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Current status of antifungal susceptibility testing methods

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Antifungal susceptibility testing is a very dynamic field of medical mycology. Standardization of *in vitro* susceptibility tests by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST), and current availability of reference methods constituted the major remarkable steps in the field. Based on the established minimum inhibitory concentration (MIC) breakpoints, it is now possible to determine the susceptibilities of *Candida* strains to fluconazole, itraconazole, voriconazole, and flucytosine. Moreover, utility of fluconazole antifungal susceptibility tests as an adjunct in optimizing treatment of candidiasis has now been validated. While the MIC breakpoints and clinical significance of susceptibility testing for the remaining fungi and antifungal drugs remain yet unclear, modifications of the available methods as well as other methodologies are being intensively studied to overcome the present drawbacks and limitations. Among the other methods under investigation are Etest, colorimetric microdilution, agar dilution, determination of fungicidal activity, flow cytometry, and ergosterol quantitation. Etest offers the advantage of practical application and favorable agreement rates with the reference methods that are frequently above acceptable limits. However, MIC breakpoints for Etest remain to be evaluated and established. Development of commercially available, standardized colorimetric panels that are based on CLSI method parameters has added more to the antifungal susceptibility testing armamentarium. Flow cytometry, on the other hand, appears to offer rapid susceptibility testing but requires specified equipment and further evaluation for reproducibility and standardization. Ergosterol quantitation is another novel approach, which appears potentially beneficial particularly in discrimination of azole-resistant isolates from heavy trailers. The method is yet investigational and requires to be further studied. Developments in methodology and applications of antifungal susceptibility testing will hopefully provide enhanced utility in clinical guidance of antifungal therapy. However, and particularly in immunosuppressed host, *in vitro* susceptibility is and will remain only one of several factors that influence clinical outcome.

Keywords antifungal susceptibility, microdilution, Etest, flow cytometry, ergosterol quantitation, disk diffusion

Introduction

Development and standardization of antifungal susceptibility tests have constituted a remarkable progress in the field of medical mycology. While reference methods for testing at least some fungal genera are now available, the field of antifungal susceptibility assays is still very dynamic. The current research topics

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target to: (i) overcome the current limitations of the available methods, (ii) provide user-friendly and practical modifications, (iii) extend the utility of antifungal susceptibility tests to other fungal genera and species, and (iv) reveal the beneficial impact of antifungal susceptibility testing at different clinical settings. This review summarizes the current status and future directions of antifungal susceptibility testing methods.

Why do we need antifungal susceptibility tests?

Invasive fungal infections are now more important and troublesome than ever before. In the last two decades, there have been remarkable changes concerning the host factors, the infecting fungi, and the antifungal agents in clinical use. These changes include: (i) the increase in number of patients with profound immunosuppression and a related increase in incidence and mortality rates of invasive fungal infections that affect these patients [1,2], (ii) the development and emergence of new antifungal drugs [1], and (iii) the emergence and recognition of antifungal resistance [3–10]. As a result of the changing face of this dynamic triangle; the host, the antifungal therapeutics, and the infecting fungi, the need for development of a standard *in vitro* antifungal susceptibility assay essentially appeared for optimization of antifungal therapy and prediction of clinical outcome.

Development of standard susceptibility testing assays and currently used reference antifungal susceptibility testing methods

Clinical and Laboratory Standards Institute (CLSI) Reference Susceptibility Testing Assays

Multicenter studies to develop a standardized antifungal susceptibility testing assay were initiated by the Clinical and Laboratory Standards Institute (CLSI, formerly 'National Committee for Clinical Laboratory Standards' – NCCLS) in 1983. These studies eventually led to the documentation of reference microdilution methodologies for yeasts (including *Candida* spp. and *Cryptococcus neoformans*) (NCCLS, M27-A2) [11] and moulds (*Aspergillus* spp., *Fusarium* spp., *Rhizopus* spp., *Pseudallescheria boydii*, and the mycelial form of *Sporothrix schenckii*) (NCCLS, M38-A) [12]. The reference CLSI documents include antifungal susceptibility testing of amphotericin B, flucytosine, fluconazole, ketoconazole, itraconazole, and the new triazoles (posaconazole, ravuconazole, and voriconazole). The method is based on visual reading of minimum inhibitory concentration (MIC, µg/ml) values. As a modification, spectrophotometric reading has been studied by several investigators, resulting in favorable agreement rates with visual evaluation in general [13–16].

Following the documentation of CLSI microdilution method for yeasts, studies have focused on development of a correlated, user-friendly and practical format of this available methodology [17–20], and a standard antifungal disk diffusion susceptibility testing method for *Candida* vs. fluconazole and voriconazole is now also available (CLSI, M44-A) [21]. This reference disk diffusion assay uses Mueller-Hinton agar supplemented with 2% glucose and methylene blue as the test medium. This medium has been shown to be superior to RPMI agar supplemented to 2% glucose since it produces clear inhibition zone edges and less intrazonal growth, enabling the easy interpretation of inhibition zone diameters [22] (Fig. 1). A global antifungal surveillance study has been carried out by using the CLSI reference disk diffusion assay to determine the *in vitro* activities of fluconazole and voriconazole against *Candida* and other yeast species collected from 39 countries over a period of 6.5 years [18].

