mycoses
Diagnosis, Therapy and Prophylaxis of Fungal Diseases

Review article

In vitro susceptibility testing in fungi: a global perspective on a variety of methods

Cornelia Lass-Flörl, Susanne Perkhofer and Astrid Mayr

Department of Hygiene, Microbiology and Social Medicine, Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Innsbruck, Austria

Summary

Candida and *Aspergillus* species are the most common causes of invasive fungal infections in immunocompromised patients. The introduction of new antifungal agents and recent reports of resistance emerging during treatment have highlighted the need for *in vitro* susceptibility testing. For some drugs, there is a supporting *in vitro–in vivo* correlation available from studies of clinical efficacy. Both intrinsic and emergent antifungal drug resistance are encountered. Various testing procedures have been proposed, including macrodilution and microdilution, agar diffusion, disk diffusion and Etest. Early recognition of infections caused by pathogens that are resistant to one or more antifungals is highly warranted to optimise treatment and patient outcome.

Key words: *In vitro* antifungal susceptibility testing, *in vitro* and *in vivo* resistance, EUCAST.

Introduction

Invasive fungal infections (IFI) constitute a significant burden in patients with impaired immunity[1,2] and the spectrum of fungal pathogens is growing.[3] The available therapeutic options are limited, particularly for pathogens that are resistant to antifungals.

The requirement for accurate and predictive susceptibility testing of fungi became a major issue in the AIDS era.[4] The use of fluconazole often at sub-therapeutic concentrations led to the emergence of fluconazole-resistant *Candida albicans* and selected for innate, resistant *Candida glabrata*. Azole-resistance in yeast was documented *in vitro* and *in vivo.*[5] Currently, the survival after mould infections has improved when compared with that of the years before, yet it is still too high.[2] The reasons for failure are multiple and one factor might be infection with drug-resistant strains.[6] Some isolates of *Aspergillus fumigatus* have been found to be resistant to itraconazole or other azoles, yet resistance to the anti-*Aspergillus* triazoles has been unusual thus far; however, recent studies suggest that the rate may be dramatically increasing.[7–10]

At any rate, early recognition of infections caused by pathogens that are resistant to one or more antifungals is highly warranted to optimise treatment and patient outcome.[5,6,11]

In this study, we will discuss and review the relevance of antifungal susceptibility testing by addressing practical viewpoints and summarising key principles.

In vitro susceptibility testing methods

Antifungal drug resistance is usually quantified using the minimum inhibitory concentration (MIC) in which growth of a microorganism in the presence of a range of drug concentrations is measured over a defined time period according to a standard protocol.[12] The lowest drug concentration that results in a significant reduction or complete lack of growth of the microorganism is the MIC.

Until the early 1990s, testing methods were not standardised and therefore intra- and interlaboratory reproducibility was poor. Numerous studies attested to the *in vitro* results being influenced by a number of technical factors, including concentration of the fungal inoculum,[13] the composition and pH of medium,[14] the incubation temperature[15,16] and the length of incubation.[17,18]

Correspondence: Cornelia Lass-Flörl, Department of Hygiene, Microbiology and Social Medicine, Fritz Pregl Str 3, 6020 Innsbruck, Austria.
Tel.: +43 90 037 0725. Fax: +43 90 037 3700.
E-mail: cornelia.lass-floerl@i-med.ac.at

Accepted for publication 5 October 2009

doi:10.1111/j.1439-0507.2009.01813.x

Table 1 Differences of CLSI and EUCAST conditions for antifungal susceptibility testing for yeast

Characteristic	CLSI M 27 – A3	EUCAST–E.DEF.7.1
Suitability	Yeast	Fermentative yeast
Inoculum	0.5–2.5 × 10^3 CFU ml^{-1}	0.5–2.5 × 10^5 CFU ml^{-1}
Inoculum standardisation	0.5x McFarland	0.5x McFarland
Test medium	RPMI 1640 0.2% glucose	RPMI 2% glucose
Buffer	MOPS	MOPS
Microtitration plates	U-shaped wells	Flat-bottom wells
Format	Microdilution	Microdilution
Temperature	35 °C	35–37 °C
Duration of incubation	24–48 h	24 h
Reading	Visually	Photometrically
Endpoint ⁄ inhibition	100% amphotericin B 50% azoles, candins	50% 5 FC, azoles, candins 90% amphotericin B

FC, flucytosine.

Table 2 Differences of CLSI and EUCAST conditions for antifungal susceptibility testing for moulds

Characteristic	CLSI M38- A2	EUCAST–E. DEF 9.1
Suitability	Conidium and spore-forming fungi	Conidia-forming moulds
Inoculum	0.4–5 × 10^4 CFU ml^{-1}	2 to 5 × 10^5 CFU ml^{-1}
Inoculum standardisation	Spectrophotometrically	Haemocytometer
Test medium	RPMI 1640	RPMI 2% glucose
Buffer	MOPS	MOPS
Microtitration plates	U-shaped wells	Flat-bottom wells
Format	Microdilution	Microdilution
Temperature	35 °C	35 °C
Duration of incubation	48 h	48 h
Reading	Visually	Visually
Endpoint	No growth	No growth

Broth-based assays

Currently, two international standard methodologies for determining the susceptibility of yeast and moulds to antifungal agents are available. The first one was published by the Clinical Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards).[19,20] The second one was developed by Antifungal Susceptibility Testing Subcommittee of EUCAST (EUCAST–AFST).[21,22] Both procedures have a high inter- and intralaboratory reproducibility, differentiating populations with low and high MICs to antifungal drugs.[23,24] These two methods differ in the inoculm, medium and MIC reading (see Tables 1 and 2); suggested breakpoints from CLSI cannot be extrapolated to the EUCAST methods and vice versa (see Table 3). The EUCAST–AFST has collated the fluconazole MICs for 26 447 strains of *Candida* spp. and proposed a clinical breakpoint of ≤2 μg ml^{-1} for *C. albicans*, *C. parapsilosis* and *C. tropicalis.*[25] This subcommittee has refrained from assigning breakpoints for fluconazole to *C. krusei* and *C. glabrata*. *Candida krusei* exhibits high MICs, and this species is considered to be inherently resistant, whereas for *C. glabrata*, the median MIC was 8 μg ml^{-1} and the range was 1–128 μg ml^{-1}, with the majority of MICs ranging from 4 to 16 μg ml^{-1}.[26] For voriconazole, a clinical response of 76% was achieved in infections caused by *Candida* spp. when the MICs were lower than or equal to the epidemiological cut-off values. Therefore, the EUCAST–AFST considered

Table 3 Breakpoints according to CLSI and EUCAST for *Candida* species

	Breakpoints (μg ml^{-1})	
Drugs	EUCAST[1]	CLSI
Fluconazole ⁄ Amphotericin B	*R* > 4 NA	*R* ≥ 64 NA
Itraconazole	NA	*R* ≥ 1
Voriconazole	>0.125 (E cut-off)	*R* ≥ 4
Posaconazole	NA	NA
Caspofungin[2]	NA	NS > 2
Anidulafungin[2]	NA	NS > 2
Micafungin[2]	NA	NS > 2

R, resistant; E cut-off, epidemiological cut-off; NA, not available; NS, there is no resistance category assigned for the echinocandin agents; isolates with higher MICs are described as non-susceptible.

