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Resistance of *Candida* spp. to antifungal drugs in the ICU: where are we now?

Danièle Maubon, Cécile Garnaud, Thierry Calandra, Dominique Sanglard, Muriel Cornet

Take-home message: The emergence of resistance is a warning signal triggering improvements in antifungal drug use, particularly in patients for whom the potential benefit of treatment is unproven. Practical proposals to detect and prevent the risk of clinical failure are (i) accurate assessments of prior antifungal exposure, (ii) close clinical monitoring of patients treated with antifungal drugs, (iii) routine surveillance of in vitro susceptibility testing and (iv) development of feasible methods for rapid detection of mutations.

Electronic supplementary material is available at the end of the present document.

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Abstract Current increases in antifungal drug resistance in *Candida* spp. and clinical treatment failures are of concern, as invasive candidiasis is a significant cause of mortality in intensive care units (ICUs). This trend reflects the large and expanding use of newer broad-spectrum antifungal agents, such as triazoles and echinocandins. In this review, we

firstly present an overview of the mechanisms of action of the drugs and of resistance in pathogenic yeasts, subsequently focusing on recent changes in the epidemiology of antifungal resistance in ICU. Then, we emphasize the clinical impacts of these current trends. The emergence of clinical treatment failures due to resistant isolates is described. We also consider the clinical usefulness of recent advances in the interpretation of antifungal susceptibility testing and in molecular detection of the mutations underlying acquired resistance. We pay particular attention to practical issues relating to ICU patient management, taking into account the growing threat of antifungal drug resistance.

Keywords Antifungal resistance · Resistance mechanisms · *Candida* resistance · Intensive care unit · Clinical resistance · Microbial resistance

Introduction

Invasive candidiasis is a major threat to intensive care unit (ICU) patients, causing significant mortality. An early initiation of antifungal therapy is crucial to improve the prognosis [1, 2]. However, the performance of current diagnostic tools for confirming the diagnosis remains limited. Increasing numbers of ICU patients without

documented candidiasis are therefore receiving prophylactic or empirical antifungal treatments in an attempt to decrease *Candida*-related mortality. This strategy has been encouraged by the introduction of new, better-tolerated antifungal drugs, such as triazoles and echinocandins, leading to stronger selective pressure [3, 4]. Antifungal drug resistance was considered less problematic in *Candida* spp. than in other pathogens, but

recent increases in resistance to both echinocandins and azoles have led to clinical failures [5, 6]. This is a matter of concern because of the limited number of drug classes targeting different fungal components and because the number of patients at risk receiving treatment is continually growing, thus further increasing antifungal drug pressure.

In this review, we firstly summarize the basis of the mechanisms of action and resistance concentrating on recent advances that improve our understanding of antifungal drugs. Then, we describe the current changes in the epidemiology of *Candida* spp. resistance. We enlighten their consequences for responses to antifungal treatments and for the optimal choice for empiric, preemptive and targeted strategies in ICU patients. The clinical relevance of the new developments for laboratory antifungal drug testing and for the detection of resistance-associated mutations is discussed with specific attention paid to practical approaches, to assess the risk of clinical treatment failure and to improve its prevention.

Targets and mechanisms of action of systemic antifungal drugs

Fungi are more closely related to humans than other pathogens, such as bacteria, limiting the number of available antifungal targets. Despite the introduction of a novel drug class exploiting a new target (echinocandins) and new azole drugs with a broader spectrum of activity (voriconazole, posaconazole), the antifungal arsenal still remains restricted.

Antifungal agents acting on the cell wall and/or plasma membrane

Echinocandins

Caspofungin, micafungin and anidulafungin block cell wall synthesis by inhibiting (1,3)- β -D-glucan synthase, which catalyses the first step in the elongation of (1,3)- β -D-glucans, a major cell wall component together with chitin and mannoproteins. Echinocandins inhibit the catalytic subunit (Fksp) encoded by two or three *FKS* genes, depending on the fungal species (Fig. 1) [7].

Azoles

Triazoles—fluconazole, itraconazole, voriconazole and posaconazole—are the azoles most commonly used to treat invasive fungal infections. Isavuconazole (ISA;

BAL4815), is a novel triazole currently in global phase 3 clinical trials for treatment of invasive fungal infections. It showed good activity against *Candida* spp. with reduced susceptibility to currently used azoles (personal communications: Smart JI, P983, ECCMID, Berlin, 2013 and Maertens J., O230, ECCMID, Barcelona, 2014). Triazoles block the synthesis of the main sterol of fungal membranes, ergosterol, by targeting the lanosterol-14 α -demethylase, also called Erg11p or Cyp51p (Fig. 1). This blockade has three major effects: (a) ergosterol depletion and changes in membrane permeability, (b) changes in the activity of membrane-bound proteins, some of which are involved in cell wall synthesis and (c) synthesis of toxic sterols as a result of Erg3p activity and accumulation of 14 α -methylated sterols (Fig. 1) [8]. Azoles have long been considered to act solely on the cell membrane, but there is growing evidence to suggest that they also act on the cell wall structure. Studies have demonstrated compensatory responses similar to those observed with cell wall-disrupting agents [9, 10].

Polyenes

Amphotericin B (AMB) and its lipid and liposomal derivatives bind ergosterol, causing pore formation and ion leakage, with fungicidal effects (Fig. 1). It has been suggested that pore formation is not required for the fungicidal effect, which is dependent only on ergosterol binding [11]. In addition, a recent study revealed that AMB is able to aggregate and to act like a “sponge”, thus extracting this key component from cell membranes [12]. Cholesterol is the major sterol of the mammalian membranes. Ergosterol and cholesterol have different structures, but drug specificity is not absolute and AMB has also been shown to bind cholesterol [13]. New formulations involving liposome encapsulation (L-AMB), AMB colloidal dispersion (ABCD) and AMB lipid complex (ABLC) have increased drug specificity and delivery, greatly reducing toxicity without decreasing efficacy [14]. However, ABCD caused a similar number of infusion-related reactions to AMB, and is no longer available.