Some studies have focused on comparison of the disk diffusion method with the reference microdilution method, particularly for *Candida* and fluconazole. The results of these studies suggest that disk diffusion is a reproducible method which in general shows good correlation with the reference microdilution antifungal susceptibility testing assay [22–24]. Discordance of the disk diffusion and microdilution results in terms of the susceptibility category may be observed in some isolates [25]. Importantly and in some studies, the agreement of disk diffusion assay with the reference microdilution method appeared to be higher particularly for *Candida* strains that are susceptible to fluconazole [26].

The standard disk diffusion assay constituted a good model to be used for investigational purposes for testing other fungal genera and drugs as well. These studies include those that tested posaconazole against filamentous fungi [27], micafungin against *Aspergillus* [28], and caspofungin against *Aspergillus* and *Fusarium* [29]. Intrazonal growth was observed as a consistent finding when echinocandin disks were used against *Aspergillus.* The microscopic examination of these intrazonal colonies yielded short, stubby hyphal branchings [28,29].CLSI MIC breakpoints (µg/ml) and/or disk diffusion inhibition zone (mm) interpretive guidelines are currently available only for fluconazole, itraconazole, voriconazole, and flucytosine against *Candida* [11,12] (Table 1). There are no definitive

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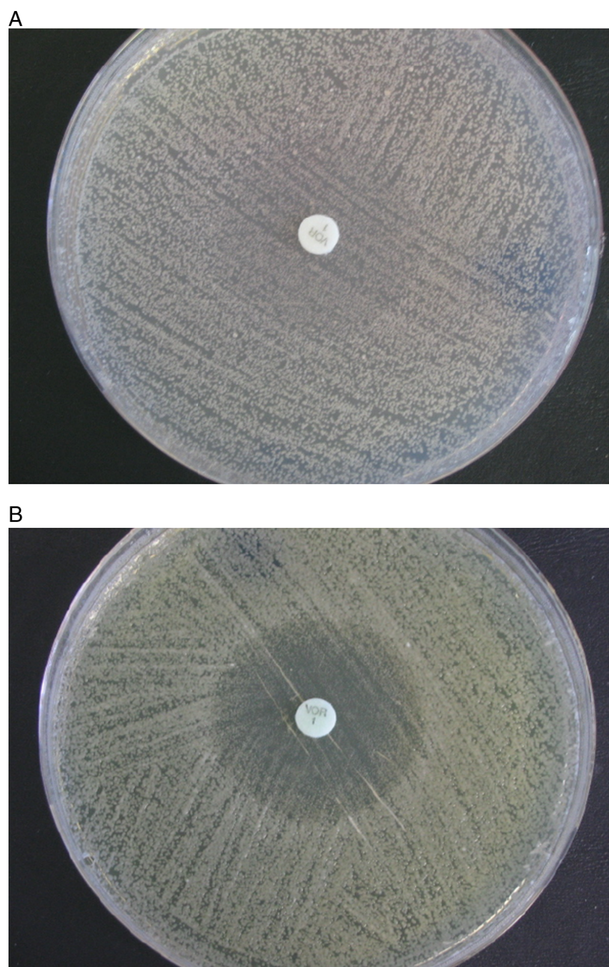

Fig. 1 Voriconazole disk diffusion assay for a *Candida albicans* strain (item code: 317). The assay was performed on two media; (a) RPMI agar supplemented to 2% glucose, and (b) Mueller-Hinton agar supplemented with 2% glucose and methylene blue. Mueller-Hinton agar supplemented with 2% glucose and methylene blue produces clearer inhibition zone edge and less intrazonal growth. (From the collection of Hacettepe University Medical School Mycology Laboratory).(Please see colour online)

guidelines yet for determination of the susceptibility categories of other fungal genera-antifungal drug combinations. The available proposed breakpoint data for fungi other than *Candida* and for drugs other than those noted above remain investigational [23,30].

Utility of CLSI Antifungal Susceptibility Testing Methods for Other Drugs

The reference CLSI methodologies do not include fungi and/or antifungal drugs other than those noted above. However, these methodologies constitute a good model and are being commonly investigated also for testing other yeast and mould genera, and other drugs including new echinocandins (caspofungin, micafungin, anidulafungin) and terbinafine [27–29,31–39]. These data remain at least partially experimental and will hopefully and eventually serve for standardization of the method for the remaining fungal genera and antifungal drugs as well.

For echinocandins against *Candida*, the currently accepted protocol applies CLSI M27-A2 as the test method, 24 h as the incubation period, and MIC-2 (~50% reduction in turbidity as compared to the growth control well) as the MIC endpoint [30,40–43]. For echinocandins against *Aspergillus* and possibly other moulds, the use of minimum effective concentration (MEC, μg/ml; lowest concentration of the drug yielding conspicuously aberrant, short, stubby hyphal growth) as the MIC endpoint appears to produce the most reproducible MIC data [28,43–46].

European Committee for Antimicrobial Susceptibility Testing (EUCAST) Standard Susceptibility Testing Assays

Following the documentation of CLSI methodology, studies have been carried out by Subcommittee on Antifungal Susceptibility Testing (AFST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), EUCAST to develop a broth dilution assay for susceptibility testing of yeasts. The EUCAST reference method for determination of minimum inhibitory concentration (MIC, μg/ml) by broth dilution for fermentative yeasts has been documented in EUCAST discussion document E.Dis 7.1 [47]. The documented EUCAST microdilution method has been developed for testing clinically significant yeasts that can ferment glucose. It is validated primarily for *Candida* spp. and does not cover *Cryptococcus neoformans* and other nonfermentative yeasts.