[1] EUCAST defined species-related breakpoints.

[2] Tentative breakpoints.

wild-type populations of *C. albicans*, *C. tropicalis* and *C. parapsilosis* as susceptible ($R > 0.125$ μg ml^{-1}).[27]

The CLSI supports fluconazole, voriconazole, itraconazole and flucytosine breakpoints for *Candida* spp. and the CLSI methodology. A dose/MIC ratio of approximately 25 was supportive of breakpoints for fluconazole and *Candida* spp.[28] For voriconazole, an analysis of 249 patients demonstrated a statistically significant correlation between MIC and outcome.[29] For the candins, the CLSI subcommittee has decided to recommend a ''susceptible only'' breakpoint MIC of ≤2 μg ml^{-1} because of the lack of echinocandin resistance in the population of *Candida* isolates thus far. Isolates for which MICs exceed 2 μg ml^{-1} should be designated 'non-susceptible'.[30] Table 3 summarises CLSI and EUCAST breakpoints for *Candia* spp.

In vitro antifungal susceptibility testing of azoles vs. *Aspergillus* spp. has been standardised by both the CLSI and the EUCAST.[19,31,32] Breakpoints based upon the correlation of *in vitro* data with clinical outcome have not been established for any *Aspergillus*–drug combination. In the absence of the necessary clinical data, one practical approach to the use of susceptibility testing data in detecting resistance or decreased susceptibility has been to define the wild-type (WT) distribution of MICs for the relevant drug–organism combinations and to set epidemiological cut-off values (ECV) that would discriminate WT strains from those with acquired resistance mechanisms.[33] ECVs could nonetheless serve as the foundation for the laboratory detection of acquired resistance and be used to monitor resistance development. Rodriguez-Tudela *et al.* [34] employed the EUCAST method to define the WT MIC distribution of four triazole antifungal agents (itraconazole, posaconazole, ravuconazole and voriconazole) for *A. fumigatus*. ECVs of <1 μg ml^{-1} for itraconazole, ravuconazole and voriconazole and <0.25 μg ml^{-1} for posaconazole identified the WT strains and distinguished WT population from strains with resistance mutations in the *cyp51A* gene. Similar differentiation was obtained by others using CLSI methodology.[33] ECVs will be very useful in resistance surveillance and serve as an important step in the establishment of clinical breakpoints.

Defining breakpoints for amphotericin B is not easy because of the narrow ranges of MICs, fungi cluster between 0.5 and 2 μg ml^{-1}. This does not allow the distinction of susceptible isolates from potentially resistant ones. However, MICs >1 μg ml^{-1} for *A. terreus* seems to be indicative of worse outcome.[35]

Assessment of *in vitro* activity of echinocandins against *Aspergillus* spp. is complicated by the fact that the MIC often exceeds safely achievable plasma concentrations[36,37] and the phenomenon of trailing endpoints makes MICs for *Aspergillus* poorly reproducible. The minimum effective concentration (MEC) defined as the lowest drug concentration at which short, stubby and highly branched hyphae are observed on microscopic examination has been shown to generate more consistent susceptibility results than the MIC and is currently the suggested endpoint for determining the *in vitro* activity of caspofungin against *Aspergillus* spp.[36–40] Furthermore, with mould infections, antifungal exposure detects activity against conidia rather than activity against the more clinically relevant hyphal structures.[41]

Disk-based assays

Disk-based susceptibility testing is convenient, simple and economical. A CLSI reference method (M 44A)[42] exists for *in vitro* susceptibility testing of *Candida* spp. and disk breakpoints have been suggested: for fluconazole (disks with 25 μg fluconazole) $S \geq 19$ mm; SDD = 15–18 mm and $R \leq 14$ mm.[28,42] The corresponding disk test breakpoints for voriconazole (disks with 1 μg voriconazole) are as follows: $S \geq 17$ mm; SDD = 14–16 mm and $R \leq 13$ mm.[29,42] The choice of growth medium appears critical; some investigators use RPMI-1640 agar supplemented with 0.2% glucose, whereas the CLSI recommends the use of Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg ml^{-1} methylene blue.[42] Disk diffusion is also suitable for determining the activity of echinocandins against yeast as it produces easy to read and sharp zones of inhibition.[43]

For moulds, the correlation between zone size and MIC is somewhat variable.[44] However, with the use of YNB medium, authors concluded the technique to be reliable, cost effective and easy to perform, with consistent results.[45] A multicentre evaluation was performed to correlate inhibition zone diameters with broth dilution MICs of five antifungal agents.[46] Based on these results, the optimal testing conditions for *Aspergillus* disk diffusion testing were: (i) plain MH agar, (ii) incubation times of 24 h for *A. fumigatus*, *A. flavus* and *A. niger* and 48 h for other species and (iii) posaconazole 5 μg, voriconazole 1 μg, itraconazole 10 μg, caspofungin 5 μg and amphotericin B 5 μg disks. Agar-based methods hold promise as simple and reliable methods for determining susceptibilities of filamentous fungi.[47]

Commercial kits

Etest (AB Biodisk, Solna, Sweden) directly quantifies antifungal susceptibility in terms of discrete MIC values.

The choice of growth medium appears critical with the Etest technique, and RPMI-based agars seem to be the most useful.[48] Others apply Mueller-Hinton agar supplemented with 2% glucose and $0.5\ \mu g\ ml^{-1}$ methylene blue, which appears to enhance the formation of inhibition ellipses with clear edges and less intra-elliptic growth.[49] The method is suitable for yeast and moulds, and is a reliable and reproducible method. The results correlate well with the CLSI methodology.[50] Alexander *et al.* [51] evaluated the Etest with Sensititre Yeastone against CLSI methodology for yeast and seven antifungals, and obtained an excellent agreement (95%) between the reference test method and the Etest. Categorical agreement was the lowest for *C. glabrata* and *C. tropicalis*. Etest provided better agreement at 24 h compared to that at 48 h for *C. glabrata*.