Antifungal agents acting on nucleic acids and protein synthesis

Flucytosine is a pyrimidine analogue that is converted to 5-fluorouracil, which inhibits both RNA and DNA synthesis. Cytosine permease (Fcy2p), cytosine deaminase (Fcy1p), and uracil phosphoribosyl transferase (Fur1p) activities are required for antifungal activity (Fig. 1).

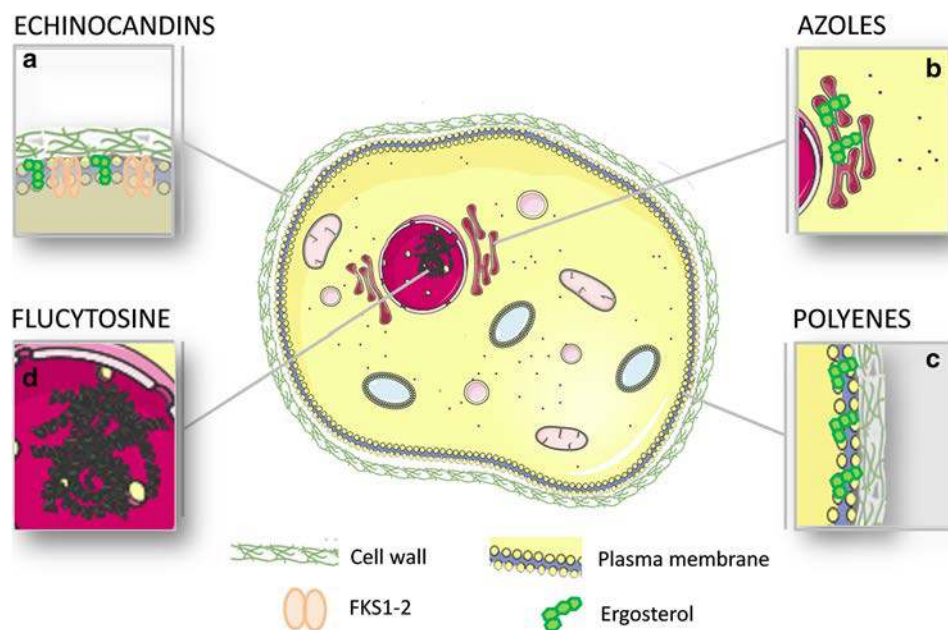


Fig. 1 Targets and mechanisms of action of systemic antifungal drugs. Sites and modes of action of the current classes of systemic antifungal drugs used to treat invasive candidiasis. **a** Echinocandins target cell wall synthesis, inhibiting (1,3)- β -D-glucan synthesis, which occurs on the inner side of the plasma membrane. **b** Azoles target the ergosterol biosynthesis pathway in the endoplasmic reticulum. They block 14 α -demethylase (also called Erg11p or

Cyp51p), resulting in ergosterol depletion in the membrane and activation of the Erg3p alternative pathway, leading to the synthesis of toxic sterols. **c** Polyenes bind to cell membrane ergosterol creating pores and aggregate, to act as a “sponge”, thus resulting in ion depletion. **d** Flucytosine acts in the nucleus, where its toxic metabolites inhibit nucleic acid synthesis

Antifungal drug resistance in *Candida* spp.

Tolerance and resistance due to cellular stress responses

An increase in cell wall chitin content has been shown to occur in response to the exposure to echinocandin and azoles in *C. albicans* [10, 15]. The blockade of this cell wall compensatory mechanism with calcineurin or protein kinase C (PKC) inhibitors restores the fungicidal activity of both azoles and echinocandins consistent with the hypothesis that chitin accumulation plays a role in tolerance to these drugs [16, 17]. Furthermore, a high chitin content has been associated with resistance to echinocandins in mice and in ‘paradoxical growth’, defined as the ability to develop in vitro at high, but not intermediate concentrations of a drug [18]. The clinical impact of this paradoxical growth in vitro remains unclear, as it is also related to lower virulence [19].

Molecular mechanisms of antifungal drug resistance in *Candida* spp.

The molecular mechanisms of antifungal drug resistance are presented in Fig. 2 and Table S1 (electronic supplementary material).

Echinocandins

Molecular resistance to echinocandins is mediated principally by mutations in *FKS* genes: *FKS1* in *Candida* spp., and *FKS1* and *FKS2* in *C. glabrata*. These mutations are located in two “hotspot” regions, HS1 and HS2 and are mostly S645F/P/Y and S629P in *FKS1* of *C. albicans* and *C. glabrata* respectively, and S663F/P in *C. glabrata* *FKS2* [7, 20]. These mutations confer cross-resistance to all three echinocandins, by modifying the catalytic and kinetic properties of the target enzyme.

