Issues in antifungal susceptibility testing

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In line with the availability of an increasing array of systemic antifungal agents, there is a need for accurate, reproducible and predictive susceptibility testing of fungal isolates in order to help inform clinical choice. Much early attention on antifungal susceptibility testing focused on defining test parameters that produced reproducible and reliable intra- and inter-laboratory results and there are now standardized methods for the testing of yeast and mould isolates. The application of this standardized approach produces susceptibility results that are comparable between laboratories and allow epidemiological analyses at the national and even international level. In addition, monitoring and prediction of emerging susceptibility trends are now possible. As methodology improved, attention shifted to clinical outcome data in order to establish breakpoints for antifungal agents. However, there are a large number of confounding factors that must be considered when trying to assess the in vivo activity of antifungal agents in invasive disease. An approach based on defining normal susceptibility ranges, together with pharmacokinetic and pharmacodynamic analyses and identification of resistance mechanisms, has helped to establish breakpoint data for at least some of the systemic agents active against yeast isolates. Moreover, for some drugs, there is a supporting in vitro–in vivo correlation available from studies of clinical efficacy. Application of susceptibility testing has helped to define the spectrum of activity of all the currently available antifungal agents. Both intrinsic and emergent antifungal drug resistance are encountered and the predictability of innate resistance has meant that species identification is often sufficient to alert the clinician to the likelihood of in vitro and often-associated in vivo resistance. Currently, emergence of resistance in a previously susceptible strain during a course of treatment remains rare.

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Introduction

The requirement for accurate and predictive susceptibility testing of fungi did not become a major issue until the start of the AIDS era, and ironically, it was HIV-infected patients who provided the best in vivo test bed for the development of in vitro tests. The use of oral fluconazole, often at sub-therapeutic concentrations, for the treatment of oropharyngeal candidiasis in HIV-infected individuals led to the widespread emergence of fluconazole-resistant Candida albicans and selected for innately resistant strains of Candida glabrata. Moreover, such treatment regimens encouraged the rapid emergence of resistance in the haploid species C. glabrata.1 Previously, the only significant antifungal drug resistance encountered was emergent azole resistance documented in a handful of yeast isolates from patients with chronic mucocutaneous candidiasis treated for prolonged periods and flucytosine resistance occurring in patients with invasive candidiasis or cryptococcosis treated with flucytosine monotherapy. Resistance to other agents was reported only rarely.

In vitro susceptibility testing

Early reports of resistance were questioned on the grounds that different methods had been used to assess pre- and post-treatment isolates, and in the late 1980s, testing methods were not standardized and unsurprisingly inter- and even intra-laboratory reproducibility was poor.2 Numerous subsequent papers documented the importance of standardized test conditions and the impact on MIC determinations of test variables including, but not limited to, inoculum size, test medium composition and pH, test format, temperature, duration of incubation and endpoint determination. One of the most comprehensive papers detailing the effect of procedural variables on MICs is that by Rambali et al.,3 who examined multiple combinations of 10 test variables and documented their effect on itraconazole susceptibility of a series of test isolates including Candida spp. and filamentous fungi in two growth media. It was clear that by modifying test conditions, isolates could be made to appear susceptible or resistant to itraconazole, thus highlighting the crucial importance of establishing a set of standardized testing criteria.
while acknowledging that any unified method will represent a compromise rather than an ideal system.

**Broth-based test methods**

The first properly optimized and standardized method for yeast was a broth macrodilution method developed by the NCCLS (now the CLSI). This proved unwieldy for testing large numbers of isolates, and it was later adapted to allow a microdilution format in microtitre plates, and the method reported in NCCLS M27-A2 is now a widely accepted standard. Further suggested modifications of this microtitre method adopted by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) allow reading after 24 h on a spectrophotometer to provide a more objective endpoint analysis. However, multicentre analysis of the two methods in tandem have suggested that the NCCLS breakpoints will not be suitable for interpreting results obtained by the EUCAST method. Susceptibility testing of filamentous isolates has lagged behind that of yeast, but broth microdilution testing of moulds is addressed in NCCLS standard M38-A.

Antifungal agents of the echinocandin class, caspofungin, anidulafungin and micafungin, presented a particular challenge. In an extensive inter-laboratory comparison of susceptibility testing with caspofungin against *Candida* and *Aspergillus* spp., considerable variation was observed. The conditions producing the greatest level of inter-laboratory agreement for testing yeast isolates were incubation in RPMI 1640 medium at 35°C with endpoint determination after 24 h and the MIC defined as a ‘prominent growth reduction’ (≥50% inhibition relative to control). These conditions have now been widely validated and provide reproducible results. This method can also detect phenotypic resistance in isolates with mutation in the *FSK1* gene, which gives reduced susceptibility to the echinocandins both in vitro and in animal models.