A clear benefit of utilising Etest is assessing the susceptibility to amphotericin B, as this method gives much broader MIC ranges. Etest is also highly suitable for determining the activity of echinocandins against yeast as it produces easy to read, sharp zones of inhibition.[43]

MIC reading of echinocandins against *Aspergillus* sp. might be troublesome because of heavy growth (macro- and microcolonies) within a discrete ellipse. The meaning of the growth within the zone of inhibition is not clear (Fig. 1).

Sensititre YeastOne (TREK Diagnostic Systems), a colorimetric antifungal panel, has been favourable compared with the CLSI methodology. Yeast has proved to be easy to interpret. Voriconazole, anidulafungin, caspofungin, micafungin and posaconazole are recently included on the test plates, making this methodology useful.[4,52] Excellent agreement between the reference test method and Sensititre (91%) was observed. Sensititre showed a ≥92% agreement for MICs for itraconazole, flucytosine, amphotericin B and caspofungin, but 82% for fluconazole and 85% for voriconazole. Categorical agreement was the lowest for *C. glabrata* and *C. tropicalis*, and Sensititre provided better agreement at 24 h compared to that at 48 h for *C. glabrata*.[51]

Avolio *et al.* [53] tested the turnaround time for susceptibility testing directly from the bottle of blood culture positive for yeast, determining MIC as quickly as possible. Of a total of 40 strains tested, no very major errors or major errors occurred.

Sensititre YeastOne has also been favourable compared with CLSI methodology with amphotericin B,[54] itraconazole,[54] voriconazole[54,55] and posaconazole for *Aspergillus* spp.[56] Slight discrepancies were found because of higher Sensititre MICs. Overall, Sensititre YeastOne method could have potential value for susceptibility testing of *Aspergillus* spp. to voriconazole and is able to detect resistance to itraconazole.[54,56]

The ATB Fungus 2 (bioMérieux, La Balme-les Grottes, France) was compared with the SensititreYeastOne for antifungal susceptibility testing of yeast;[57] it was concluded that this method could be used as an alternative for susceptibility testing of common *Candida* spp.[58] The agreement between these two methods was

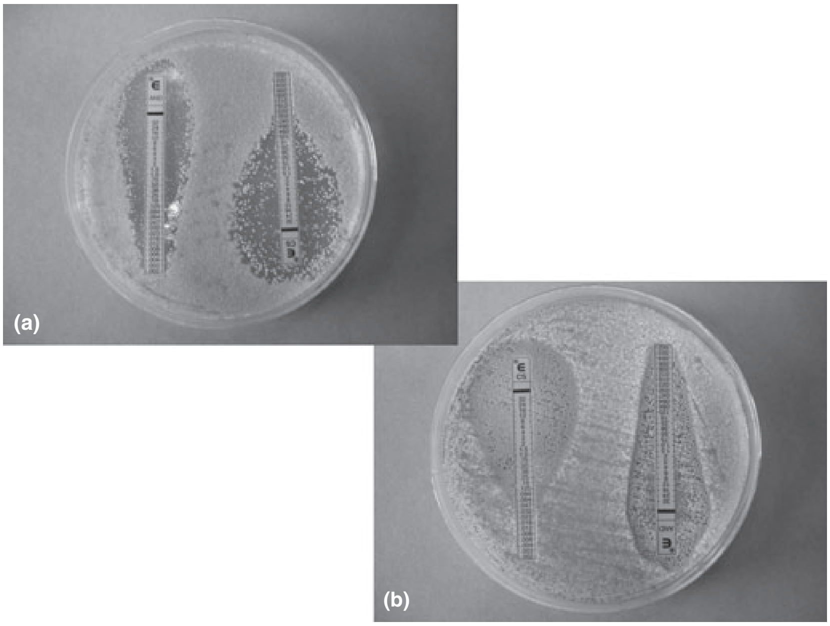

Figure 1 Etest MICs of $0.002\ \mu g\ ml^{-1}$ and $0.047\ \mu g\ ml^{-1}$ for caspofungin and anidulafungin for *Aspergillus fumigatus* read at 24 h (a). Microcolonies and macrocolonies within the ellipse, MICs $>8\ \mu g\ ml^{-1}$ for both candins (b).

assessed with a total of 133 *Candida* strains and MIC endpoints were read after 24 h. Overall agreement between ATB Fungus 2 and Sensititre YeastOne was 91–97% for amphotericin B, 5-fluorocytosine and itraconazole, and 82% for fluconazole. The categorical agreement for the ATB Fungus 2 was lower for the triazoles (72.9–75.9%) when compared with that for SensititreYeastOne.

The VITEK 2, a fully automated commercial antifungal susceptibility testing system (bioMérieux, Inc., Hazelwood, MO, USA), was compared with the CLSI reference broth microdilution method by testing 2 quality control strains, 10 reproducibility strains and 426 isolates of *Candida* spp. against amphotericin B, flucytosine and voriconazole.[59] The system reliably detected flucytosine and voriconazole resistance among *Candida* spp. and demonstrated excellent quantitative and qualitative agreement with the reference method. Similar result was observed for fluconazole.[60] In another study, an excellent categorical agreement of Vitek 2 with the CLSI broth microdilution method was observed (97.5% for fluconazole and voriconazole). The Vitek 2 was able to identify all but 2 of 59 investigated fluconazole-resistant organisms.[61]

A method using a commercially prepared colorimetric microdilution panel (ASTY; Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) was compared in four different laboratories with the CLSI reference microdilution method by testing 802 clinical isolates of *Candida* spp. (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae*, *C. guilliermondii*, *C. lipolytica*, *C. rugosa* and *C. zeylanoides*) against amphotericin B, 5-fluorocytosine, fluconazole and itraconazole.[62] The ASTY colorimetric microdilution panel method appears to be comparable with the CLSI method for testing the susceptibility of *Candida* spp. to a variety of antifungal agents. The ASTY method was thus determined to be comparable with the CLSI method when testing the susceptibility of *Trichosporon asahii* to a variety of antifungal agents.[63]

Others

Other susceptibility tests are available, yet not for the daily routine. Incorporation of the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] or XTT (2,3-bis(2methoxy-4-nitro-5-desulfophenyl)-5-[(phenylamino) carbonyl] 2H-tetrazolium hydroxide) as a colorimetric marker for redox potential has been found to offer convenient possibilities for MIC reading for *Aspergillus*.[64,65] This approach generates MICs comparable with those in CLSI method and presents substantial opportunities for automation. Flow cytometry has been found to be a possible tool for antifungal susceptibility testing[66] and has been developed for yeast and moulds.[67] Staining or lack of staining with suitable dyes permits the rapid detection of damaged or inactive fungi. This method can distinguish *Aspergillus* isolates susceptible to amphotericin B from those that are resistant.[68] In conceptually related studies, fluorescent viability dyes have been used to examine the nature of drug-induced damage and to estimate minimum fungicidal concentrations (MFCs) for aspergilli.[41] Given that azole antifungal agents act by inhibition of ergosterol synthesis, direct measurement of alterations in ergosterol synthesis appears relevant. Arthington-Skaggs *et al.* [69] have described a workable laboratory method for antifungal susceptibility testing for *Candida* spp. and *Aspergillus* spp.