Azoles

Decreased susceptibility or resistance to azoles in *Candida* spp. is mediated by various mechanisms, which may operate simultaneously in a given isolate, following sequential acquisition under drug pressure [21, 22]. Drug efflux is a major mechanism, mediated by mutations of genes encoding regulators of transporters of the ATP-binding cassette (ABC) superfamily or the major facilitator superfamily (MFS) [22, 23]. ABC transporter overexpression is associated with cross-resistance to diverse azoles, whereas MFS transporter overexpression is limited to resistance to fewer

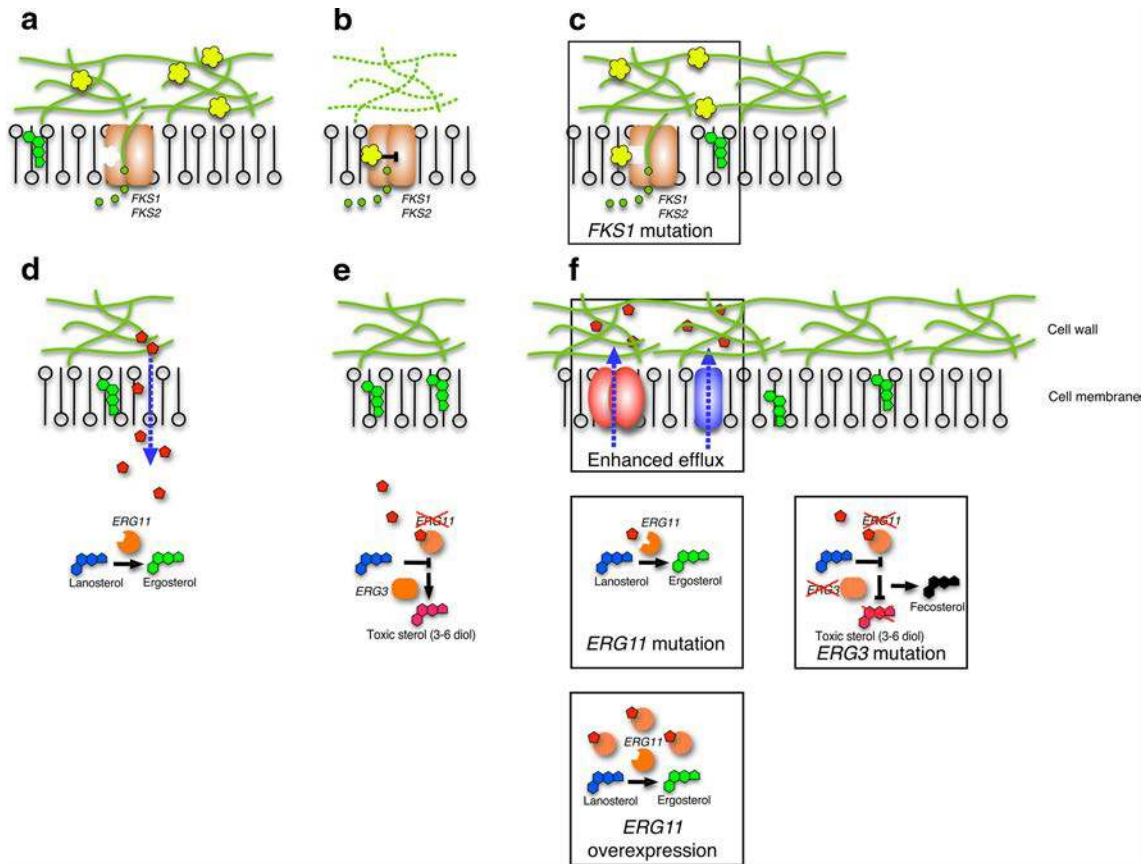


Fig. 2 Molecular mechanisms of echinocandin and azole resistance in *Candida* spp. **a** Regular β -1,3-glucan synthesis on the inner side of the fungal membrane. **b** Typical echinocandin activity. These compounds block cell wall synthesis by inhibiting the Fksp subunit of the β -1,3-glucan synthase. **c** Echinocandin resistance due to *FKS* mutations. The target enzyme is less sensitive to echinocandins, allowing the production of β -1,3-glucans. **d** Typical ergosterol synthesis at the endoplasmic reticulum and uptake of azole antifungal drugs into the cytosol of the fungal cell. **e** Typical azole

activity. These molecules inhibit the lanosterol-14 α -demethylase (Erg11p), leading to (1) membrane ergosterol depletion and (2) the production of toxic sterols via Erg3p. **f** Azole resistance due to (1) the overproduction of transporters, increasing azole efflux, (2) alteration of the target enzyme by mutations of *ERG11*, (3) Erg11p overproduction, (4) mutations of *ERG3* preventing the azole-mediated production of toxic sterols which are substituted by the non-toxic fecosterol

azoles (fluconazole, voriconazole) (Table S1). A second major mechanism is overproduction of the target enzyme Erg11p [22]. Amino acid substitutions in Erg11p may also decrease the affinity of the drugs for this enzyme [24]. Finally, *ERG3* mutations are associated with cross-resistance to azoles through a metabolic bypass leading to the synthesis of fecosterol which is able to replace ergosterol (Fig. 2) [25].

Other major genetic alterations may decrease azole susceptibility. Aneuploidy, through chromosomal duplication or loss of heterozygosity, increases the copy number of genes involved in azole resistance in *C. albicans* and *C. glabrata* [22, 23, 26]. Respiratory and mitochondrial deficiencies may also contribute to azole resistance in these species [27].

Polyenes

Polyene resistance has been little described and the exact mechanisms involved remain unclear, partly because of the small number of clinical isolates displaying altered susceptibility in vitro. Resistance is associated with changes in membrane sterol composition due to mutations in the genes of the ergosterol biosynthesis pathway: *ERG2*, *ERG3*, *ERG5*, *ERG6* and *ERG11* [28].

Flucytosine

Two main mechanisms of flucytosine resistance are known: (1) decreased uptake of the drug due to mutations

of the *FCY2* gene encoding the cytosine permease, and (2) impaired metabolism of the drug or its active metabolite (5-FU) due to mutations of *FCY1* or *FURI*. Such mutations have been described in clinical isolates of *C. albicans* and *C. lusitanae* [29, 30].