Owing to their mode of action, which involves disruption of cell wall synthesis but with no effect on the pre-formed cell wall, echinocandins often do not produce a clear-cut endpoint in broth-based susceptibility tests with filamentous fungi. Therefore, a new interpretative endpoint had to be defined, and in place of the MIC, the activity marker for mould isolates is described as the minimum effective concentration. This relies on a microscopic observation of a change in growth form from a mat of hyphae in control wells to small, discrete micro-colonies with truncated, swollen, distorted ends to the hyphae in the presence of inhibitory concentrations of echinocandin. A similar effect has been observed in disc tests and Etest with *Aspergillus* isolates where small intrazonal colonies demonstrating short and stubby branches and a star-like morphology appear within the zone of inhibition. When subcultures from the intrazonal colonies are re-tested, an identical pattern is observed to the initial testing, thus excluding the possibility of heterogeneous resistance.

The commercial test format that equates most closely with the NCCLS methodology is Sensititre® YeastOne (TREK Diagnostic Systems), which is based on microbroth dilution but with a chromogenic substrate to facilitate endpoint interpretation. This has proved easy to interpret and results correlate closely with those obtained by the NCCLS method. Voriconazole, caspofungin and posaconazole are recent inclusions on the test plates making this methodology useful for all the systemically active antifungal agents.

**Etest**

The Etest (AB BIODISK, Solna, Sweden) is one of the commercially available tests for the detection of antifungal resistance. It is a simple method involving surface inoculation of an agar plate followed by the application of a plastic strip, which contains a concentration gradient of the antifungal under test. After incubation of a susceptible organism, a zone of inhibition can be seen and the MIC is read at the point at which the zone intersects the strip. This method is suitable for yeast and mould isolates, and once the operator has experience in reading the test, it is a reliable and reproducible method that produces results that correlate well with the NCCLS methodology. An exception to this close correlation is seen with amphotericin B for which the Etest appears to produce results over a greater concentration range that more readily distinguishes susceptible and resistant strains. One proviso with Etest is that if resistance is detected in a species in which it is not expected, it should be confirmed by a second method until the operatives have become proficient at differentiating true resistance from background intrazonal growth. Such problems are particularly encountered with strains of *Candida tropicalis*, which can also often show heavy trailing in broth formats, and with *Cryptococcus neoformans*, which may grow poorly on the test agar.

**Disc diffusion**

A simple method that is suitable for water-soluble agents such as fluconazole, fluconazole and voriconazole is disc diffusion and there is an NCCLS method for in vitro susceptibility testing of *Candida* species. This provides a zone of inhibition, the measurement of which can be correlated with the MIC value as has been demonstrated with several thousands of yeast isolates in a multinational study. Disc diffusion and Etest methods have also been shown to be suitable for determining the activity of echinocandins against yeast isolates producing easy to read, sharp zones of inhibition.

**Establishing breakpoints for antifungal agents**

As for antibacterial agents, breakpoints can be established for antifungal agents based on a number of factors including distribution curves of MIC values for wild-type populations of particular organisms, as well as their pharmacokinetic and pharmacodynamic properties. In terms of defining the relationship between breakpoints for particular agents and clinical outcomes in patients treated with these drugs, there has been evaluation of both the duration of the dosing interval where the drug concentration in plasma remains above the MIC for the infecting pathogen and the ratio of the area under the time–concentration curve to the MIC, when assessing the potential activity of antifungal drugs in vivo, with different pharmacodynamic properties being suitable for different classes of agents. However, the proof of breakpoint validity comes from analysis of in vitro–in vivo correlation in clinical practice. HIV-positive individuals with recalcitrant oropharyngeal candidosis proved suitable in vivo test subjects for correlating clinical outcome with the development of in vitro antifungal susceptibility tests to detect in vitro resistance. This group of patients had a homogeneous predisposing condition, very visible and therefore easily
evaluable disease, and differing fluconazole treatment regimens led to recalcitrant infections with azole-resistant organisms. It was therefore mainly in mucosal infection that the first azole breakpoints were evaluated.22

Owing to the relative infrequency of resistant isolates and the confounding factors in invasive disease, it proved much harder to validate breakpoints in patients with systemic infections. However, data have recently been presented to support the earlier fluconazole breakpoints.23 There are also in vivo data supporting an interpretive breakpoint for voriconazole, although not as yet for posaconazole.24 Currently, validated breakpoints include those for fluconazole, itraconazole, flucytosine and voriconazole.

