]	3351/122 [3.5]	Espinel-Ingroff et al. (2012)
	VNI	1104/33 [2.9]	1132/13 [1.1]	757/2 [0.3]	1063/26 [2.4]	
	VNIII	53/1 [1.9]	–	–	50/1 [2]	
	VNIV	–	–	–	50/3 [5.7]	
C. neoformans FLC (285) PSC (285) VRC (285)	Non genotyped	276/9 [3.1]	–	275/10 [3.5]	271/14 [4.9]	Pfaller et al. (2011)
C. gatt FLC (827) ITC (504 PSC (250) VRC (649)	Non genotyped	124/13 [9.5]	70/0 [0]	67/1 [1.5]	93/5 [5.1]	Espinel-Ingroff et al. (2012)
	VGI	257/3 [1.2]	256/1 [0.4]	181/1 [0.5]	258/0 [0]	
	VGII[b]	94/7 [6.9]	46/1 [2.1]	–	92/4 [4.1]	

(continued)

Table 4 (continued)

Species/total no of isolates used in the study	Genotype	Antifungal agents				References
		Fluconazole	Itraconazole	Posaconazole	Voriconazole	
		WT/Non-WT [% of isolates with MICs > ECV]	WT/Non-WT [% of isolates with MICs > ECV]	WT/Non-WT [% of isolates with MICs > ECV]	WT/Non-WT [% of isolates with MICs > ECV]	
	VGIIa	192/8 [4]	–	–	192/5 [2.5]	
	VGIII	42/1 [2.3]	44/0 [0]	–	–	
	VGIV	82/4 [4.7]	86/0 [0]	–	–	

[b]Isolates identified as VGII molecular type and not being one of the VGIIa subtypes examined as a separate group

(–) Data not available

cross-resistance was attributed to a missense mutation [involving tyrosine to phenylalanine (Y145F) substitution] in the *ERG11* gene (Espinel-Ingroff et al. 2012). In addition, the level of heteroresistance to FLC was found to be more in *C. gattii* than in *C. neoformans* isolates (Kwon-Chung and Rhodes 1986). Heteroresistance is the expression of different resistance profiles in subpopulations of a strain (Nunes et al. 2007).

6 Molecular Mechanism of Drug Resistance to Azoles

Resistance to first line of therapy such as FLC and VRC has increased in recent years. Several high-throughput studies with clinical isolates and in vitro-developed resistant isolates of *Candida* spp. have been carried out in order to understand the genes/proteins and pathways involved in drug resistance. Table 5 represents high-throughput studies using transcriptomic, proteomic, and lipidomic analysis to understand molecular pathways involved in drug resistance. Here, we discuss the factors associated with drug resistance against azoles in major pathogenic fungi.

6.1 Alteration in Ergosterol Biosynthesis Pathway Enzyme

As discussed earlier, azoles generally target lanosterol 14α-demethylase which is a cytochrome P450-dependent enzyme encoded by *CYP51* or ERG11 gene. This is an oxidative process which involves the removal of 14α-methyl group from lanosterol. Azole binds to the ferric ion moiety of the heme-binding site and blocks the enzyme's natural substrate lanosterol, disrupting the biosynthetic pathway (Odds et al. 2003). Amino acid substitutions in the drug target that inhibit drug binding are common azole drug resistance mechanisms in fungi.

Azole resistance in *Candida* species is attributed to the point mutation in ERG11 gene resulting in the alteration of drug target that inhibits the azole drug from binding to its target (Vandeputte et al. 2012). In a study on *Candida*, five different mutations in CYP51A1 gene (G129A, Y132H, S405F, G464S, and R467K) were observed. In the clinical isolates of *C. albicans*, combined mutations in CYP51A1 gene resulted in a greater decrease in binding affinity of the drugs and then single mutations (Sanglard et al. 1998). Studies on *C. albicans* and *C. glabrata* also found that overproduction of drug target (lanosterol demethylase) which resulted from genome rearrangement and chromosome duplication was another factor for an increased azole resistance (Marichal et al. 1997; Selmecki et al. 2008). In another study, a zinc transcription factor, CaUpc2, was essential for controlling the regulation of ERG genes in the subsistence of ergosterol biosynthesis inhibitors (Vandeputte et al. 2012). Further, in other study the binding capacity of CaUpc2 to the promoter region of ERG11 in *C. albicans* was confirmed (MacPherson et al. 2005).