Antifungal drug resistance in *Candida* spp. biofilms

In ICUs, candidiasis may be favoured by biofilms formation, mostly on catheters but also on other implanted medical devices. Only a few antifungal drugs (L-AMB and echinocandins) have some efficacy against yeasts embedded in such complex structures [31]. Echinocandins are active against biofilms, but are more effective against biofilms containing *C. albicans* or *C. glabrata* than against biofilms of *C. tropicalis* or *C. parapsilosis* [32, 33]. Conversely, yeast cells in biofilms are up to 1,000 times more resistant to azoles than their planktonic counterparts [34].

The resistance of biofilms combines both planktonic and biofilm-specific resistance mechanisms. Efflux pump upregulation is involved in the early stages of biofilm development, whereas the greater resistance of mature biofilms is due to the presence of an extracellular matrix (ECM) and persister cells, changes to the sterol composition of the membrane and the activation of stress-induced pathways [35–37]. The ECM plays a key role, by sequestering antifungal agents and preventing their interaction with the target. This action is mediated at least by (1,3)- β -D-glucan polymers [38]. Stress responses, mediated by the PKC, calcineurin and heat shock protein 90 (HSP90) pathways, also control ECM production [35, 39]. Extracellular DNA also affects biofilm resistance and the treatment of *C. albicans* biofilms with DNase potentiates the antifungal activity of echinocandins and polyenes, but not fluconazole [40]. Persister cells have been described in *Candida* spp. biofilms, particularly those formed by *C. krusei* and *C. albicans*. These cells are phenotypic variants able to survive in the presence of antifungal agents. They can again proliferate when drug pressure is released and may cause relapses often described in clinical situations [35, 37].

Laboratory detection of antifungal resistance

Antifungal drug susceptibility testing assays

Methods for in vitro susceptibility testing are available from the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [41, 42]. Other commercially available standardised tests, such as Etest[®] (bioMérieux), Sensititre YeastOne[®] (TREK diagnostic

systems), ATB fungus 2[®] (bioMérieux) and Vitek-2[®] (bioMérieux), are more appropriate for routine clinical use [43–45]. These tests determine the minimum inhibitory concentration (MIC) or directly classify isolates as susceptible (S), intermediate (I) or resistant (R), corresponding to a high probability of treatment success (S), an uncertain effect of treatment (I) or a high probability of treatment failure (R). This classification is based on the clinical breakpoints (CBPs) established for MIC interpretation. Previous CBPs were not species-specific and were too high to distinguish between *C. glabrata* isolates susceptible and resistant to azoles and to detect emerging resistance in *C. albicans*, *C. tropicalis* and *C. parapsilosis*. In addition, clinical resistance to echinocandins due to *FKS* mutations was increasingly being reported in patients infected with “S” strains, defined with a former CBP of 2 μ g/ml or less. Up to 45 % of *FKS* mutants have been incorrectly considered as susceptible [7, 46, 47].

The revised CBPs in current use are species-specific and were established on the basis of five parameters: dose regimens; MIC distributions from multiple laboratories; epidemiologic cut-off values defined with respect to the higher MIC of wild-type isolates; pharmacokinetic/pharmacodynamic parameters and clinical outcome [20]. The key changes concern the susceptibility of *C. glabrata* and *C. parapsilosis* to fluconazole and echinocandins, respectively. The “S” category was abolished by CLSI and EUCAST for *C. glabrata* and fluconazole, considering all isolates to be intermediate or resistant. EUCAST was even more severe in its approach, recommending, as for *C. krusei*, that *C. glabrata* should not be tested with fluconazole and that fluconazole should not be used for *C. glabrata* infections [48]. The same removal of the “S” category was recommended, albeit only by EUCAST, for *C. parapsilosis* and echinocandins [20]. One other major difference between CLSI and EUCAST is that this latter does not determine CBPs for caspofungin. Indeed, CLSI and EUCAST agree that there is a lack of interlaboratory reproducibility in MIC values for caspofungin. Until this problem, not seen with other echinocandins, is resolved, neither CLSI nor EUCAST recommends caspofungin resistance testing [49–52]. EUCAST specifies that some mutations decrease susceptibility to anidulafungin and caspofungin but not micafungin, and thus recommends the use of anidulafungin as a marker for echinocandin resistance [20] (see Table 1 for simplified CLSI and EUCAST updated CBPs). Both CLSI and EUCAST also determined epidemiological cut-off values which are more sensitive than CBP to detect non-wild-type isolates exhibiting potential resistance mutations and mechanisms.

AMB testing remains particularly challenging and microbiological resistance is rarely detected [53]. Etest[®] (bioMérieux) was found to be superior to both CLSI and EUCAST reference methods for identifying resistant and intermediately susceptible *C. glabrata* isolates [54]. Thus,

Table 1 EUCAST and CLSI antifungal breakpoints for the main *Candida* species

Antifungal agent	MIC breakpoint (mg/l)									
	<i>C. albicans</i>		<i>C. glabrata</i>		<i>C. krusei</i>		<i>C. parapsilosis</i>		<i>C. tropicalis</i>	
	≤S	>R	≤S	>R	≤S	>R	≤S	>R	≤S	>R
Amphotericin B										
EUCAST	1	1	1	1	1	1	1	1	1	1
CLSI	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluconazole										
EUCAST	2	4	0.002	32	–	–	2	4	2	4
CLSI	2	4	0.002	32	–	–	2	4	2	4
Voriconazole										
EUCAST	0.12	0.12	IE	IE	IE	IE	0.12	0.12	0.12	0.12
CLSI	0.12	0.5	–	–	0.5	1	0.12	0.5	0.12	0.5
Anidulafungin										
EUCAST	0.03	0.03	0.06	0.06	0.06	0.06	0.002	4	0.06	0.06
CLSI	0.25	0.5	0.12	0.25	0.25	0.5	2	4	0.25	0.5
Micafungin										
EUCAST	0.016	0.016	0.03	0.03	IE	IE	0.002	2	IE	IE
CLSI	0.25	0.5	0.06	0.12	0.25	0.5	2	4	0.25	0.5

Adapted from Arendrup et al. [20] drug resistance updates (doi:10.1016/j.drug.2014.01.001) with permission. For complete data, see Arendrup et al. [20]

ND not done, IE insufficient evidence

AMB resistance is mainly identified through clinical failure.