The EUCAST method is principally similar to the CLSI M27-A2 assay with modifications concerning some of the test parameters (Table 2). Multicenter evaluation of the EUCAST microdilution assay validated the reproducibility of the assay [48]. Comparative studies showed that the MIC-based correlation between CLSI and EUCAST methods within a 3-dilution range are in general good (varying between 85 and 95%, depending on the species, drug, and incubation time) when testing amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, and flucytosine against *Candida* spp. [49,50]. However, the EUCAST method tended to produce lower MICs as compared to the CLSI method against *Candida*. When the comparison was based on the susceptibility category using the CLSI MIC breakpoints for fluconazole, itraconazole, and voriconazole, the agreement between the two

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Table 1 CLSI: (a) MIC breakpoints and (b) disk diffusion inhibition zone interpretive guidelines for *Candida* [11,17,21]
(a)

Antifungal drug	MIC (μg/ml)			
	Susceptible (S)	Dose-dependent susceptible (S-DD)**	Intermediate (I)***	Resistant (R)
Fluconazole*	≤8	16–32	–	≥64
Itraconazole	≤0.125	0.25–0.5	–	≥1
Voriconazole	≤1	2	–	≥4
Flucytosine	≤4	–	8–16	≥32

(b)

Antifungal drug	Inhibition zone diameter (mm)		
	Susceptible (S)	Dose-dependent susceptible (S-DD)**	Resistant (R)
Fluconazole (25 μg)	≥19	15–18	≤14
Voriconazole (1 μg)	≥17	14–16	≤13

**Candida krusei* is intrinsically resistant to fluconazole and this scale should not be used for *C. krusei*.
**S-DD ('dose-dependent susceptible'): Maximal blood levels of the related drug needs to be achieved to treat infections due to S-DD isolates. For fluconazole, doses of ≥400 mg/day may be required in adults with normal renal functions. For itraconazole, plasma itraconazole concentrations of >0.5 μg/ml may be required for optimal clinical outcome. For voriconazole, taking into account the nonlinear pharmacokinetics and the dosing flexibility of the drug, the infection may be appropriately treated in body sites where the drug is physiologically concentrated or when a high dosage of the drug can be used.
*** ('intermediate'): Isolates with uncertain susceptibility category. These isolates cannot be classified as S or R.

methods was occasionally poor [49]. These findings revealed that the CLSI MIC breakpoints should not be used to interpret EUCAST MIC data. The EUCAST AFST Subcommittee is in current work of documentation of EUCAST MIC breakpoints for *Candida*.

The EUCAST-AFST subcommittee is in continuing work of content development and documentation of EUCAST standards for testing fungi other than *Candida* as well. Test parameters have been studied and determined for *Aspergillus* [51]. The degree of overall agreement between EUCAST and CLSI M38-A methods for voriconazole and posaconazole against *Aspergillus* was found to be 92.5%. For isolates with discrepant results, EUCAST method tended to produce higher posaconazole and voriconazole MICs as compared to CLSI M38-A method [52].

Specific growth patterns observed in antifungal susceptibility testing: Trailing growth and paradoxical (Eagle) effect

Trailing growth. Azoles, such as fluconazole and itraconazole produce incomplete and partial growth inhibition of *Candida*. As a result of this effect, some *Candida* isolates show reduced but persistent growth over an extended range of the concentrations of the drug in susceptibility testing. This phenomenon is referred to as 'trailing' and results in difficulties in interpretation of visual endpoints in azole susceptibility testing. Trailing for azoles has mostly been reported for strains of *C. albicans* and *C. tropicalis*. It has been observed also for other *Candida* spp. by some investigators [53–55].

'Heavy' trailers are the most problematic subset of isolates for interpretation of azole susceptibility.

Table 2 The major differences in test parameters of CLSI M27-A2 [11] and EUCAST E.Dis 7.1 [47] broth dilution methods

Test parameter	CLSI M27-A2	EUCAST E.Dis 7.1
Test medium	RPMI 1640 with glutamine, without bicarbonate glucose concentration: 0.2%	RPMI 1640 with glutamine, without bicarbonate glucose concentration: 2%
Inoculum density	$0.5–2.5 \times 10^3$ cfu/ml	$1–5 \times 10^5$ cfu/ml
Microdilution plates	96 U-shaped wells	96 flat-bottom wells
MIC reading time point	48 h	24 h
MIC reading method	Visual	Spectrophotometric (530 nm)

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Trailing growth appears to increase as the incubation period is extended and it may be so heavy that a *Candida* isolate which appears to be susceptible at 24 h may be misinterpreted as resistant at 48 h by visual readings. Such isolates are described as having a 'low-high' phenotype [56,57]. The 48 h CLSI MIC results classify these isolates as resistant. However, the *in vivo* results following fluconazole therapy in a murine model of invasive candidiasis suggest that these low-high phenotype strains are virtually susceptible to fluconazole and 24 h MIC results – but not the 48 h values – correlate with therapeutic outcome [56].

Trailing growth is observed as heavy growth of microcolonies inside the inhibition zone or inhibition ellipse in disk diffusion assay and Etest, respectively. For some of the trailer isolates, these agar-based methods may offer easier and more accurate interpretation of the azole susceptibility results as compared to the microdilution methodology [58] (Figs. 1 and 2). In addition and as discussed also elsewhere, ergosterol quantitation and spectrophotometric reading of azole MICs may ease the determination of accurate susceptibility categories for the trailer low-high phenotype isolates [53].