Table 5 Various studies carried out to understand the molecular mechanisms of azole resistance in *Candida* Species

Candida species/strains	Azoles tested	Techniques used	No. of differentially expressed proteins/genes	Verifications/functional studies	References
Transcriptomic studies					
C. albicans (FLC susceptible and resistant isogenic strains showing overexpression of CDR1 and CDR2)	FLC	RNA sequencing	228 genes	Transcription factor encoding gene, CZF1, overexpressed in FLC resistant isolate, was further studied and its role in drug resistance was shown in *C. albicans* and proposed as a potential target for FLC resistant isolates of *C. albicans*	Dhamgaye et al. (2012)
C. albicans (Azole-resistant clinical strains and strains with disrupted TFs encoding genes)	FLC (MIC for resistance—64 µg/ml)	Microarray and qRT-PCR	209 genes were upregulated in response to FLC	To analyze the role of CAS5 transcriptonal regulator in azole resistant isolates of *C. albicans,* CAS5 mutants (exhibiting the resistance mutations in TAC1, MRR1 and ERG11) were generated and it was shown that there was reduction of both MIC and MFCs values in these isolates. These data suggests that in absence of CAS5 regulator, susceptibility to FLC was increased in resistant isoates having specific resistance mutations whereas in the presence of CAS5, resistance was persisted in these isolates. Further, genome-wide transcriptional analysis revealed the role of CAS5 in cell wall organization, iron transport and homeostasis	Vasicek et al. (2014)

C. glabrata *(Seven pairs of susceptible and resistant isogenic isolates)*	FLC (MIC range for resistance—128–512 μg/ml)	DNA microarray and qRT-PCR	Expression of 45 genes was significantly altered in at least one clinical pairs. Of these 19 were upregulated in majority of the resistant isolates	All the resistant isolates had acquired mutation in *C. glabrata* pleiotropic drug resistance (CgPDR1) open reading frame. Transcript analysis showed twofold upregulation of CgPDR1 and its known target genes in all the seven resistant isolates. The study showed that gain-of-function mutations in CgPDR1were associated with azole resistance	Tsai et al. (2010)
C. glabrata *(FLC susceptible and resistant isogenic strains)*	FLC (MIC for resistance—> 256 μg/ml)	Microarray and qRT-PCR	379 genes	qRT-PCR analysis of FLC susceptible and resistant isogenic strains showed the resistant isolate exhibited mitochondrial dysfunction and upregulation of the ABC transporter genes, C. glabrata CDR1 (CgCDR1), CgCDR2, and CgSNQ2, involved in drug resistance. Further, the resistant isolate showed increased virulence in vivo in both systemic and vaginal murine infection models. Microarray analysis of FLC susceptible and resistant isogenic strains further confirmed the overexpression of ABC transporter genes, *C. glabrata* CDR1 (CgCDR1), CgCDR2, and CgSNQ2 and cell wall proteins, GPI anchored proteins, yapsins (CgYps1, 3, 5, 8-11). Overall, this study showed that mitochondrial dysfunction was one of the factor in *C. glabrata* virulence and may serves a foundation for identification of virulence factors in *C. glabrata*	Ferrari et al. (2011)

(continued)

Table 5 (continued)

Candida species/strains	Azoles tested	Techniques used	No. of differentially expressed proteins/genes	Verifications/functional studies	References
C. parapsilosis (FLC resistant,VRC resistant, PSC resistant strains) (*invitro* study)	FLC (MIC for resistance —≥64 μg/ml), VRC (MIC for resistance —≥4 μg/ml), POS (MIC for susceptibility —≤1 μg/ml)	cDNA microarray and qRT-PCR	1128 genes (in FLC resistant), 210 genes (in VRC resistant), 598 genes (in PSC resistant strain)	Resistant *C. parapsilosis* strains were obtained after constant exposure to VRC, FLC, POS. Microarray analysis showed increased expression of MDR1 and other efflux pump members, MFS, and transcription factor MRR1, involved in regulation of MDR, to be associated with VRC and FLC resistance in *C. parapsilosis* resistant strains, while increased expression of ergosterol biosynthesis genes and transcription factors, UPC2 and NDT80, involved in regulation of ergosterol biosynthesis genes,were associated with PSC resistance. Some of the genes involved in ergostrol biosynthesis, ERG5, 11, MDR1, MRR1 and NTD80, were further confirmed by qRT-PCR	Silva et al. (2011)
Proteomic studies					
C. albicans (FLC resistant and sensitive strains, clinical isolates)	FLC (MIC for resistance—≥64 μg/ml)	2D-PAGE and MALDI-TOF MS	15 proteins	The comparative proteomic analysis showed altered expression of proteins was majorly involved in energy metabolism and amino acid biosynthesis in resistant strain. Proteomic analysis revealed upregulation of alcohol dehydrogenase (Adh1p), involved in biofilm formation and interaction with host, in FLC resistant strain, which was earlier reported to be associated with FLC resistance in *C. albicans* using differential display-PCR technique	Wang et al. (2012)