Combination antifungal susceptibility

The high rate of mortality from mould infections and the relatively limited efficacies of current agents have produced a significant interest in the use of antifungal combinations in these difficult-to-treat infections.[70] *In vitro* antifungal combination testing is controversial; tests are difficult to assess and the results depend on the methodology and analysis used.[70] The chequerboard dilution method and time–kill studies have become the most widely accepted techniques. In the classic checkerboard dilution scheme, all testing parameters remain the same, including medium, inoculum and incubation. The final result is the lowest concentration of drug A plus the lowest concentration of drug B in which the endpoint criteria are met. The MIC of each drug within the combination is expressed as a fraction of each drug alone. The fractions are then added to arrive at the fractional inhibitory concentration index (FICI).[71] Synergism, indifference and antagonism are achieved when the FICI is ≤ 0.5, >0.5 to ≤ 4 and >4. Time–kill studies can help elucidate the pharmacodynamics of an antifungal combination by measuring the effects of the antifungal interaction on the rate and extent of fungal killing.[72]

Lewis *et al.* [73] examined the utility of Etest for testing antifungal combinations (amphotericin B–fluconazole) against *Candida* spp. and indicated that this method could be used as an alternative to time–kill studies. Criteria were recommended by the manufacturer: synergy was defined as a decrease of ≥ 3 dilutions in the resultant MIC, additivity as a decrease of ≥ 2 but <3 and indifference as a decrease of <2 dilutions in the MIC. Antagonism was defined as an increase of ≥ 3 dilutions of the MIC for the antifungal combination.

Determination of MFCs

Lots of discussion are taking place on whether MICs or MFCs should be taken into account for patient management. This topic is still in progress and needs further attention. All of the issues of standardisation that occur with MICs also apply to MFCs. Many variables such as size of inoculum, incubation period, drug carry over, sample volume and endpoint influence the test outcome.[74,75]

Minimum fungicidal concentrations have the potential for being more relevant to clinical outcome, especially in the context of profoundly immunosuppressed hosts. The poor *in vitro* fungicidal activity of amphotericin B appears to correspond with the refractory nature of *A. terreus* infections to therapy with this agent.[76] Both Johnson *et al.* [77,78] and Walsh *et al.* [53] correlated the low *in vitro* fungicidal resistance of *A. terreus* to amphotericin B with *in vivo* resistance in a persistently neutropenic rabbit model of experimental invasive aspergillosis.

Challenges in the interpretation of susceptibility results

Microbiologists and clinicians are still faced with the challenge of interpreting the results of *in vitro* antifungal susceptibility tests. MIC values do not always directly associate with response to antifungal therapy.[44,79]

The discordance between *in vivo* and *in vitro* data is illustrated by the '90–60 rule', which maintains that infections caused by susceptible strains respond to appropriate therapy in ~90% of cases, whereas infections caused by resistant strains respond in ~60% of cases.[80]

The most important factors associated with poor outcome are negative host status, delay of early diagnosis and a lack of adequate antifungal therapy. Another factor might be infection with drug-resistant fungal pathogens.[3] The immune reconstitution inflammatory syndrome for example is associated with prominent signs and symptoms of inflammation and can therefore be confused with failure to control fungal growth. One data support that early treatment of fungal infection with a lower burden of organisms reduces the number of treatment failures.[81,82] On the contrary, toxicities from polyenes (nephrotoxicity) and azoles can be a cause of treatment failure,[83] drug–drug interactions can contribute to morbidity and mortality[3,84] and finally, the ability of fungi to form biofilms on foreign bodies is a primary reason for clinical failure.

The frequency of *in vitro* and *in vivo* resistance to antifungal drugs

A significant antifungal drug resistance has emerged by azole-resistant yeast isolates from patients with chronic mucocutaneous candidosis treated for prolonged periods, by flucytosine resistance occurring in patients with invasive candidosis or by cryptococcosis treated with flucytosine monotherapy.[85–87]

In general, azole resistance in *C. albicans* is less common among patients with other diseases, such as vaginal candidiasis and candidaemia.[88–90] Reported rates are about 1.0–2.1% in *C. albicans*, 0.4–4.2% in *C. parapsilosis* and 1.4–6.6% in *C. tropicalis*.[88–90] A clear exception is *C. glabrata*, which is second to *C. albicans* in causing systemic fungal infections in Europe.[43,91] The incidence of fluconazole resistance in *C. glabrata* increased from 7% in 2001 to 12% in 2004.[91]

There have been recent reports of echinocandin resistance in patients with *Candida* infections (caused by *C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis*).[88,90] Resistance to echinocandins developed during therapy and was associated with treatment failure.[92] Resistance mechanisms other than Fks1 mutations were involved in some cases.[93]

Although resistance to amphotericin B among *Candida* strains remains rare, there have been recent reports of increasing MICs to amphotericin B among *C. krusei* and *C. glabrata* isolates.[85] In addition, intrinsic polyene resistance is frequently noted in *Candida lusitaniae* and *T. asahii*.[85,94]

Although *Aspergillus* species, particularly *Aspergillus fumigatus*, account for the largest proportion of invasive mould infections, the last decade has witnessed the emergence of new opportunistic pathogens, including non-fumigatus *Aspergillus* species, *Fusarium* species, *Paecilomyces* species, *Scedosporium* species, the dematiaceous fungi (*Alternaria*, *Bipolaris*, *Curvularia*, *Cladosporium* and *Exserohilum* species) and the agents of zygomycosis (mucormycosis).[95]

Filamentous fungi are more likely than yeasts to have reduced susceptibility to polyenes. Among *Aspergillus* species, *Aspergillus terreus* is generally resistant to amphotericin B.[96] Polyene resistance is increasingly encountered in other *Aspergillus* species, such as *Aspergillus flavus* and even *A. fumigatus*, which traditionally exhibits the highest susceptibility to amphotericin B.[97,98] A total of 10 variants of multidrug-resistant *A. fumigatus* clinical isolates were identified, all of which had an unusual sporulation pattern and a unique mitochondrial cytochrome *b* sequence.[98] These isolates exhibited increased MICs against all the triazoles tested.