Trailing has been observed with flucytosine and echinocandins as well for various *Candida* spp. Trailing in echinocandin susceptibility testing frequently appears to be slight and less common and does not much interfere with interpretation of MICs [55].

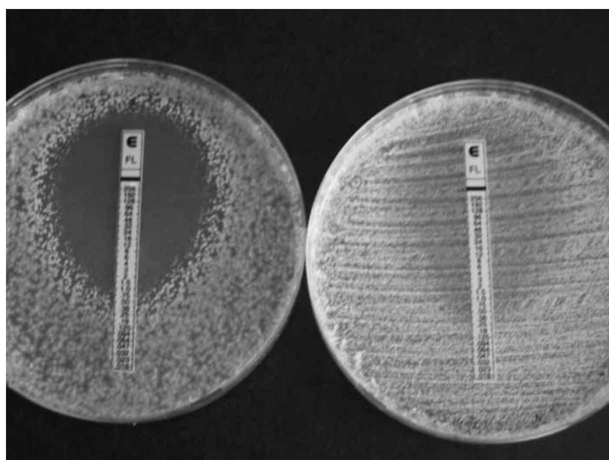

Fig. 2 Fluconazole Etest performed on two different media for a *Candida tropicalis* strain (item code: 4715). *Left*: Mueller-Hinton agar supplemented with 2% glucose and methylene blue produces sharp and clear inhibition ellipse. *Right*: RPMI agar supplemented to 2% glucose. (From the collection of Hacettepe University Medical School Mycology Laboratory).

Paradoxical (Eagle) effect. This growth pattern, defined as the paradoxical growth of the fungal strain *in vitro* at high concentrations that are above the MIC of the drug has so far been observed with echinocandins [55,59–62] and itraconazole [63] for *Candida*. Decrease in beta-1,3- and beta-1,6-glucan and a rapid shift of fungal cell wall polymer to chitin has been proposed to be responsible for the paradoxical effect of echinocandins for *Candida* [61].

The *in vivo* significance of Eagle effect for echinocandins has been investigated in a systemic murine candidiasis model of *C. albicans* treated with caspofungin. The results of the study failed to prove the presence of a consistent, reproducible paradoxical effect *in vivo* for isolates that showed paradoxical growth *in vitro* [60]. Clinical significance of the Eagle effect remains unclear and has yet to be further investigated.

When do we need to perform antifungal susceptibility tests?

Standardization of antifungal susceptibility testing and development of reference methods constitute a remarkable progress in the field of mycology. *In vitro* antifungal susceptibility tests are now mainly used for:

1. Epidemiological surveys for determination of susceptibility profiles and resistance rates of the infecting strains against commonly used antifungal drugs at a particular center,
2. Determination of the degree of antifungal activity of the newly developed compounds,
3. Prediction of clinical outcome and optimization of antifungal therapy in routine mycology laboratory practice.

Performance of antifungal susceptibility tests is not required for every individual clinical fungal strain in routine practice [64,65]. The decision to perform an antifungal susceptibility test for a particular clinical fungal strain in routine clinical laboratory practice requires the presence of specified indications [65] (Table 3). These indications have mostly been documented for yeasts and mostly for *Candida*.

Based on these indications and more specifically, antifungal susceptibility tests may be of help at some clinical settings for directing antifungal therapy. Accordingly, routine antifungal susceptibility testing appears be beneficial in: (i) determination of local antifungal resistance rates in a particular center for rationale selection of empirical antifungal regimen, (ii) assessment of susceptibility profiles of *Candida* strains isolated from candidemia or deep-seated *Candida*

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Table 3 Well-defined indications for application of routine antifungal susceptibility testing for clinical isolates* [65–67,84]

Indications
Invasive infection; *Candida* strain isolated from a sterile body site
Any *Candida* species for which high rate of resistance to an antifungal drug or a class antifungal drug is well-known to be possible (e.g., *C. glabrata*-fluconazole and other triazoles)
Unexpected clinical failure during standard therapy of a *Candida* infection
Surveillance antifungal susceptibility testing to detect/rule out emergence of secondary resistance following prior antifungal therapy

*These indications are valid and remain established mostly for *Candida*.

infections for optimal selection of long-term therapies, and (iii) determination of susceptibility profiles of *Candida* strains isolated from recurrent mucosal infections for rationale selection of alternative regimens [66,67].

For clinically significant filamentous fungi, the validity and benefit of routine antifungal susceptibility testing remains poorly defined and the available data are still only investigational [68,69].

To what extent can antifungal susceptibility tests predict clinical outcome: *in vitro-in vivo* correlation studies

The major *in vitro-in vivo* correlation data reported so far for each individual antifungal drug-fungal genus combination will be shortly reviewed below.

Amphotericin B

Determination of *in vitro* resistance to amphotericin B has been one of the most difficult issues so far. Available data in general fail to validate significant *in vitro-in vivo* correlation and emphasize the significance of other factors, specifically the host status in determination of clinical outcome.