C. glabrata (FLC resistant and susceptible strains, clinical isolates)	FLC (MIC for resistance—64 µg/ml)	2D SDS-PAGE and LC-MS/MS	65 proteins 39 intracellular and 26 membrane proteins	The study analyzed membrane and cellular proteins differentially expressed in FLC resistant isolate. Resistant strains showed upregulation of membrane proteins while cellular proteins were downregulated. Heat shock protein and stress response proteins were observed to be upregulated in membrane fractions of resistant strains	Yoo et al. (2012)
C. glabrata (VRC resistant,susceptible, susceptible dose-dependent strains, clinical isolates)	VRC (MIC for resistance—4 µg/ml)	2D SDS-PAGE and LC-MS/MS	46 proteins 15 intracellular and 31 membrane proteins	The study analyzed membrane and cellular proteins differentially expressed in VRC resistant isolate. This study showed that there was overexpression of heat shock protein 70 (Hsp70) isoforms in intracellular fraction and decreased expression of nine Hsp70 protein isoforms in membrane fractions of susceptible, susceptible dose-dependent and resistant *C. glabrata* strains suggesting that this protein may be associated with VRC resistance in resistant *C.glabrata* strains	Yoo et al. (2013)
C. glabrata (FLC resistant strain) (in vitro study)	FLC (MIC for resistance—> 256 µg/ml)	2D-PAGE, MALDI-TOF MS, qRT-PCR	25 proteins	FLC resistant strains of *C. glabrata* were generated and eight of the selected mutants with large (n=4) and small colonies (n=4) analyzed by CHEF showed four genotypes for these mutants. Two of the randomly selected stable FLC resistant mutants further analyzed by proteomic analysis showed	Samaranayake et al. (2013)

(continued)

Table 5 (continued)

Candida species/strains	Azoles tested	Techniques used	No. of differentially expressed proteins/genes	Verifications/functional studies	References
				a total of 25 proteins to be differentially expressed in resistant mutants, some of them, ERG11, CDR1, CDR2, MFS, MTI, TPR, VPS and EFT2, were further confirmed by qRT-PCR analysis. Interestingly, FLC resistant isolates also showed resistance to other antifungal azole-ITC (MIC for resistance —>32 μg/ml), Ketoconazole (MIC for resistance—>32 μg/ml) and VRC (MIC for resistance—>32 μg/ml). The study also showed increased bud formation of yeast and metallothionein production in resistant mutants and proposed these phenotypes to be associated with drug resistance in *C. glabrata*	
Lipidomics studies					
C. albicans (*Eight pairs of FLC susceptible and resistant clinical isolates*)	FLC (MIC range for resistance—16 to 128 μg/ml)	ESI-MS/MS	>200 lipids species[a]	This study performed comparative lipidomic analysis of FLC susceptible and resistant clinical isolates (with over-expression of an ABC transporter encoding gene CaCDR1 or MFS encoding gene, CaMDR1) by mass spectrometry. Molecular lipid species ranging from monounsaturated to	Singh and Prasad (2011)