Defining breakpoints for amphotericin B has proved problematic as most susceptibility testing methods result in quite a narrow range of MICs. It had previously been widely thought that amphotericin B MICs >1.0 mg/L might be indicative of resistance, and there is some supporting evidence for such a breakpoint in invasive aspergillosis.25 However, later studies failed to support this finding, once again illustrating the complexity of factors influencing treatment outcome.26 By utilizing Etest strips for assessing susceptibility to amphotericin B, a much broader range of MIC results is obtained. By this method, susceptible isolates usually display very low MICs, whereas results at the resistant end of the spectrum tend to be higher than those obtained under other test conditions. However, even when isolates are tested by this methodology, susceptibility data for Candida spp. do not appear to correlate with treatment failure or success, suggesting that factors other than MIC may have a greater impact on the outcome of invasive candidiasis.27

Breakpoints for the echinocandin class of agents have yet to be established due largely to the dearth of resistant isolates for analysis. Kartsonis et al.28 failed to establish any relationship between baseline caspofungin MIC and clinical outcome with isolates from oesophageal and invasive Candida infections; however, their data set only included three isolates with reduced susceptibility to caspofungin (MIC ≥4.0 mg/L). Strains of Candida parapsilosis are less susceptible to echinocandins in vitro than other Candida spp., although caspofungin appears to be as effective as amphotericin B in the treatment of candidemia due to this species.29 However, any in vivo analysis of outcome data should also take account of the relative virulence of different Candida spp. Epidemiological and outcome data suggest that C. parapsilosis is associated with a lower mortality rate than other yeast species and may therefore be a weaker pathogen and easier to eradicate.30,31 It is clear that although breakpoints for susceptibility to the echinocandins have yet to be established, any proposed susceptibility cut-off point should encompass the normal ranges of the majority of Candida spp. without bisecting any species group. A caspofungin MIC of ≤2.0 mg/L, which is a blood concentration that can be achieved in vivo under normal dosing, would encompass 99.7% of all clinical isolates of Candida spp.8

Resistance to antifungal agents

Amphotericin B

Although amphotericin B has a wide spectrum of activity that encompasses the majority of yeast and mould isolates, there have been reports suggesting that some strains of Candida lusitaniae and Trichosporon spp. are less susceptible to amphotericin B than other yeast species.32,33 Widespread innate resistance of C. lusitaniae has not been substantiated but there have been reports of emergent resistance leading to clinical failure in this species. Moreover, recent reports have suggested that C. glabrata may be less susceptible to amphotericin B than most other yeast species, with only 75.2% requiring an MIC of ≤1.0 mg/L, whereas 2% required MICs >2.0 mg/L by Etest.34 Infections with this species are associated with higher mortality than those involving other common pathogenic Candida species30,31 but that may be more a reflection of the types of patients that acquire the infection than any reduction in antifungal susceptibility. Candida krusei also demonstrates reduced susceptibility to amphotericin B when tested by Etest, with only 8% of 234 isolates being susceptible (MICs ≤1.0 mg/L), whereas more than 50% required MICs ≥4.0 mg/L.34 Aspergillus terreus and Scedosporium apiospermum are among the mould isolates that often demonstrate reduced susceptibility to amphotericin B and therefore respond better to other agents in vivo.34

Flucytosine (5-fluorocytosine)

Innate resistance is seen in a high percentage of isolates of C. tropicalis and C. krusei15 and few mould isolates are susceptible in vitro. However, it is emergent resistance that has limited the clinical utility of this agent. It has been recognized for many years that flucytosine should not be used as monotherapy due to the high levels of emergent resistance among the isolates of Candida and Cryptococcus.36–38

Azoles

Intrinsic resistance to one or more of the azole antifungal agents, which is generally consistent and predictable, may be encountered in new and emerging yeast and mould pathogens and highlights the need for accurate and rapid identification of infecting organisms. Treatment with azole derivatives has been associated with the emergence of isolates of C. krusei that are innately resistant to fluconazole and the haploid yeast C. glabrata that often demonstrates either innate or rapidly emergent resistance due to up-regulation of CDR drug efflux pumps.36 Scatterplots of voriconazole or posaconazole MICs versus fluconazole MICs for C. glabrata show high levels of correlation (R = 0.9 and R = 0.8, respectively) suggesting that isolates that are resistant to fluconazole often show reduced susceptibility, although not necessarily overt resistance, to the other azoles.8 This does not hold true for isolates of C. krusei, which although innately resistant to fluconazole are susceptible to voriconazole and posaconazole.