These *A. fumigatus* variants were tested in a guinea pig model and were found to retain virulence *in vivo*. Phylogenetic analysis based on genetic studies indicated that most of these isolates belong to a new *Aspergillus* species, *A. lentulus.*[97] Within the aspergilli, the resistance of *A. fumigatus* to itraconazole is admitted, where an isolate with an MIC value of greater than 8 $\mu g\ ml^{-1}$ to itraconazole is considered as resistant.[7,99] In that case, *in vitro* resistance has been correlated with resistance *in vivo.*[15,100] Multiple-azole (itraconazole, voriconazole, posaconazole and isavuravuconazole) resistant *A. fumigatus* clinical isolates have been reported with increasing frequency[8,101] and *in vivo* correlation. For *A. fumigatus*, MICs of voriconazole and posaconazole of $>4\ \mu g\ ml^{-1}$ and $>1\ \mu g\ ml^{-1}$ respectively seem to be elevated when compared with that for wild-type population and therefore are referred to as resistant strains (tentative breakpoints).[102] There have been recent reports of echinocandin resistance (MIC $>16\ \mu g\ ml^{-1}$) in patients with *Aspergillus* infections.[103]

Other moulds, such as *Scedosporium apiospermum*, *Scedosporium prolificans*[104,105] and *Fusarium* species, are typically resistant to amphotericin B.[106]

How and when to use antifungal susceptibility testing

Susceptibility testing helps to define the spectrum of activity of an available antifungal agent.

Clinically relevant fungi need to be addressed as follows:

- Identify the isolate at least to the genus level, better to species level.
- For *Candida* species from sterile sites, perform routine susceptibility testing for fluconazole and according to the local epidemiology include other azoles.
- Perform susceptibility testing as an adjunctive to treatment for patients with invasive disease and clinical failure of initial therapy, or with break-through infection.
- Isolates with a high rate of intrinsic resistance need not usually to be tested; *C. krusei* and fluconazole, and *A. terreus* and amphotericin B.
- Perform susceptibility testing as an adjunctive to treatment for patients with invasive disease, long-term azole treatment and/or recurrent cultivation of a fungus.
- Perform susceptibility testing as an adjunctive to treatment for patients with invasive disease and infection with rare moulds or other fungi.
- Take into account the role of cross-resistance and broaden the agents to be tested, if necessary.

For choosing the best drug, take into account the identified fungus, the local epidemiology, antifungal pretreatment, the severity of the infection, the patient's immune status, the ability of a drug to reach levels at infection site, the ability to identify and control the site of infection, the speed of clinical response, the consequences of recurrence of infection, drug's safety and toxicity, drug–drug interactions and the magnitude of the resistance.

Conclusion

Overall, each *in vitro* susceptibility testing method has its own advantages and disadvantages. The reference EUCAST and CLSI standard methods are cumbersome and not directed for daily routine; the Etest is a relatively expensive, yet an attractive alternative method so far. MICs can be useful in the selection and monitoring of the best therapeutic agent, yet MIC is not the only predictor of *in vivo* response to therapy.

References

1 Patterson T. Advances and challenges in management of invasive mycoses. *Lancet* 2005; **17**: 1013–1025.

2 Neofytos D, Horn D, Anaissie E *et al.* Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. *Clin Infect Dis* 2009; **48**: 265–273.

3 Kanafani Z, Perfect J. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. *Clin Infect Dis* 2008; **46**: 120–128.

4 Johnson E. Issues in antifungal susceptibility testing. *J Antimicrob Chemother* 2008; **61**: i13–i18.

5 Weiler S, Lass-Flörl C, Auberger J, Bellmann-Weiler R, Stein M, Joannidis M. Triazole-resistant candidaemia following posaconazole exposure. *Int J Antimicrob Agents* 2008; **33**: 494–495.

6 Auberger J, Lass-Flörl C, Clausen J *et al.* First case of breakthrough pulmonary *Aspergillus niveus* infection in a patient after allogeneic hematopoietic stem cell transplantation. *Diagn Microbiol Infect Dis* 2008; **62**: 336–339.

7 Howard S, Webster I, Moore BC *et al.* Multi-azole resistance in *Aspergillus fumigatus*. *Int J Antimicrob Agents* 2006; **28**: 450–453.

8 Snelders E, van der Lee H, Kuijper J *et al.* Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *Plos Med* 2009; **5**: e 219.

9 Gulshan K, Moye-Rowley W. Multidrug resistance in fungi. *Eukaryot Cell* 2007; **6**: 1933–1942.

10 Moore C, Walls C, Denning D. In vitro activity of the new triazole BMS-207147 against *Aspergillus* species in

comparison with itraconazole and amphotericin B. *Antimicrob Agents Chemother* 2000; **44**: 441–443.

11 Pavie J, Lacroix C, Hermoso D *et al.* Breakthrough disseminated *Aspergillus ustus* infection in allogeneic hematopoietic stem cell transplant recipients receiving voriconazole or caspofungin prophylaxis. *J Clin Microbiol* 2005; **43**: 4902–4904.

12 Warnock D, Arthington-Skaggs BA, Ren-Kai L. Antifungal drug susceptibility testing and resistance in *Aspergillus*. *Drug Resist Update* 1999; **2**: 326–334.

13 Gehrt A, Peter J, Pizzo P, Walsh T. Effect of increasing inoculum sizes of pathogenic filamentous fungi on MICs of antifungal agents by broth microdilution method. *J Clin Microbiol* 1995; **33**: 1302–1307.

14 Meletiadis J, Meis J, Mouton J, Verweij PE. Analysis of growth characteristics of filamentous fungi in different nutrient media. *J Clin Microbiol* 2001; **39**: 478–484.

15 Gomez-Lopez A, Aberkane A, Petrikkou E, Mellado E, Rodriguez-Tudela JL. Analysis of the influence of tween concentration, inoculum size, assay medium, and reading time on susceptibility testing of *Aspergillus* spp. *J Clin Microbiol* 2005; **43**: 1251–1255.

16 Rodriguez-Tudela JL, Chryssanthou E, Petrikkou E, Mosquera J, Dennind DW, Cuenza-Estrella M. Interlaboratory evaluation of hematocytometer method of inoculum preparation for testing antifungal susceptibilities of filamentous fungi. *J Clin Microbiol* 2003; **41**: 5236–5237.

17 Aberkane A, Cuenca-Estrella M, Gomez-Lopez A *et al.* Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. *J Antimicrob Chemother* 2002; **50**: 719–722.