Candida. In one of the studies which investigated the *in vitro-in vivo* correlation in candidemic patients treated with amphotericin B, the use of microdilution method, RPMI 1640 medium, and an MIC breakpoint of 1 µg/ml appeared to correlate well with microbiological outcome [70]. Other reports suggested the use of Antibiotic Medium 3 (AM3) as the test medium to enhance the discrimination of amphotericin B-resistant *Candida* [71]. However, further studies emphasized the remarkable impact of the technical factors on the issue, since the results varied even from one lot of AM3 to other [72]. Utility of Etest as the susceptibility assay also proved to be an independent beneficial factor. Etest was found to be superior in detection of amphotericin B-resistant isolates as compared to the reference microdilution method [73]. Moreover, MIC breakpoints were proposed for amphotericin B Etest using AM3 supplemented to 2% glucose (MIC breakpoint: 1 µg/ml) and RPMI supplemented to 2% glucose (MIC breakpoint: 0.38 µg/ml) [74,75].

However, these preliminary data were not strengthened and not validated by the results of other studies. A recent analysis which evaluated the validity of five previously studied *in vitro* susceptibility testing settings (1-CLSI microdilution method, RPMI 1640 medium, MIC determination; 2-CLSI microdilution method, AM3 supplemented to 2% glucose, MIC determination; 3-CLSI microdilution method, RPMI 1640 medium, followed by determination of minimum fungicidal concentration-MFC; 4-CLSI microdilution method, AM3 supplemented to 2% glucose, followed by determination of MFC; 5-Etest, RPMI 1640 supplemented to 2% glucose, MIC determination) failed to reveal evidence of correlation between *in vitro* resistance and clinical failure [76]. Another correlation study in cases with neonatal candidemia also failed to correlate MICs with clinical outcome [77]. Conclusively, current data suggest amphotericin B susceptibility tests remain to be further investigated for development of relevant test methods and parameters that correlate with clinical and microbiological outcome.

Aspergillus. *In vitro-in vivo* correlation data for cases of invasive aspergillosis treated with amphotericin B remain more limited. The data obtained from patients with cancer and invasive aspergillosis failed to demonstrate significant correlation between *in vitro* susceptibility and clinical efficacy [78]. Likewise, no correlation could be detected between in vitro susceptibility to amphotericin B and in vivo outcome in a murine model of aspergillosis [79].

C. neoformans. In one of the studies, the correlation between clinical outcome and in vitro susceptibility to various antifungal drugs, including amphotericin B, flucytosine, and fluconazole was investigated for C. neoformans as well. This multicenter prospective study used CLSI method, Etest, and broth microdilution in yeast nitrogen base (YNB) medium as the susceptibility assays. None of the methods used could predict early clinical outcome in patients with cryptococcosis [80].

Testing lipid amphotericin B formulations. Previous investigations have focused on the determination of comparative *in vitro* activities of various lipid formulations

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of amphotericin B (liposomal amphotericin B, amphotericin B lipid complex, amphotericin B colloidal dispersion) and conventional (free) amphotericin B against various fungal genera, including *Candida*, *Aspergillus, C. neoformans,* and *Fusarium*. While conventional amphotericin B MICs were similar to those of lipid formulations in some studies [81], others have yielded discrepant results [82,83]. Due primarily to the fact that the compound, amphotericin B, which is responsible for antifungal activity is same in conventional and lipid formulations, the currently accepted recommendation is to test conventional amphotericin B only in antifungal susceptibility tests.

Azoles

Candida, C. neoformans, and *Histoplasma*. The limits and the extent of the benefit of routine antifungal susceptibility testing were best demonstrated by a meta-analysis, which included the data reported in major *in vitro-in vivo* correlation studies [65]. This meta-analysis included cases of *Candida*, *Cryptococcus neoformans*, and *Histoplasma* infections treated with fluconazole, itraconazole or ketoconazole. The results showed that clinical success rate was 91% for infections due to isolates susceptible to the antifungal agent used for treatment and 48% for infections due to isolates that were resistant. These percentages were similar to those previously found for bacterial infections and the corresponding susceptibility categories, and by approximation, the concept is known to as '90–60 rule'. In other words, clinical response is achieved in 90% of the time for infections due to susceptible isolates. On the other hand, the expected response rate in infections due to resistant isolates is not 0%; it may even be as high as 60% on average. This is primarily due to the influence of other factors, such as the immune status of the host, pharmacokinetic properties of the antifungal drug, severity of the infection, presence of prosthetic devices and catheters, and surgical interventions. Conclusively, *in vitro* susceptibility assay is a beneficial adjunct in predicting clinical outcome, but remains as only one of several factors that have impact on clinical response.

Recently, further evaluation of the interpretive breakpoints for fluconazole and *Candida* by expanded MIC data and clinical efficacy studies has validated antifungal susceptibility tests as a beneficial adjunct in optimizing treatment of candidiasis [84].

Aspergillus. In previous studies, an MIC breakpoint (>16 μg/ml) has been proposed for itraconazole and *Aspergillus*. This breakpoint appeared to predict clinical outcome in a limited number of patients with invasive aspergillosis [85,86]. However, further studies to support and validate this breakpoint are lacking.

Echinocandins

Candida. MIC breakpoints for echinocandins (caspofungin, micafungin, anidulafungin) against *Candida* have not yet been validated. While the echinocandin MICs against *Candida parapsilosis*, *Candida guilliermondii* and *Candida famata* are higher than those detected for other *Candida* spp. [46,62,87], clinical outcomes with caspofungin across different *Candida* spp. remained similar in a limited number of neutropenic patients with documented invasive candidiasis [88].