				polyunsaturated fatty acid-containing phosphoglycerides were identified to be among the commonalities in the lipid profiles of these pairs. While fluctuation in phosphatidyl serine, mannosylinositol phosphory-lceramides, and sterol esters levels indicated their compensatory role in maintaining lipid homeostasis among most AR isolates. This sudy establishes the versatility of lipid metabolism in handling azole stress among various matched azole resistant isolates	
C. albicans (Six isolates collected from 3 different time points of FLC therapy from HIV patients)	FLC (MIC for resistance—> 125 μM)	ESI-MS/MS,RT-PCR and HP-TLC	242 lipids species[a]	The study compared the lipidome profiling of FLC suceptible, intermediate and resistant isogenic isolates. They observed differential levels of plasma membrane microdomain specific lipids such as mannosylinositolphosphorylceramides and ergosterol, and in a mitochondrial specific phosphoglyceride, phosphatidyl glycerol in resistant isolates. They further confirmed the expression of key genes involved in lipid metabolism by RT-PCR. Further, the lipid contents were observed to be altered in resistant isolates. This study revealed correlation of development of FLC resistance	Singh et al. (2012)

(continued)

Table 5 (continued)

Candida species/strains	Azoles tested	Techniques used	No. of differentially expressed proteins/genes	Verifications/functional studies	References
				with lipid remodelling in *C. albicans*. Further, mitochondrial dysfunction and defective cell wall was observed in clinical azole resistant isolates of *C. albicans* providing an evidence of a cross-talk between mitochondrial lipid homeostatis,cell wall integrity and azole resistance	

MIC minimum inhibitory concentration, *FLC* fluconazole, *ITC* itraconazole, *VRC* voriconazole, *PSC* posaconazole, *qRT-PCR* quantitative real-time polymerase chain reaction, *ABC* ATP-binding cassette transporter, *2D-PAGE* two-dimensional polyacrylamide gel electrophoresis, *MALDI-TOF MS* matrix-assisted laser desorption ionization-time of flight mass spectrometry, *SDS* sodium dodecyl sulfate, *LC-MS/MS* liquid chromatography tandem mass spectrometry, *ESI-MS/MS* electron spray ionization tandem mass spectrometry, *HP-TLC* high performance thin layer chromatography, *RT-PCR* reverse transcriptase polymerase chain reaction, *MDR* multidrug resistance, *CHEF* contour-clamped homogeneous electrophoretic field analysis

The azole resistance in *A. fumigatus* is majorly mediated by mutations in gene CYP51A1. Target gene, cyp51 (ERG11), has two copies, i.e., CYP51A1 and CYP51B, each encoding a different protein (Mellado et al. 2001); however, mutations in CYP51A1 gene are reported as major cause of resistance in *A. fumigatus* (Odds et al. 2003; Diaz-Guerra et al. 2003; Warrilow et al. 2010). In *A. fumigatus* the first mutation to be identified was the glycine 54 (G54) point mutation detected in clinical isolates resistant to ITC and POS (Mellado et al. 2007). A high-throughput multiplex RT-PCR has been developed for detecting mutations in *A. fumigatus* CYP51A1 that leads to ITC resistance (Balashov et al. 2005). In another study, clinical isolates of *A. fumigatus* with reduced susceptibility were identified, and it was seen that the resistance developed among these species may be linked to two factors: firstly due to a point mutation involving substitution of leucine by histidine at 98th position (L98H) in CYP51A1 gene and secondly due to the presence of two copies of a 34-bp tandem repeats (TR) in the CYP51A1 promoter region (Snelders et al. 2010). Methionine 220 (M220) and glycine 138 (G138) are the less common mutations in *A. fumigatus* azole resistance strains (Slaven et al. 2002). TR/L98H genotype is the most ubiquitous mutation responsible for resistance mechanism observed for azole-resistant strain (Willger et al. 2008). A recently identified mechanism in the CYP51A1 gene that decreases the susceptibility of *A. fumigatus* against voriconazole consists single polymorphisms mainly substitutions in tandem repeat of 46-bp in the promoter region (Dirr et al. 2010).

But a limited number of mutations in *ERG11* has been reported in *C. neoformans*, including Y145F (Espinel-Ingroff et al. 2012) and G484S (Rodero et al. 2003). Study by Espinel-Ingroff et al. showed by sequencing the ERG11 gene from clinical isolates of MRL862 (*C. neoformans* strain isolated from a FLC-treated patient) that the strain MRL862 contained five unique mutations compared to reference strain H99. Triazole susceptibility coupled with the molecular changes in the ERG11 gene revealed that a single missense Y145F, tyrosine replaced by phenylalanine, mutation resulted in high FLC resistance of the strain (Espinel-Ingroff et al. 2012). The study by Rodero et al. analyzed five *C. neoformans* isolates that were sequentially isolated from an AIDS patient with frequent meningitis and found that out of five isolates, four were FLC susceptible while FLC resistance was seen to be developed in the fifth isolate. The analysis further revealed that a point mutation G484S (involving the glycine to serine substitution at 484th position) in the ERG11 gene was the key to the development of FLC resistance in the fifth isolate (Rodero et al. 2003). Studies by various groups have demonstrated that this substitution confers an orientation change in the P450 heme-binding domain that leads to a decrease in the binding affinity of azole drug as well as a decrease in the enzyme catalytic efficiency (Sanglard et al. 1998; Kelly et al. 1999).