18 Petrikkou E, Rodriguez-Tudela JL, Cuenca-Estrella M, Gomez A, Molleja A, Mellado E. Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. *J Clin Microbiol* 2001; **39**: 1345–1347.

19 Clinical Laboratory Standard Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi*. Approved standard. M38-A2. Wayne, PA: Clinical Laboratory Standard Institute, 2008.

20 Clinical Laboratory Standard Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. Approved standard, M27-A3. Wayne, PA: Clinical Laboratory Standard Institute, 2008.

21 Subcommittee of Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases. EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. *Clin Microbiol Infect* 2008; **14**: 398–405.

22 Subcommittee of Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology Infectious Diseases. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. http://www escmid org 2007.

23 Cuenca-Estrella M, Arenderup M, Chryssanthou E *et al.* Multicentre determination of quality control strains and quality control ranges for antifungal susceptibility testing of yeasts and filamentous fungi using the methods of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST). *Clin Microbiol Infect* 2007; **13**: 1018–1022.

24 Espinel-Ingroff A, Bartlett M, Bowden R *et al.* A multicenter evaluation of the standardization of antifungal susceptibility testing for filamentous fungi. *J Clin Microbiol* 2004; **35**: 139–143.

25 European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST). EUCAST technical note on fluconazole. *Clin Microbiol Infect* 2008; **14**: 193–195.

26 Arendrup M, Kahlmeter G, Rodriguez-Tudela JL, Donnelly JP. Breakpoints for susceptibility testing should not divide wild-type distributions of important target species. *Antimicrob Agents Chemother* 2009; **53**: 1628–1629.

27 Subcommittee of Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases. EUCAST technical note on voriconazole. *Clin Microbiol Infect* 2008; **14**: 985–987.

28 Pfaller MA, Diekema DJ, Sheehan DJ. Interpretive breakpoints for fluconazole and *Candida* revisited: a blueprint for the future of antifungal susceptibility testing. *Clin Microbiol Rev* 2006; **19**: 435–447.

29 Pfaller M, Diekema DJ, Rex J *et al.* Correlation of MIC with outcome for *Candida* species tested against voriconazole: analysis and proposal for interpretive breakpoints. *J Clin Microbiol* 2006; **44**: 819–826.

30 Pfaller M, Diekema DJ, Ostrosky-Zeichner L *et al.* Correlation of MIC with outcome for *Candida* species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints. *J Clin Microbiol* 2008; **46**: 2620–2629.

31 Lass-Flörl C, Cuenca-Estrella M, Denning D, Rodriguez-Tudela J. Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology. *Med Mycol* 2006; **44**(Suppl 1): 319–325.

32 Chryssanthou E, Cuenca-Estrella M. Comparison of the EUCAST-AFST broth dilution method with the CLSI reference broth dilution method (M38-A) for susceptibility testing of posaconazole and voriconazole against *Aspergillus* spp. *Clin Microbiol Infect* 2006; **12**: 901–904.

33 Pfaller M, Diekema DJ, Ghannoum A *et al.* Wild type MIC distribution and epidemiological cutoff values for *Aspergillus fumigatus* and three triazoles as determined by the Clinical and Laboratory Standards Institute

Broth Microdilution Methods. *J Clin Microbiol* 2009; **47**: 3142–3146.

34 Rodriguez-Tudela J, Cazar-Fuoli L, Mellado E, Astruey-Izquierdo A, Monzon A, Cuenca-Estrella M. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 2008; **52**: 2468–2472.

35 Lass-Flörl C, Griff K, Mayr A *et al.* Epidemiology and outcome of infections due to *Aspergillus terreus*: 10-year single centre experience. *Br J Haematol* 2005; **131**: 201–207.

36 Kartsonis N, Nielsen J, Douglas C. Caspofungin: the first in a new class of antifungal agents. *Drug Resist Update* 2003; **6**: 197–218.

37 Kurtz M, Heath I, Marrinan J, Dreikorn S, Onishi J, Douglas C. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-ß-d-glucan synthase. *Antimicrob Agents Chemother* 1994; **38**: 1480–1489.

38 Arikan S, Lozano-Chiu M, Paetznick V, Rex J. In vitro susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. *Antimicrob Agents Chemother* 2001; **45**: 327–330.

39 Odds F, Motyl M, Andrade R *et al.* Interlaboratory comparison of results of susceptibility testing with caspofungin against *Candida* and *Aspergillus* species. *J Clin Microbiol* 2004; **42**: 3475–3482.

40 Espinel-Ingroff A. Utility of mould susceptibility testing. *Curr Opin Infect Dis* 2003; **16**: 527–532.

41 Lass-Flörl C, Nagl M, Speth C, Ulmer H, Dierich MP, Würzner R. Studies of in vitro activities of voriconazole and itraconazole against *Aspergillus* hyphae using viability staining. *Antimicrob Agents Chemother* 2001; **45**: 124–128.

42 Clinical Laboratory Standard Institute. *Methods for Antifungal Disk Diffusion Susceptibility Testing of Yeasts: Approved Standard M44-A*. Wayne, PA: Clinical Laboratory Standard Institute, 2004.

43 Pfaller M, Boyken L, Hollis RJ, Messer SA, Tendolkar S, Diekema DJ. In vitro susceptibilities of *Candida* spp. to caspofungin: four years of global surveillance. *J Clin Microbiol* 2006; **44**: 760–763.

44 Rex JH, Pfaller M, Rinaldi MG, Polak A, Galgani NJ. Antifungal susceptibility testing. *Clin Microbiol Rev* 1993; **6**: 367–381.

45 Therese K, Bagyalakshmi R, Madhavan N, Deepa P. In-vitro susceptibility testing by agar dilution method to determine the minimum inhibitory concentrations of amphotericin B, fluconazole and ketoconazole against ocular fungal isolates. *Indian J Med Microbiol* 2007; **24**: 273–279.

46 Espinel-Ingroff A, Arthington-Skaggs BA, Igbal N *et al.* A multicenter evaluation of a new disk agar diffusion method for susceptibility testing of filamentous fungi with voriconazole, posaconazole, itraconazole, amphotericin-B and caspofungin. *J Clin Microbiol* 2007; **45**: 1811–1820.

47 Messer SA, Diekema DJ, Hollis RJ *et al.* Evaluation of disk diffusion and Etest compared to broth microdilution for antifungal susceptibility testing of posaconazole against clinical isolates of filamentous fungi. *J Clin Microbiol* 2007; **45**: 1322–1324.

48 Pfaller M, Messer SA, Mills K, Bolmstrom A. In vitro susceptibility testing of filamentous fungi: comparison of Etest and reference microdilution methods for determining itraconazole MICs. *J Clin Microbiol* 2000; **38**: 3361.