In vitro-in vivo correlation data on echinocandins remain sparse. Case reports on clinical failure together with reduced susceptibility to echinocandins are present [89,90]. Similarly, increased MICs for all three echinocandins and concordant and progressive lack of clinical response to micafungin therapy have been reported for four *C. albicans* strains with identical allelic homology isolated from a patient with HIV infection and oesophagitis [91]. However, some other reports fail to suggest correlation of clinical response with MIC values for caspofungin [92]. The difficulties in demonstration of *in vitro-in vivo* correlation for echinocandins vs. *Candida* might have originated from the fact that isolates with virtually high echinocandin MICs are yet rare. Further data are required for clarification of the issue. The molecular basis of echinocandin resistance is also under investigation. Specific amino acid changes in Fks1p subunit of 1,3-beta-D-glucan synthase have been found to be associated with reduced susceptibility of *C. albicans* and *C. krusei* strains to echinocandins [93].

Drawbacks of dilution-based reference antifungal susceptibility testing methods

Certain drawbacks limit the optimal use of reference microdilution methods particularly in routine laboratory practice. These limitations are summarized in Table 4.

Methods other than reference assays to detect *in vitro* antifungal susceptibility

Etest

This practical, agar-based diffusion method, which enables the determination of MIC values has been studied for assessment of the activity of various antifungal drugs against various fungal genera and species. Etest strips (AB BioDisk, Solna, Sweden) are commercially available for amphotericin B, fluconazole,

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Table 4 Current drawbacks of reference dilution-based antifungal susceptibility testing methods

Drawbacks	Notes/Specifications	References
Needs a long time Time needed to report the result is 24–72 h *following performance of the test*	The issue starts with isolation of the infecting fungus from the clinical specimen. Time required for culture growth may vary between 24 h to days, depending on the genus of the fungus. Antifungal susceptibility test adds 24–72 h more to this, which means the test result is frequently late in directing *initial* antifungal therapy. However, identification of the fungus to species level may provide meaningful information in prediction of resistance for that particular species and the following antifungal susceptibility test result for that particular strain may provide further evidence for guidance of therapy.	[11,12,21,47,201]
Fails to discriminate amphotericin B-resistant isolates from the susceptible ones	The use of Antibiotic Medium 3 as the test medium and/or Etest as the test method was proposed to enhance the ability to detect amphotericin B-resistant strains. However these data could not be fully verified and the issue still remains unresolved.	[71–76,78–80]
MIC breakpoints for several fungus-antifungal drug combinations are yet unestablished	MIC breakpoints have been verified for fluconazole, itraconazole, voriconazole, and flucytosine against *Candida* spp. only. For the rest of the fungus (including all moulds) – antifungal drug combinations, the data remain investigational and the results of antifungal susceptibility tests can only serve to determine the degree of *in vitro* activity and to detect isolates with relatively high MICs. Clinical trials are required to establish the clinical utility of antifungal susceptibility testing for these settings.	[11,12,17]
Azole and flucytosine MICs may be hard to read and-interpret particularly for heavy trailing isolates	Some *Candida* isolates may give unclear endpoints in reading MICs by using the CLSI recommended visual MIC-2 endpoint. This has been most commonly reported for *C. albicans* and is more pronounced at 48 h readings as compared to those at 24 h.	[30,53,160,202,203]

itraconazole, flucytosine, voriconazole, posaconazole, and caspofungin. As already noted, the most remarkable advantage of Etest is its user-friendly format. In addition, regardless of the test medium used, Etest has once been found to be more efficacious as compared to the reference microdilution method in detection of amphotericin B resistance in *Candida* [75]. However, further studies failed to validate the correlation between clinical outcome and *in vitro* resistance determined by Etest or CLSI microdilution method [76].

RPMI 1640 supplemented to 2% glucose is the most commonly used medium for Etest. Other media, including Casitone agar (particularly for azoles) [94–97], yeast nitrogen base (particularly for testing *C. neoformans*) [98,99], AM3 [94,95,99], and Mueller-Hinton agar supplemented with 2% glucose and methylene blue have also been used by some investigators. AM3 has been once recommended particularly for testing amphotericin B due to the potential ability of this medium to enhance discrimination of amhotericin B-resistant isolates from the susceptible ones [99,100]. Mueller-Hinton-methylene blue agar, on the other hand, is now being used more commonly [23,101,102] and produces sharp ellipses of inhibition (Fig. 2).

The agreement of Etest with the CLSI reference microdilution method is variable but frequently above acceptable limits. The dilution range criterion used for defining agreement between two methods also varies from one study to other, most of the results being interpreted within either ± 1 or ± 2 dilution. Moreover, several factors may influence the extent of agreement, including the fungal species and the incubation time point used to interpret the results. For *Candida* spp. in general, the percentage agreement rates between Etest