In *C. albicans*, point mutations in ERG3 gene result in the alteration of C5 sterol desaturase enzyme; therefore, 14α-methyl-3,6-diol (toxic sterol) cannot be synthesized. However, in the presence of azoles, ergosterol is replaced by sterol species resulting in functional fungal cell membrane (Sanguinetti et al. 2015).

6.2 Drug Efflux

There are two different drug efflux systems in fungi that contribute to azole drug resistance. They belong to the superfamily ATP-binding cassette (ABC) and the major facilitator superfamily (MFS). ABC proteins are the ATP-dependent transporters, usually arranged in duplicates, comprise of two transmembrane span (TMS) domains and the two cytoplasmic nucleotide-binding domains (NBDs) which facilitate the ATP hydrolysis. In *C. albicans* drug resistance isolates, it was reported that CDR1 and CDR2 are main contributors of azole resistance which belongs to ABC transporters superfamily (Vandeputte et al. 2012). Studies on regulation of CDR1 and CDR2 showed cis-acting regulatory elements: a basal expression element (BEE), a drug-responsive element (DRE), two steroid responsive elements (SREs), and a negative regulatory element (NRE) in CDR1, while the CDR2 promoter contains only a DRE motif (de Micheli et al. 2002; Karnani et al. 2004; Gaur et al. 2005). Among these DRE was reported to be the exclusively responsible element for overexpression/upregulation of both CDR1 and CDR2. In azole-resistant clinical isolates of *C. glabrata*, three transporters mainly CgCDR1, CgCDR2, and ABC transporter co-regulated with CgCDR1 and CgCDR2, called SNQ2, were found to be upregulated and involved in azole resistance (Torelli et al. 2008). In *A. fumigatus*, atrF and AfuMDR4 were found to be upregulated in itraconazole-resistant strains (Vandeputte et al. 2012). In *C. albican*s, a gene encoding a protein CaNdt80p that was found to regulate CDR1 participating in drug resistance mechanism was reported (Chen et al. 2004). In a study, it was demonstrated that mutations lead to hyperactive alleles in *C. albicans* and consequent loss of heterozygosity (LOH) at the transcriptional activator of CDR gene (TAC1) and multidrug resistance 1 (MRR1) loci (Coste et al. 2009).

In azole resistance clinical isolates of *C. albicans*, multidrug resistance 1 (MDR1) transporter was reported as MFS transporter (Ben-Yaacov et al. 1994). Among FLC-resistant isolates of *C. albicans*, nucleotide region, called MDR1 drug resistance element (MDRE), was found to be responsible for the overexpression of MDR1 (Riggle and Kumamoto 2006; Rognon et al. 2006). A study by Hiller et al. reported three different cis-activating regions (regions 1, 2, and 3) in MDR1, and regions 1 and 3, close to the MDRE region, were reported to be necessary for controlling the expression of MDR1 in an azole-resistant isolate (Vandeputte et al. 2012). Studies of MRR1 gene deletion in azole-resistant strains were shown to diminish the overexpression of MDR1 suggesting MRR1p identification as a main controller of MDR1. The CdMDR1 and CtMDR1 are homologues of MDR1 in *C. dubliniensis* and *C. tropicalis*, respectively, which are found to be upregulated in azole-resistant strains (Pinjon et al. 2003, 2005; Vandeputte et al. 2005).