While current MIC testing protocols are adapted for planktonic cells, these protocols are still not implemented in biofilms. Since biofilms can be detected in infected tissues, this is clearly another limitation in the interpretation of susceptibility tests for predicting patient outcome.

The performance of direct antifungal drug susceptibility testing, through the use of Etest[®] (bioMérieux) on positive blood samples, has been evaluated. Agreement between direct and standard methods was high and false-positive results for resistance to fluconazole and voriconazole were obtained for 7 % of isolates, with false-negative results obtained for 0.6 % of blood samples. No errors were detected for caspofungin, but the method was not reliable for AMB [55]. The new CBPs are species-specific, so this approach requires a rapid identification tool. Direct antifungal drug susceptibility testing should therefore be re-evaluated, according to the current standards, for both categorisation and identification.

Molecular detection of mutations conferring antifungal drug resistance

Molecular methods have been developed for the characterisation of resistance-causing mutations. Culture-based susceptibility assays take at least 24 h, but molecular tools can assess resistance more rapidly and with greater sensitivity. Both azole and echinocandin resistance mutations are accurately detected with next-generation sequencing platforms, allele-specific real-time probes,

melt curve analysis or microarrays, or microsphere-based technologies, such as Luminex Mag Pix (Austin, TX) [56–60] (C. Garnaud, personal communication). Moreover, as in *Aspergillus fumigatus* azole resistance, the direct detection of mutations in clinical samples may make it possible to detect mutations earlier by eliminating the time required for culture [61].

Update on the epidemiology of *Candida* spp. antifungal resistance

When focusing on species distribution and antifungal resistance, recent epidemiological studies, including the SENTRY cohort, did not show major differences between ICU and non-ICU patients [62, 63]. In both ICU and non-ICU, the five main species (i.e. *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei*) are responsible for more than 90 % of invasive fungal infections [62–65]. *C. albicans* still stands in first place, even if, since the early 2000s, a shift towards non-*albicans* species was clearly noticed [66, 67]. The fluconazole drug pressure may explain this trend but other factors, mainly underlying conditions or antibacterial therapy, have been suggested [68]. A main difference in species distribution is related to geographical location. In southern countries (Italy, Spain, South America) *C. parapsilosis* ranks second while in northern countries *C. glabrata* is the most frequent species after *C. albicans* [69]. These site specificities highlight the importance of local data on *Candida* epidemiology, specific to each health-care centre.

Table 2 Spectrum of activity of the antifungal agents used to treat invasive candidiasis

<i>Candida</i> spp.	Polyenes	Azoles				Echinocandins			Flucytosine
	AMB formulations	FLU	ITRA	VOR	POSA	CAS	MIC	ANI	
<i>C. albicans</i>	++	++	++	++	++	++	++	++	++
<i>C. glabrata</i>	+ ^a	+/-	+/-	+	+	++	++	++	++
<i>C. parapsilosis</i>	++	++	++	++	++	+	+	+	++
<i>C. tropicalis</i>	++	++	++	++	++	++	++	++	++
<i>C. krusei</i>	+	-	+/-	+	+	++	++	++	-
<i>C. rugosa</i>	+ ^a	+	+	++	++	+	+	+	++
<i>C. guilliermondii</i>	++	++	++	++	++	+	+	+	++
<i>C. lusitaniae</i>	++	++	++	++	++	++	++	++	++
<i>C. inconspicua</i>	++	-	+/-	+	+	++	++	++	NS
<i>C. norvegensis</i>	++	-	+/-	+/-	+/-	++	++	++	NS

Adapted with permission from Denning DW, Hope WW (2010) Trends Microbiol (doi:10.1016/j.tim.2010.02.004). In vitro inherent activity: ++ good activity, + mild activity, +/- slight activity, - no activity; NS not specified

AMB amphotericin B, FLU fluconazole, ITRA itraconazole, VOR voriconazole, POSA posaconazole, CAS caspofungin, MIC micafungin, ANI anidulafungin

^a This slight decrease in susceptibility to AMB is more pronounced in North America than in Europe

Indeed, some non-*albicans* species show intrinsic resistance. For example *C. glabrata* and *C. krusei* are less susceptible to azoles than other species (Table 2; [70–72]) and *C. parapsilosis* is less susceptible to echinocandins owing to naturally occurring polymorphisms of the *FKS* genes [73, 74]. Breakthrough infections with these species may therefore occur during azoles or echinocandin treatment [75, 76].

Another major and increasing threat is the risk of becoming infected with a strain which has acquired a resistant phenotype. Acquired resistance is thought to be rare in *Candida* spp., or at least less frequent than intrinsic resistance. Fortunately, yeasts, unlike bacteria, do not display the horizontal transmission of resistance genes [77]. Moreover, cross-contamination between patients and health-care workers has been described mostly for *C. albicans* and *C. parapsilosis* but remains rare [69, 78, 79]. Acquired resistance thus results principally from the selection of mutants subjected to drug pressure in patients.