Although emergent resistance has previously been encountered with C. albicans and C. glabrata, mainly as a consequence of inappropriate prescribing practices for specific infections in certain patient groups, there are currently few problems.38 Emergent resistance to azole drugs among isolates of C. albicans from the oral cavities of patients with HIV infection was a serious problem until the introduction of highly active antiretroviral therapy (HAART) for the treatment of HIV infection in the late 1990s, which has led to declining rates of oropharyngeal candidosis. Concomitantly, there has been a recognition that higher dose, shorter course regimens of azole therapy are less likely to induce resistance. There have also been reported cases of emergent resistance in isolates of Cryptococcus neoformans.
from HIV-infected patients on prolonged fluconazole maintenance therapy.\textsuperscript{40} However, despite the widespread use of long-term suppressive fluconazole therapy in this group of patients, this has not emerged as a serious clinical problem and resistance is rarely the cause of relapse.

**Echinocandins**

Pfaffer and Diekema\textsuperscript{8} have reported caspofungin susceptibility test results for thousands of *Candida* isolates, undertaken as part of a global surveillance programme to examine geographical and temporal trends. Results indicate that >99% of isolates tested each year since the introduction of caspofungin (2001–04) have MICs of $\leq 1.0 \text{ mg/L}$, which mirrors the susceptibility profiles encountered in the years prior to its introduction, suggesting that to date there is little problem with innate or emerging resistance in this genus.

Given their shared mechanism of action, it is not surprising that caspofungin, anidulafungin and micafungin demonstrate similar potency. Scatterplots of anidulafungin or micafungin MICs versus caspofungin MICs show high levels of correlation ($R = 0.83)$,\textsuperscript{8} In vitro time–kill assays have also demonstrated similar concentration-dependent fungicidal activity against most *Candida* spp.\textsuperscript{41} Although it has been observed that caspofungin is not fungicidal for isolates of *C. parapsilosis* or *C. guilliermondii*, the clinical ramifications, if any, of this in vitro observation have yet to be established.\textsuperscript{42}

Caspofungin, anidulafungin and micafungin have good in vitro activity against *Candida* spp. and *Aspergillus* spp. but little activity against certain other yeast genera. In particular, the basidiomycete yeasts such as *Cryptococcus* spp., *Rhodotorula* spp. and *Trichosporon* spp., moulds of the genus *Fusarium* and the zygomycetes, demonstrate innate resistance to this class of agents, as they possess insufficient or altered forms of the target enzyme.\textsuperscript{11,34} To date, there have been few reports of clinical failure due to emergent resistance to this class of agents; such reports have recently been summarized by Rogers et al.\textsuperscript{35}

**Current challenges**

A major challenge to antifungal chemotherapy is in the treatment of some zygomycoses and emerging mycoses including infections due to *Fusarium* spp. and *Scedosporium prolificans*. Zygomycosis has traditionally been treated with lipid forms of amphotericin B and there is anecdotal evidence to suggest that this is one indication where doses higher than the licensed doses may be beneficial. *In vivo* data on the most recently licensed agent posaconazole also look promising but it is clear that surgical resection and resolution of the underlying predisposing condition are also important components of any treatment strategy.\textsuperscript{44} There are data to suggest favourable outcomes of infections with *Fusarium* spp. and *Scedosporium apiospermum* treated with voriconazole,\textsuperscript{45} but *S. prolificans* is resistant to all of the systemically active antifungal agents *in vitro* and is responsible for particularly recalcitrant infections.\textsuperscript{46} Anecdotal reports suggest that combination of voriconazole and terbinafine may be beneficial.

Combination therapy may be of value in some cases, although there is only good *in vitro* and *in vivo* evidence in cryptococcal meningitis, and synergy testing in the laboratory is another area in need of standardization.\textsuperscript{47} Moreover, laboratory synergy testing will never be able to reflect the differential pharmacokinetics and tissue penetration properties of the different agents and therefore may not be able to accurately predict potential clinical benefits of combination therapy. The issue of fungicidal versus fungistatic activity may be pertinent in immunocompromised patients where host immune defences presumably make little contribution to fungal eradication. However, it should be recognized that most invasive fungal infections will not resolve unless patient’s immunological status improves, and the principal function of antifungal therapy is to ensure that the patient survives the infection until their underlying condition improves. There is a suggestion that even though caspofungin is not fungicidal for mould species it causes release of $\beta$-1-glucans, which appears to stimulate the production of pro-inflammatory cytokines, which may in turn stimulate phagocytic cells to remove any remaining viable fungal fragments.\textsuperscript{48} At present, although there have been many publications, there is no accepted standard for assessing the fungicidal activity of an agent *in vitro* and little understanding of the potential clinical implications of the results.

**Summary**

The interaction between antifungal drug and fungus, fungus and host and antifungal drug and host is a complex one. Establishing the antifungal activity of an agent *in vitro* therefore provides only a fragment of the information necessary to predict the likely outcome of treatment of the infection. However, as test methods improve and the choice of therapeutic agents broadens, there is increasing evidence to suggest that *in vitro* testing does and will make a significant contribution to the selection of the most appropriate agent.

**Transparency declarations**

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**References**

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