Drug efflux pumps are also responsible for resistance in *A. fumigatus* as they mediate reduced accumulation of intracellular drugs (Coleman and Mylonakis 2009). A probe derived from CDR1 gene, ABC transporter genes for drug efflux in *C. albicans*, was used to clone atrF gene from *A. fumigatus* with characteristics of multidrug resistance motifs. In *A. fumigatus* isolate, AF72 has approximately

fivefold higher levels of expression of atrF compare to the susceptible isolates AF10 and H06-03 with sub-MIC levels of ITC (Slaven et al. 2002). In *A. fumigatus*, overexpression of multidrug resistance 3 (AfuMDR3) or AfuMDR4 is linked to high-level resistance against itraconazole due to the mutations at the drug target site (Denning et al. 1997; Nascimento et al. 2003). In the biofilms of azole resistance of *A. fumigatus* efflux pump, AfuMDR4 pump was seen to be upregulated that is responsible for resistance to VRC (Rajendran et al. 2011).

In *A. nidulans*, high levels and differential expression of AtrA to AtrD genes were reported in the presence of drugs such as camptothecin, imazalil, ITC, hygromycin, and 4-nitroquinoline oxide using real-time RT-PCR. Further, AtrA to AtrD expression levels were verified in the *A. nidulans* imazalil-resistant mutants (Semighini et al. 2002). One of the four ABC-type transporter genes, abcD, was reported to have two- to sixfold increased mRNA level expression following exposure to miconazole, camptothecin, methotrexate, and ethidium bromide (do Nascimento et al. 2002).

Many ABC proteins are found in *C. neoformans* in comparison to other pathogenic fungi (Lamping et al. 2010), but only a few are linked to azole drug resistance. The upregulation of the ABC transporter-encoding gene *AFR1* in *C. neoformans* is contributing to the in vitro resistance to FLC (Coleman and Mylonakis 2009) and corelated to less azole accumulation. Sanguinetti et al. generated a set of recombinant strains of *C. neoformans* BPY22.17 (FLC-resistant mutant strain), BPY444 (*afr1* mutant strain), and BPY445 (AFR1-overexpressing mutants), all derived from a FLC-susceptible isolate of *C. neoformans* strain BPY22. In infectious mice model exposed to these strains showed, strain BPY445 was more virulent than BPY22 and displayed enhanced intracellular survival due to upregulation of *AFR1* (Sanguinetti et al. 2006). In spite of several studies on the role of ABC multidrug efflux transporter drug resistance, little is known about the contribution of the Drug:H^+ Antiporter (DHA) family in azole resistance. There are nine DHA1 and seven DHA2 transporters present in *C. neoformans* which play role in antifungal drug resistance, but their role in azole drug resistance has yet not been studied (Costa et al. 2014).

Influx of Drugs into the Cell Import of azoles occurs via facilitated diffusion mediated by transporters in fungi such as *C. albicans* and *C. neoformans*, and mutations in the transporter may greatly influence resistance. A study by Mansfield et al. demonstrated that azole compounds utilized the same mechanism for incorporating drugs inside the cell membrane which was carried out by a transporter. The mutation in that putative transporter resulted in the azole cross-resistance. Among 35 studied clinical isolates of *C. albicans*, 4 isolates showed overexpression of genes; ERG11, MDR1, CDR1, and/or CDR2. A mutation is reported in ERG11 which significantly alters [3H]-FLC import suggesting that modification of azole import mediated by mutated transporter may be associated with antifungal resistance (Mansfield et al. 2010). Composition alterations in plasma membrane are some other factor that affects fluidity and asymmetry of the membrane that leads to a decreased drug uptake (Parks and Casey 1996).

6.3 Chromosomal Abnormalities: Loss of Heterozygosity and Aneuploidy

Azole drug resistance has been associated with multiple genomic alterations, including loss of heterozygosity (LOH) of specific genomic regions (Coste et al. 2007; Selmecki et al. 2010), increase in the chromosome copy number, as well as segmental or chromosomal aneuploidies.

Comparative genome hybridization (CGH) analysis showed that there were 37 aneuploid chromosomes in *C. albicans* strains (FLC-resistant and FLC-sensitive strains). This aneuploidy was frequent on chromosome 5, while segmental aneuploidy was present in eight FLC-resistant strains with extra copies of chr5L which was confirmed by Southern blot analysis (Selmecki et al. 2006). In *C. albicans*, events of LOH were observed antifungal drug-resistant strains (Sanguinetti et al. 2015). It was shown in *C. albicans* that LOH events were increased during in vitro exposure to heat stress (onefold to 40-fold), oxidative stress (threefold to 72-fold), and treatment with antifungal agents (FLC, 285-fold) (Forche et al. 2011) and led to the development of antifungal drug resistance. Further, it was demonstrated that increasing extremity of stress was associated with increased rates of LOH. Chromosomal rearrangements and duplications also have a role in increased resistance toward azoles.