Acquired resistance to echinocandins is increasingly reported for most of the clinically important *Candida* spp. It remains uncommon in *C. albicans* (<1 %), *C. tropicalis* (<5 %) and *C. krusei* (<7 %), but is now becoming frequent in *C. glabrata* (8–15 %) [5, 63, 80, 81]. One recent study showed that the frequency of echinocandin resistance in *C. glabrata* increased from 4.9 to 12.3 % between 2001 and 2010 [5]. It has been shown that 7 days of exposure to echinocandin is sufficient to induce *FKS* mutations in *C. glabrata* [5, 6]. The haploid trait of this species may partly explain the higher level of expression of molecular resistance exhibited by *C. glabrata*. *FKS* mutations have been described in almost all the clinically important *Candida* species: *C. albicans*, *C. glabrata*, *C. tropicalis* [82, 83],

C. krusei [84] and *C. kefyr* [85] and breakthrough infections are also increasingly reported [7, 75, 76, 86–90]. A recent study focusing on *C. glabrata* candidemia described 18 % of *FKS* mutation, with prior echinocandin exposure as the only independent risk factor for the development of these mutations [91] confirming the results obtained previously by Alexander et al. [5]. Interestingly, the nature and/or the number of *FKS* mutations in *C. glabrata* and *C. albicans* influences the level of resistance in vivo [91, 92]. Even if the microbiological resistance to echinocandins is still uncommon, the growing incidence of *FKS* mutations is worrying and needs to be very closely monitored. *FKS* resistance mutations also need to be more deeply studied.

Azoles and especially fluconazole are widely prescribed for ICU patients. Acquired fluconazole resistance is frequent in *C. glabrata* (from 4 to 16 %), which increasingly displays cross-resistance to voriconazole. So far, multidrug-resistant phenotype against azole and echinocandins has only been described for *C. glabrata* and is a matter of serious concern [5, 63, 66, 80, 81, 93, 94]. Fluconazole resistance remains uncommon in *C. albicans* (<5 %), but is more prevalent in *C. parapsilosis* (4–10 %) and *C. tropicalis* (4–9 %) [63, 64, 81]. However, the recent China-SCAN study reported higher rates of fluconazole resistance in *C. albicans* (9.6 %) and *C. parapsilosis* (19.3 %) which may reflect geographical differences [93]. Again, most studies report that a previous history of azole pre-exposure increases the risk of in vitro azole resistance (from 2 to 58 % in a 2013 study by Montagna et al. [65]).

Resistance to AMB remains rare despite its use in monotherapy for years. This may be due to its inherently fungicidal effect, limiting the selection of mutants. However, resistant isolates of *C. glabrata* and *C. krusei*

are increasingly being reported and this new entity also needs to be closely monitored [54, 95–98].

Even if it remains uncommon, *Candida* spp. drug resistance is clearly becoming an ‘every-day’ concern in the mycology laboratory. Determining initial but also subsequent MICs is necessary to assess microbial resistance emergence.

Clinical impact of antifungal resistance

Clinical resistance

The failure of antifungal therapy or clinical resistance is defined as a steady-to-worse infectious syndrome with no improvement of attributable symptoms during the evaluation, death being the “ultimate” failure. However, it remains difficult to assess whether the patient dies with or of fungal infection. These criteria classify clinical outcome in trials, but can also be applied for bedside management [99]. Breakthrough infections are considered as clinical resistance and are microbiologically documented. They have frequently, but not exclusively, been described in cases of echinocandin exposure [5, 7, 75, 76, 86–90, 100, 101].

Despite its recent spread, microbiological resistance is not the major factor underlying clinical resistance. Indeed, underlying diseases, immunosuppression, complicated abdominal surgery, extreme age and renal failure, all frequently encountered in ICU patients, are known to be predictors of mortality in cases of invasive candidiasis [102, 103]. Clinical failure may also occur when the effective concentration of the chosen drug is not reached at the infected site. This situation frequently occurs for biofilms on prosthetic devices or catheters, abscesses, chorioretinitis or endophthalmitis, or other sanctuary foci. As a result of their multiple comorbidities and management strategies, ICU patients may display higher pharmacokinetic (PK) instability than other patients. Thus, regular, complete investigations of deep infections and assessments of the PK/pharmacodynamic properties of antifungal drugs are essential for correct appraisal of the clinical response. For example, given their poor penetration into the eye, echinocandins are not recommended in cases of suspected ocular secondary dissemination of *Candida*, whereas echinocandins or L-AMB are the drugs of choice when central catheters cannot be removed [104]. Given the lower frequency of primary resistance than initially thought and its beneficial penetration properties, 5-fluorocytosine may be administered in combination with other drugs to treat invasive candidiasis at deep secondary sites [105]. A recent review has provided a comprehensive analysis of the tissue penetration properties of current antifungal agents [106].

Clinical relevance of in vitro antifungal drug susceptibility testing and of the molecular detection of mutations

Crude MICs are not sufficient to predict clinical outcome. Candidemia due to *C. tropicalis*, for which the MICs of all antifungal agents are very low, has been associated with a higher mortality than for other species [102]. By contrast, *C. parapsilosis* isolates have high MICs for echinocandins, although treatment failure remains rare [104, 107]. These discrepancies between laboratory tests on antifungal drugs and clinical outcome have been extensively reported and are due to several factors, including a species-dependent virulence traits and patient-dependent conditions. The use of the revised CBPs, partly taking these factors into account, may improve the clinical predictive value of in vitro susceptibility tests. Thus, close monitoring of MICs together with accurate interpretation based on revised CBPs is always warranted to ensure appropriate specific treatment (Fig. 3). Antifungal susceptibility testing (AST) on *Candida* strains isolated from deep sites is recommended by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [104]. Reference methods are preferred but commercial techniques can be used if verification has been made that the endpoint for each species mirrors those of reference methods [104].