49 Pfaller M, Boyken L, Messer SA, Tendolkar S, Hollis RJ, Diekema DJ. Evaluation of the etest method using Mueller-Hinton agar with glucose and methylene blue for determining amphotericin B MICs for 4,936 clinical isolates of *Candida* species. *J Clin Microbiol* 2004; **42**: 4977–4979.

50 Szekely A, Johnson E, Warnock D. Comparison of E-test and broth microdilution methods for antifungal drug susceptibility testing of moulds. *J Clin Microbiol* 1999; **37**: 1483.

51 Alexander BD, Byrne T, Smith K *et al.* Comparative evaluation of Etest and sensititre yeastone panels against the Clinical and Laboratory Standards Institute M27-A2 reference broth microdilution method for testing *Candida* susceptibility to seven antifungal agents. *J Clin Microbiol* 2007; **45**: 698–706.

52 Pfaller M, Chaturvedi V, Diekema DJ *et al.* Clinical evaluation of the Sensititre YeastOne colorimetric antifungal panel for antifungal susceptibility testing of the echinocandins anidulafungin, caspofungin, and micafungin. *J Clin Microbiol* 2008; **46**: 2155–2159.

53 Avolio M, Grosso S, Bruschetta G, De Rosa R, Camporese A. Direct antifungal susceptibility testing of positive *Candida* blood cultures by sensititre YeastOne. *New Microbiol* 2009; **32**: 179–184.

54 Guinea J, Pelaez T, Alcala L, Bouza E. Correlation between the E test and the CLSI M-38 A microdilution method to determine the activity of amphotericin B, voriconazole, and itraconazole against clinical isolates of *Aspergillus fumigatus*. *Diagn Microbiol Infect Dis* 2006; **56**: 53–55.

55 Castro C, Serrano MC, Flores B, Espinel-Ingroff A, Martin-Mazuelos E. Comparison of the Sensititre YeastOne colorimetric antifungal panel with a modified NCCLS M38-A method to determine the activity of voriconazole against clinical isolates of *Aspergillus* spp. *J Clin Microbiol* 2004; **42**: 4358–4360.

56 Martin-Mazuelos E, Peman J, Valverde A, Chaves M, Serrano MC, Canton E. Comparison of the Sensititre YeastOne colorimetric antifungal panel and Etest with the NCCLS M38-A method to determine the activity of amphotericin B and itraconazole against clinical isolates of *Aspergillus* spp. *J Antimicrob Chemother* 2003; **52**: 365–370.

57 Torres-Rodriguez J, Alvarado-Ramirez E. In vitro susceptibilities to yeasts using the ATB FUNGUS 2 method, compared with Sensititre Yeast One and standard CLSI

(NCCLS) M27-A2 methods. *J Antimicrob Chemother* 2007; **60**: 658–661.

58 Eraso E, Ruesqa M, Villar-Vidal M, Carrillo-Muñoz A, Espinel-Ingroff A, Quindos G. Comparative evaluation of ATB Fungus 2 and Sensititre YeastOne panels for testing in vitro *Candida* antifungal susceptibility. *Rev Iberoam Micol* 2008; **25**: 3–6.

59 Pfaller M, Diekema DJ, Procop GW, Rinaldi M. Multicenter comparison of the VITEK 2 antifungal susceptibility test with the CLSI broth microdilution reference method for testing amphotericin B, flucytosine, and voriconazole against *Candida* spp. *J Clin Microbiol* 2007; **45**: 3522–3528.

60 Pfaller M, Diekema DJ, Procop GW, Rinaldi M. Multicenter comparison of the VITEK 2 yeast susceptibility test with the CLSI broth microdilution reference method for testing fluconazole against *Candida* spp. *J Clin Microbiol* 2007; **45**: 802.

61 Posteraro B, Martucci R, la Sorda M *et al.* Reliability of the Vitek 2 yeast susceptibility test for detection of in vitro resistance to fluconazole and voriconazole in clinical isolates of *Candida albicans* and *Candida glabrata*. *J Clin Microbiol* 2009; **47**: 1927–1930.

62 Pfaller M, Arikan S, Lozano-Chiu M *et al.* Clinical evaluation of the ASTY colorimetric microdilution panel for antifungal susceptibility testing. *J Clin Microbiol* 1998; **36**: 2609–2612.

63 Kalkanci A, Mekha N, Poonwan N, Makimura K, Sugita T. Comparative evaluation of *Trichosporon asahii* susceptibility using ASTY colorimetric microdilution and CLSI M27-A2 broth microdilution reference methods. *Microbiol Immunol* 2008; **52**: 435–439.

64 Jahn B, Stuben A, Bhakdi S. Colorimetric susceptibility testing for *Aspergillus fumigatus*: comparison of menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide and Alamar blue tests. *J Clin Microbiol* 1996; **34**: 2039–2041.

65 Meletiadis J, Meis JFGM, Mouton JW, Donnelly JP, Verweij PE. Comparison of NCCLS and 3-(4,5-Dimethyl-2-Thiazyl)-2,5-Diphenyl-2H-Tetrazolium Bromide (MTT) methods of in vitro susceptibility testing of filamentous fungi and development of a new simplified method. *J Clin Microbiol* 2000; **38**: 2949–2954.

66 Vale-Silvia L, Buchta V. Antifungal susceptibility testing by flow cytometry: is it the future? *Mycoses* 2006; **49**: 261–273.

67 Ramani R, Gangwar M, Chaturvedi V. Flow cytometry antifungal susceptibility testing of *Aspergillus fumigatus* and ocmparison of mode of action of voriconazole vis-a-vis amphotericin B and itraconazole. *Antimicrob Agents Chemother* 2003; **47**: 3627–3629.

68 Balajee SA, Marr KA. Conidial vability assay for rapid susceptibility testing of *Aspergillus* species. *J Clin Microbiol* 2002; **40**: 2741–2745.

69 Arthington-Skaggs BA, Jradi H, Desai T, Morrison CJ. Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol* 1999; **37**: 3332–3337.

70 Steinbach W, Stevens D, Dennind DW. Combination and sequential therapy for invasive aspergillosis: review of published in vitro and in vivo interactions and 6281 clinical cases from 1996 to 2001. *Clin Infect Dis* 2003; **37**: S188–S224.

71 Johnson M, MacDougall C, Ostrosky-Zeichner L, Perfect J, Rex J. Combination antifungal therapy. *Antimicrob Agents Chemother* 2004; **48**: 693–715.