Numerous analyses of azole-resistant and azole-sensitive strains obtained by comparative genome hybridization (CGH) from clinical and laboratory sources reflects a clear link between "heteroresistance" (an azole-associated acquisition of aneuploidy) and azole resistance (Kwon-Chung and Chang 2012). Among the screened isolates of *C. neoformans* and *C. gatti*, it was showed that both the species displayed intrinsic heteroresistance phenotype to FLC (Sionov et al. 2009), which was observed to diminish upon release of drug stress. It was observed that the strain resistance to FLC was always disomic for Chr1, and further elevation in drug level resulted in disomy of Chr4. Infrequent doubling of chromosome 1 has been reported in *erg11* or *afr1* mutants suggesting their role during times of stress (Sionov et al. 2010). Since both *ERG11* and *AFR1* were present on Chr1 in *C. neoformans*, it was seen that the duplication of the chromosome has been an advantage during the development of a resistant strain during FLC stress.

6.4 Mitochondrial Dysfunction

Mitochondrial dysfunction contributes greatly toward the virulence as well as drug tolerance of fungal pathogens. Loss of mitochondrial genome and changes in mitochondria membrane structures lead to the potential activation of drug resistance pathway. In a study, FLC-susceptible and FLC-resistant strains of *C. glabrata* showed upregulation of the ABC transporter genes, CgCDR1, CgCDR2, and CgSNQ2 (Ferrari et al. 2011). A study by Vazquez et al. showed that deletion of

the mitochondrial inner membrane translocase gene, OXA1, essential gene for the assembly of mitochondrial-encoded subunits, activates the drug resistance pathway in *C. glabrata*. In addition, *C. glabrata* modification of phosphatidylglycerol synthase gene CgPGS1 responsible for synthesis of the phospholipids in mitochondria has been associated with drug resistance (Shingu-Vazquez and Traven 2011).

6.5 High-Osmolarity Glycerol Pathway

The HOG pathway is often reported to play an important role in controlling various activities like stress response, biosynthesis of ergosterol, production of virulence factor, and differentiation in pathogenic fungi. In *C. neoformans*, transcriptome analysis of the HOG pathway discovered Hrk1 (gene regulated by Hog1), encoding a putative protein kinase. This gene plays a role in stress, virulence of the fungus in Hog1-dependent and Hog1-independent manner, and thus antifungal drug susceptibility (Kim et al. 2011). The HOG pathway controls expression levels of ergosterol biosynthesis genes and in turn affects azole drug susceptibility. Consequently, the HOG mutants exhibit a decrease in drug sensitivity toward azoles such as FLC and KTC (Ko et al. 2009).

6.6 Other Mechanisms

Stress response can regulate azole resistance in *A. fumigatus* but very little is known about it. Sterol regulatory element-binding protein, SrbA, a highly conserved transcription factor, was found to be mediating stress responses under hypoxic conditions. It was further reported to be implicated in maintenance of sterol biosynthesis, hyphal morphology, and specifically in azole resistance, such as FLC and VRC (Willger et al. 2008). Another study involving mutants of MAP kinase kinase 2 (Mkk2), which is generally associated with positive regulation of calcium-mediated signaling, cellular response to salt stress, and cell wall integrity pathway, showed increased sensitivity to both *POS* and VRC (Dirr et al. 2010). The inhabitation of Hsp90 increases the efficiency of VRC in *A. fumigatus* suggesting that it may have significant role in the azole resistance in *A. fumigatus* (Cowen 2009). There are several other mutations in protein-coding regions which were found to favor azole resistance in *A. fumigatus*. HapE gene encodes for CCAAT-binding transcription factor; mutation in this gene that substitutes proline to leucine is one such example (Camps et al. 2012). However, the mechanism of resistance is not clearly understood.

7 Novel Strategies to Combat Drug Resistance

Due to the limited antifungal treatments available, antifungal agents with broad spectrum of fungicidal activity using versatile mechanism of action are often required. Specific treatment of resistant organisms with less toxicity is of paramount importance (Messer et al. 2009). Novel strategies as discussed below may be considered for combating drug resistance.