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and CLSI reference microdilution method were found to be 96% for fluconazole [24,103], 95% for voriconazole [103], and 65–83% for posaconazole [23]. For *C. glabrata*, the agreement rates between the two methods were detected as 91–96% for fluconazole and 93–95% for voriconazole [102]. Notably, when testing isolates of *Trichosporon asahii*, Etest tended to generate lower amphotericin B and higher fluconazole and itraconazole MICs as compared to CLSI microdilution [31]. On the other hand, Etest and the CLSI reference microdilution methods appeared to be well correlated for amphotericin B and voriconazole (agreement rates of 99 and 94%, respectively) against *C. neoformans* [104] as well as for itraconazole and voriconazole (agreement rates of 74.2–96 and 98%, respectively) against *Aspergillus* [105]. Posaconazole MICs obtained by CLSI microdilution method and Etest were well correlated for *Aspergillus* (84%) and less common moulds (including *Cladosporium* spp., *Curvularia* sp., *Exophiala* sp., *Fusarium* spp., *Paecilomyces* spp., *Pithomyces* sp. and *Scedosporium apiospermum*) (100%), with the exception of *Penicillium* spp. (67%) [106]. Etest and CLSI microdilution was in good agreement (88%) also for testing amphotericin B against filamentous fungi, including *Aspergillus*, *Fusarium*, *Paecilomyces lilacinus*, *Rhizopus arrhizus*, *Scedosporium*, dematiaceous fungi, and *Trichoderma longibrachiatum* [107]. The overall agreement between the EUCAST microdilution method and Etest for fluconazole and *Candida* was also investigated and found to be 90.4% [108].

The virtual MIC breakpoints to be used for interpretation of the Etest MIC results remain unestablished. Some studies have questioned the categorical agreement between Etest and CLSI microdilution method for *Candida* by using the CLSI MIC breakpoints for interpretation of the Etest results as well or by applying identical investigational MIC breakpoints for both methods [23,102]. In one of these studies where the issue was investigated for fluconazole and voriconazole against *C. glabrata*, categorical agreement rates between the two methods were found to be 52–62% for fluconazole and 95–96% for voriconazole. While the categorical agreement rates for fluconazole were remarkably poor, most (37–46%) of the discrepant results were minor errors (major errors: 2%, very major error: 0%) and Etest tended to generate slightly higher MICs as compared to CLSI microdilution method [102]. These results suggest that validation of MIC breakpoints for Etest is required and discordant results might be obtained by Etest particularly for less susceptible *Candida* species and isolates with relatively higher MICs that are close to the categorical breakpoints.

Colorimetric microdilution

The difficulties in reading the MICs of azole compounds particularly for trailing isolates have led to the development and utility of assays which incorporate colorimetric indicators to ease accurate visual reading. Sensititre Yeast One (TREK Diagnostic Systems, Cleveland, Ohio) is now the mostly studied, commercially available dried colorimetric panel used for this purpose and is based on the CLSI methodology. ASTY colorimetric microdilution panel (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo) is another commercial product which has been investigated to a lesser extent [109].

The modifications in Sensititre Yeast One panel as compared to the CLSI test parameters include the use of RPMI medium supplemented to 2% glucose and incorporation of Alamar blue as the oxidation-reduction colorimetric indicator. In this system, red color indicates growth, while purple indicates growth inhibition and blue indicates no growth. Sensititre Yeast One was investigated for testing amphotericin B, fluconazole, itraconazole, and flucytosine against *Candida* spp. Acceptably high agreement rates were obtained in general (92.9, 68.2, 77.6 and 80% for amphotericin B, fluconazole, itraconazole, and flucytosine, respectively). However and importantly, very major discrepancies between Sensititre Yeast One and CLSI microdilution MICs were also noted in this study (7.6 and 7% of the isolates for fluconazole and itraconazole, respectively) [110]. The panel was found to be in very good agreement (percentage agreement rates: 92.3–98) with the CLSI microdilution method for testing voriconazole, posaconazole, and ravuconazole against *Candida* spp. [111].

The Sensititre Yeast One system has also been investigated and proved useful for testing amphotericin B, fluconazole, itraconazole, ketoconazole and flucytosine against filamentous fungi [112]. High levels of agreement between the panel and the CLSI reference method have been detected for amphotericin B (percentage agreement rates: 86.4–87.7) and itraconazole (percentage agreement rates: 91.4–93.8) [113] for these fungi. The percentage agreement rates between Sensititre Yeast One and CLSI method for testing dermatophytes, on the other hand, were found to be 81.6% for amphotericin B, 87.7% for itraconazole, 67.3% for fluconazole, and 69.4% for ketoconazole [114]. The overall agreement rate between the two methods was found to be 89% when testing amphotericin B, fluconazole, itraconazole, ketoconazole and flucytosine against *C. neoformans*. The poorest agreement was

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noted for ketoconazole for *C. neoformans* (50%) [115].

Another colorimetric method, which is noncommercial and is based on the reduction of the tetrazolium salt, 2,3-bis {2-methoxy-4- nitro-5-[(sulfenylamino) carbonyl]-2H-tetrazolium-hydroxide} (XTT) by mitochondrial dehydrogenases is also under investigation for its utility for antifungal susceptibility testing. This metabolic assay specifically quantifies fungal growth by measuring fungal metabolism. Conversion of XTT to its formazan derivative, as indicated by change of the color from yellow to purple, is measured by spectrophotometric evaluation of optical density.

XTT assay was studied for susceptibility testing of *Candida* spp. and *C. neoformans* and the results were found to be in agreement with those of CLSI method for amphotericin B, fluconazole, itraconazole, ketoconazole, and flucytosine [116]. It has also been investigated for testing amphotericin B and itraconazole against *Aspergillus* and the formazan production was found to be linearly correlated with the fungal biomass [117]. The percentage agreement rates between XTT assay and CLSI method for *Aspergillus* spp. were found as 97% for amphotericin B and 83% for itraconazole [118]. Preliminary data suggest that it may be beneficial for susceptibility testing of echinocandins as well against *Aspergillus* [119].