72 Kiraz N, Dag I, Yamac M, Kiremitci A, Kasifoglu N, Akgun Y. Antifungal activity of caspofungin in combination with amphotericin B against *Candida glabrata*: comparison of disk diffusion, Etest, and time-kill methods. *Antimicrobial Agents and Chemptherapy* 2009; **53**: 788–790.

73 Lewis R, Diekema DJ, Messer SA, Pfaller M, Klepser ME. Comparison of Etest, chequerboard dilution and time–kill studies for the detection of synergy or antagonism between antifungal agents tested against *Candida* species. *Journal of Antimicrobial Agents* 2002; **49**: 345–351.

74 Pfaller M, Sheehan D, Rex J. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. *Clin Microbiol Rev* 2004; **17**: 280.

75 Peterson L, Shanholtzer C. Tests for bactericidal effects of antimicrobial agents: technical performance and clinical relevance. *Clin Microbiol Rev* 1992; **5**: 420–432.

76 Espinel-Ingroff A, Chaturvedi V, Fothergill A, Rinaldi MG. Optimal testing conditions for determining MICs and minimum fungicidal concentrations of new and established antifungal agents for uncommon molds: NCCLS collaborative study. *J Clin Microbiol* 2002; **40**: 3776–3781.

77 Walsh T, Petraitis V, Petraitiene R *et al.* Experimental pulmonary aspergillosis due to *Aspergillus terreus*: pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. *J Infect Dis* 2003; **188**: 305–319.

78 Johnson EM, Oakley KL, Radford SA *et al.* Lack of correlation of in vitro amphotericin B susceptibility testing with outcome in a murine model of *Aspergillus* infection. *J Antimicrob Chemother* 2000; **45**: 85–93.

79 Rex J, Pfaller M, Walsh T *et al.* Antifungal susceptibility testing: practical aspects and current challenges. *Clin Microbiol Rev* 2001; **14**: 643–658.

80 Rex J, Pfaller M. Has antifungal susceptibility testing come of age? *Clin Infect Dis* 2002; **35**: 982–989.

81 Sharma O, Chwogule R. Many faces of pulmonary aspergillosis. *Eur Resp J* 1998; **12**: 705–715.

82 Hope W, Walsh T, Denning D. The invasive and saprophytic syndromes due to *Aspergillus* spp. *Med Mycol* 2005; **43**: S207–S238.

83 Zmeili O, Soubani A. Pulmonary aspergillosis: a clinical update. *J Med* 2007; **100**: 317–334.

84 Smith J, Safdar N, Knasinski V *et al.* Voriconazole therapeutic drug monitoring. *Antimicrob Agents Chemother* 2006; **50**: 1570–1572.

85 Perlroth J, Choi B, Spellberg B. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med Mycol* 2007; **45**: 321–346.

86 Perea S, Patterson TF. Antifungal resistance in pathogenic fungi. *Antimicrobial Resistance* 2002; **35**: 1073–1080.

87 Marr KA, Seidel K, Slavin MA *et al.* Prolonged fluconazole prophylaxis is associated with persistent protection against candidiasis-related death in allogeneic marrow transplant recipients: long-term follow-up of a randomized, placebo-controlled trial. *Blood* 2000; **96**: 2055–2061.

88 Pfaller M, Rinaldi M, Diekema D. Results from the ARTEMIS DISK global antifungal surveillance study: a 6.5-year analysis of the worldwide susceptibility of yeasts to fluconazole and voriconazole using standardized disk diffusion testing. *J Clin Microbiol* 2005; **43**: 5848–5849.

89 Pfaller M, Pappas P, Wingard J. Invasive fungal pathogens: current epidemiological trends. *Clin Infect Dis* 2006; **43**: 3–14.

90 Pfaller M, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 2007; **20**: 133–163.

91 Bassetti M, Righi E, Costa A *et al.* Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infect Dis* 2006; **6**: 1–6.

92 Arendrup M, Garcia-Effron G, Buzina W *et al.* Breakthrough *Aspergillus fumigatus* and *Candida albicans* double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. *Antimicrob Agents Chemother* 2009; **53**: 1185–1193.

93 Perlin D. Resistance to echinocandin-class antifungal drugs. *Drug Resist Update* 2007; **10**: 121–130.

94 Lass-Florl C, Mayr A, Perkhofer S *et al.* The activities of antifungal agents against yeasts and filamentous fungi: assessment according to EUCAST methodology. *Antimicrob Agents Chemother* 2008; **52**: 3637–3641.

95 Lass-Flörl C. The changing face of epidemiology of invasive fungal diseases in Europe. *Mycoses* 2009; **52**: 197–205.

96 Lass-Flörl C, Alastruey-Izquiderdo A, Cuenca-Estrella M, Perkhofer S, Rodriguez-Tudela JL. In vitro activities of various antifungal drugs against *Aspergillus terreus*: Global assessment using the methodology of the European committee on antimicrobial susceptibility testing. *Antimicrob Agents Chemother* 2009; **53**: 794–795.

97 Balajee S, Gribskov J, Hanley E, Nickle D, Marr K. *Aspergillus lentulus* sp. nov., a new sibling Species of *A. fumigatus*. *Eukaryot Cell* 2005; **4**: 625–632.

98 Balajee S, Nickle D, Varga J, Marr K. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryot Cell* 2006; **5**: 1705–1712.

99 Denning DW, Venkateswarlu K, Oakley KL *et al.* Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 1997; **41**: 1364–1368.

100 Denning DW, Radford SA, Oakley KL, Hall L, Johnson EM, Warnock DW. Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatus* infection. *J Antimicrob Chemother* 1997; **40**: 401–414.

101 van der Linden J, Jansen R, Bresters D *et al.* Azole-resistant central nervous system aspergillosis. *Clin Infect Dis* 2009; **48**: 1111–1113.

102 Verweij P, Howard DH, Melchers WJG, Denning D. Azole resistance in *Aspergillus*: proposed nomenclature and breakpoints. *Drug Resist Rev* 2009 (in press).

103 Ruchel R, Perske C, Glass B, Basecke J. A case of pulmonary aspergillosis with lack of response to caspofungin. *Rev Iberoam Micol* 2006; **23**: 94–96.

104 Sahi H, Avery R, Minai O *et al. Scedosporium apiospermum* (*Pseudoallescheria boydii*) infection in lung transplant recipients. *J Heart Lung Transplant* 2007; **26**: 350–356.

105 Bonatti H, Goegele H, Tabarelli D *et al. Pseudallescheria boydii* infection after liver retransplantation. *Liver Transpl* 2007; **13**: 1068–1069.

106 Richardson C, Lass-Florl C. Changing epidemiology of systemic fungal infections. *Clin Microbiol Infect* 2008; **14**(Suppl 4): 5–24.