Understanding the key molecular mechanisms in host-fungal interactions in vitro and in vivo is the key to finding novel therapies. These may also uncover the mechanisms behind the development of drug resistance. The present antifungal agents in use can be altered in a way which can target fungal virulent genes, enzyme inhibitors, and fungal metabolic pathways. Genome-wide analysis studies can be performed to identify the genes responsible in fungal survival and can serve as efficient targets. The catalogue of essential genes for *C. albicans* and *Aspergillus fumigatus* has been studied by molecular techniques such as gene replacement and conditional expression (GRACE) or conditional promoter replacement (CPR) (Roemer et al. 2003; Hu et al. 2007). Such analyses provide specific drug targets for designing effective antifungal drugs. Combinatorial antifungal therapy is used as an alternate approach for primary and salvage treatment of fungal infections, e.g., the use of amphotericin B deoxycholate and flucytosine is highly recommended for cryptococcal meningitis treatment (Pappas et al. 2009b). However, the use of combination therapy for in invasive candidiasis and in aspergillosis has mixed review and still awaits studies, which may come in near future.

The use of multifunctional approach using computational modeling and biochemical studies can be employed to screen the large libraries of chemical compounds effective against broad range of fungal pathogen. The use of in vivo model of *Caenorhabditis elegans–Candida albicans* killing assay presents rapid and economical approach for antifungal discovery (Breger et al. 2007) and presents limited and definite compounds to be tested for in vivo models or in human clinical trials. Compounds from natural sources (plants, sea, microorganisms) and chemical sources should be screened for their antifungal properties and used as another novel approach to overcome current drug resistance. A cysteine-rich antifungal protein (AFP) isolated from *Aspergillus giganteus* is reported to exert potent antifungal activity against pathogenic fungi without altering the viability of host mammalian cells (Meyer 2008); however, its bioactivity is restricted to filamentous fungi. A chitosan, a polymer isolated from crustacean exoskeletons, is another well-researched compound known to be effective against *C. neoformans* biofilms formed on indwelling surgical devices (Martinez et al. 2010). A compound named as 25-azalanosterol inhibits the growth of *C. albicans* in vitro and has potential as a new class of anti-*Candida* agents with no toxic side effects in the mammalian host (Wang and Wu 2008). Although it will be extremely useful to have new and improved antifungal drugs, the immediate focus should be on improvisation of strategies to use the antifungal treatment we possess at present.

8 Conclusions

There has been an increase in incidences of IFIs and emergence of new human pathogenic fungal species. One of the major factors for increased resistance to antifungal drugs in these pathogenic fungi is due to exposure to the antifungal drugs. In view of this, species identification and antifungal susceptibility testing to identify resistant isolate needs are urgent needs for better treatment management of IFIs. Clinical breakpoints have been defined for *Candida* species; however, more studies are required for *Aspergillus* and *Cryptococcus* species. Various studies to understand the molecular mechanisms of resistance in fungi have been carried out majorly in *C. albicans*. However, such studies need to be performed in *Aspergillus* and *Cryptococcus* species. A combinatorial therapy targeting multiple pathways involved in drug resistance might help to combat drug-resistant isolates. Further, new antifungals targeting survival genes would be helpful to in combating drug resistance and improved treatment management of IFIs. Novel strategies in search for antimycotic compounds that should be cost-effective along with high performance and limited toxicity are required.

9 Opinion

The improved diagnostic method based on molecular and serologic techniques is an urgent need for early diagnosis of IFI. Further, development of diagnostic platform detecting drug-resistant isolates from clinical samples is imperative for better IFI treatment. To improve the detection of antifungal resistance, novel assays identifying fungal isolates, which possess mutations known to be associated with antifungal resistance, should be developed and included in diagnostic platform. A reproducible and clinically relevant antifungal susceptibility testing and its use in routine clinical practice are needed. In view of the increasing resistance to antifungal drugs leading to persistent fungal infections, focused research on understanding molecular mechanisms involved in drug resistance should be promoted. Research outcomes of such studies may be beneficial for designing new antifungal drugs to combat these life-threatening infections and develop tools enabling the rapid detection of resistance in fungal population of diverse origins. Novel antifungal agents with better efficacy are required due to relative shortage of antifungal agents for treating invasive mycoses diseases.

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