There is growing evidence that the detection of mutations, and especially *FKS* mutations, could be used as a predictive marker of clinical failure. In one recent study, *FKS* mutations were found in 7.9 % of 313 *C. glabrata* isolates from blood samples, and up to 80 % of patients infected with strains with both *FKS* mutations and high MICs for caspofungin experienced clinical failure or recurrent infection [5]. Another study identified *C. glabrata FKS* mutation as the only independent risk factor associated with clinical failure and showed that the detection of *FKS* mutations was superior to MIC for predicting treatment response [108]. The same group subsequently showed that the Etest[®] method (bioMérieux, France) and a MIC greater than 0.25 µl/ml for caspofungin provided 100 % sensitivity and 94 % specificity for the identification of *FKS* mutant isolates. Prior echinocandin exposure and MIC values greater than 0.25, 0.06 and 0.03 µl/ml for caspofungin, anidulafungin and micafungin, respectively, were found to be predictive of clinical failure in 91, 89 and 78 % of patients with treatment failure, respectively [6]. These findings have led to valuable, easy-to-use algorithms for predicting the outcome of echinocandin treatment from MIC levels and prior echinocandin exposure status. Not all mycology laboratories are equipped to detect *FKS* mutations and the Etest[®] method performs well for the detection of non-wild-type strains [109, 110]; this bedside strategy can therefore be used to identify patients at risk of treatment failure, for whom other antifungal treatments should be prescribed.

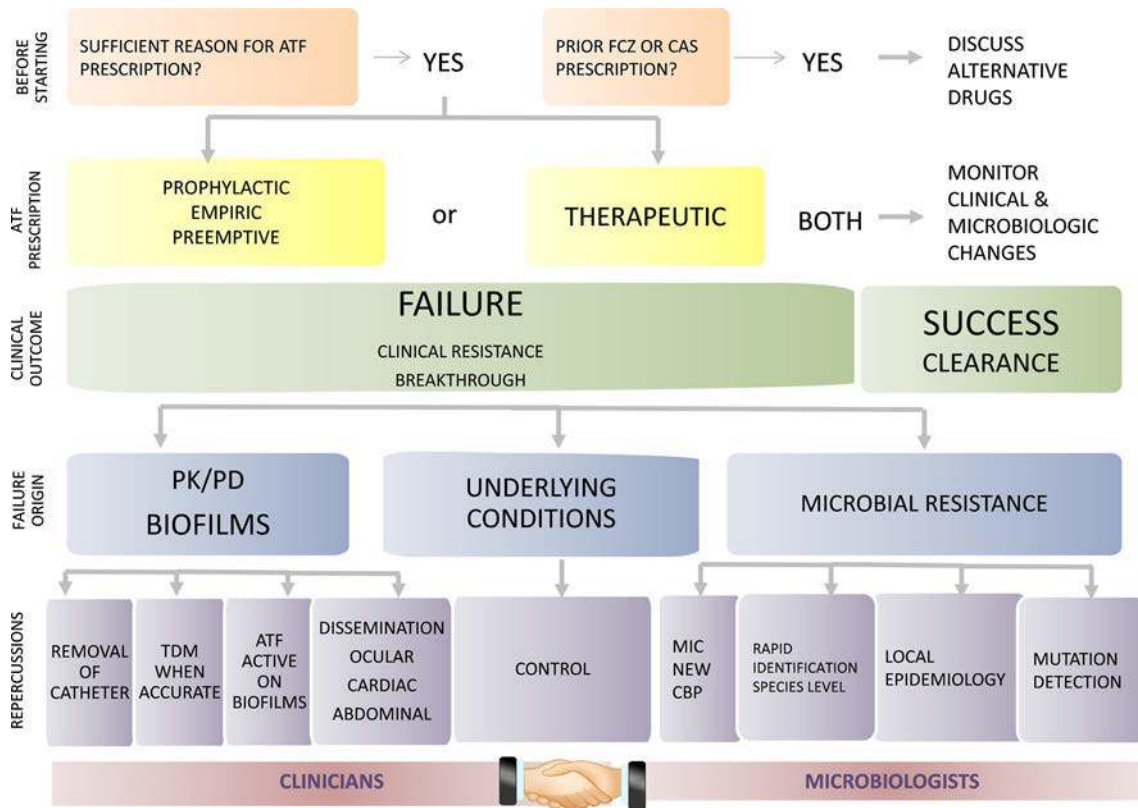


Fig. 3 Bedside strategy for circumventing antifungal drug resistance in 2014. *ATF* antifungal drug, *FCZ* fluconazole, *CAS* caspofungin, *PK/PD* pharmacokinetics and pharmacodynamics, *TDM* therapeutic drug monitoring, *MIC* minimal inhibitory concentration, *CBP* clinical breakpoint

Similar predictive markers have been suggested for azole resistance. Exposure to fluconazole in the last 30 days has been shown to have a significant impact on species distribution and MIC [4]. Algorithms have also been developed for assessment of the growing risk of *C. glabrata* infections. Cohen et al. identified six independent risk factors for *C. glabrata* fungemia in ICU patients: age greater than 60 years, recent abdominal surgery, less than 7 days between ICU admission and first positive blood culture, recent use of cephalosporins, solid tumour and absence of diabetes mellitus [111].

Integration of clinical and microbiological data (as proposed in Fig. 3) is thus crucial to improve the prediction of treatment response. Previous exposure to fluconazole and echinocandin should be accurately monitored, although the exact period to be considered remains to be defined. Patients receiving prophylactic, empirical or targeted antifungal therapy should be carefully monitored for breakthrough infections. Local epidemiological investigations and MIC determinations for *Candida* spp. isolates are also crucial and should be interpreted with the most appropriate, revised CBPs. Molecular tools are also required for the rapid detection of mutant strains.

Impact of antifungal drug resistance on patient management

Epidemiological changes have a direct impact on clinical management, leading to the updating of international expert committee recommendations [104, 112–116]. These recommendations propose consensual attitudes to the management of invasive candidiasis, but divergence remains on several crucial, contentious points [107, 117, 118], which may be confusing for clinicians treating patients.

All experts agree that patients with *Candida*-positive blood cultures should be treated with systemic antifungal drugs, but ESCMID cites echinocandins as the only initial treatment with the highest levels of strength of recommendation (A) and quality of evidence (I) [104], whereas the European Conference on Infections in Leukaemia (ECIL) and the Infectious Diseases Society of America (IDSA) consider fluconazole at the AI level of recommendation as a suitable alternative for patients with less severe or stable infection not previously exposed to azoles [10, 115, 116]. Indeed, in a recent study including 216 patients with *Candida*-induced septic shock, no difference

in mortality was observed between patients treated with fluconazole or with echinocandins [119]. In patients at risk of *C. glabrata* candidemia, echinocandins should be preferred. Voriconazole is not usually used as first-line therapy but it offers an alternate option for intrinsically less susceptible species (*C. krusei* or *C. glabrata*). Because acquired mutations can lead to cross-resistance to both fluconazole and voriconazole, a strain resistant to fluconazole should not be treated with voriconazole unless its susceptibility profile has been confirmed, or the mutation genetically characterized.

All expert panels strongly recommend the removal of central venous catheters ‘whenever possible’, but ESC-MID guidelines suggest that replacement is not formally required in patients treated with echinocandins or L-AMB [104]. Catheter exchange via a guide wire entails a risk of contaminating the new device with *Candida* and should be restricted to patients with limited venous access [107, 116, 120]. Given the specific link between *C. parapsilosis* and catheter infections and the low susceptibility of this species to echinocandins, catheter removal is appropriate in patients with invasive *C. parapsilosis* candidiasis. In stabilised patients infected with a fluconazole-susceptible isolate, with negative blood cultures, step-down therapy onto oral fluconazole is recommended, over a period of 3–10 days, depending on the guidelines considered.

Conclusions

Although drug resistance is rapidly spreading in *Candida* spp., antifungal treatments are still generally successful:

up to 80 % of *C. albicans* infections are cleared with echinocandins. Treatment success rates are also generally satisfactory for fluconazole. However, the emergence of antifungal resistance must be considered at the patient level in order to improve patient management. In ICUs, intrinsic resistance of *C. glabrata* and *C. krusei* to fluconazole can be detected and handled rapidly through correct species identification, detailed assessment of antifungal drug exposure and *Candida* spp. colonisation history. The emergence of acquired resistance during or after treatment is more worrying: it mostly involves *C. glabrata* and the echinocandins and leads to breakthrough infections or treatment failures. This highlights the need for (a) accurate assessments of prior antifungal exposure, (b) close monitoring of patients on antifungal drugs, (c) the routine surveillance of in vitro susceptibility testing and (d) the development of feasible methods for rapid detection of mutations. The emergence of resistance should also be considered at the community level as a warning sign triggering improvements in antifungal drug use, particularly in patients for whom the potential benefit of treatment is unproven. Closer monitoring of antifungal drug use is thus required.

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Table S1. Overview of the genes involved in antifungal resistance in clinical isolates of *Candida* spp (limited to the 5 most frequent species involved in invasive candidiasis in ICUs and genes described in clinical isolates)

Mechanism of resistance	Antifungals	Genes involved in resistance (species)	Comments	Reference
Increased efflux	Azole antifungals	TAC1* , CDR1 , CDR2 (CA)	ABC transporters + role in biofilms	(1–3)
	Fluconazole	CgPDR1* , CgCDR1 , CgCDR2 , CgSNOQ2 (CG)		
Alteration of antifungal target	Azole antifungals	MRR1* (CA), MDR1 (CA)	MFS transporters	(3,4)
	Echinocandins	ERG11 (CA, CG, CT)	Lanosterol-14 α -demethylase	(5–7)
Overexpression of antifungal target	Azole antifungals	FKS1 (CA, CG, CK, CT, CP) FKS2 (CG)	Beta-1,3-glucan synthase	(8)
	Metabolic by-pass	UPC2* (CA), ERG11 (CA, CT)		(3,9,10)
Changes in sterol composition of cell membranes	Azole antifungals	ERG3 (CA)	Sterol $\Delta^{5,6}$ -desaturase	(11)
	Amphotericin B	ERG2 (CG) ERG3 (CT, CG) ERG5 (CA) ERG6 (CG) ERG11 in association with ERG3 (CA)	Decrease or absence of ergosterol + role in biofilms	(4, 12–16)
Chromosomal alterations	Azole antifungals	ERG11 , TAC1 , CgPDR1 (CA, CG)	Increase of gene copy number	(3)
	Reduced drug uptake	FCY2 (CA)	/	
Alteration of drug metabolism	Flucytosine	FCY1 , FUR1 (CA)	/	(17,18)

Bold = presence of mutations, underlined = overexpression, * = GOF mutations – transcription factors

CA: *C. albicans*, CG: *C. glabrata*, CP: *C. parapsilosis*, CK: *C. krusei*, CT: *C. tropicalis*

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