XTT assay has also been studied for rapid MIC determination of Zygomycetes for amphotericin B, posaconazole, and voriconazole, and by adding XTT-menadione solutions at 6, 8, or 12 h after inoculation and further incubation for 2 h to allow conversion of XTT to its formazan derivative. At 6 h time points, the percent agreement rates between XTT assay and CLSI method were found as 93, 76, and 67% for amphotericin B, posaconazole, and voriconazole, respectively. Notably and as the percentage agreement rates indicate, early inhibition of metabolic activity was delayed with the azoles as compared to that with amphotericin B [120].

Conclusively, the results obtained with the commercially available colorimetric assays appear promising. However, isolates with discrepant results as compared to the CLSI reference method may be observed and the issue needs to be cautiously interpreted particularly for routine susceptibility testing settings. On the other hand, further considerations are required for standardization of noncommercial systems, such as XTT assay.

Agar dilution

This conventional susceptibility testing method has been studied for various antifungal agent-fungus combinations, including fluconazole, itraconazole, ketoconazole, flucytosine and amphotericin B against *Candida* [121,122], fluconazole against *C. neoformans* [123], amphotericin B, itraconazole and voriconazole against *Aspergillus* [85,124], caspofungin against *Aspergillus* [125], terbinafine and itraconazole against dermatophytes [126], and amphotericin B, fluconazole, itraconazole, miconazole, ketoconazole, and terbinafine against *Fusarium* [127]. While agar dilution method remained in good correlation with microdilution method in most of the comparative studies, it remains unstandardized and is now less commonly used, particularly for antifungal-fungus combinations that the reference methodologies cover [11,12,21,47]. Agar dilution method is under investigation for some difficult-to-grow fungi, such as *Malassezia* [128,129], for which an *in vitro* antifungal susceptibility testing method has not been yet standardized.

Determination of fungicidal activity by minimum fungicidal concentration (MFC) and time-kill assay

MFC. Whether the determination of MFC instead of MIC better correlates with clinical outcome has remained one of the major inquiries for antifungal susceptibility testing as well [130]. Test parameters for assessment of MFC are not fully standardized. MFC is mostly defined as the lowest concentration of the drug that yields $\geq$98–99.9% killing effect as compared to the starting inoculum. The starting inoculum, the volume sampled from the clear wells or tubes, and the 'percentage of killed cells' which has been used as the definition of MFC may result in great variations of the results. Using a sample volume of 10 µl and the starting inocula recommended for yeasts (10^3 cfu/ml) in CLSI M27-A2 [11] document, MFC determination is not possible since the final inoculum in the wells is insufficient to detect more than 90% killing. Modifications that used larger inocula and larger samples volumes have been proposed for amphotericin B MFC determinations of *Candida* isolates [131].

MFC determinations for filamentous fungi also remain unstandardized. Using different test conditions, collaborative work has been carried out by CLSI for evaluation of different test parameters for determination of MFC of various drugs against *Aspergillus* [132] and other moulds [37].

The impact of MFC in prediction of clinical outcome remains to be established by further studies. This issue has been explored particularly for amphotericin B against *Candida*. While the previous studies focused on the use of amphotericin B MFC as well as MIC in prediction of clinical outcome in patients with

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candidemia [70], recent studies failed to show correlation of neither MFC nor MIC with clinical outcome [76]. Other studies emphasized the fact that fungicidal activity is a species-dependent phenomenon, as exemplified by the variable fungicidal effect of voriconazole [133] and amphotericin B [131] against different species of *Candida*.

Time-kill assay. Determination of fungicidal activity by time-kill assay is a valuable tool for attaining information about the dynamic interaction between the fungal strain and the antifungal agent under study. As would be expected, the assay reveals relative rate and extent of the fungicidal activity and gives information about pharmacodynamic characteristics and postantifungal effect (PAFE) of the agent. The results are analyzed by examining the time-kill plot [130].

Time-kill kinetics have so far been studied for various antifungal agents against yeasts, including amphotericin B, fluconazole, itraconazole, voriconazole, caspofungin, micafungin, and flucytosine against *Candida* [134–143], and voriconazole and flucytosine against *C. neoformans* [142–144].

As with the other methodologies, standardization of the time-kill assay parameters is required. A time-kill method has been proposed for standardized performance of the test for *Candida* and nonmucoid strains of *C. neoformans* [145]. An inoculum size of $1\text{–}5 \times 10^5$ cfu/ml, RPMI 1640 medium, a transfer volume of 30 µl, agitation of the tubes during the course of the test, and the criterion of $\geq$99.9% or 3-$\log_{10}$-unit reduction in cfu/ml from the starting inoculum were used as the test parameters in this proposed assay. For echinocandins, utility of RPMI 1640 as the test medium resulted in variable fungicidal activity, depending on the concentration as well as the species and strain of *Candida*. However, when AM3 was used instead of RPMI 1640 as the test medium, uniformly fungicidal activity was observed for echinocandins against *Candida* spp. [140,146].

The proposed time-kill assay appeared to be reproducible, suggesting that standardization of time-kill studies for *Candida* is possible. For filamentous fungi, such as *Aspergillus* spp., on the other hand, time-kill studies are scarce [147] and need further evaluation. Among the reasons why there are very few studies that have used time-kill method for filamentous fungi are the nonhomogeneous growth pattern, the difficulties in CFU evaluation when the fungus is growing as hyphae, and the problems in sampling of the antifungal-free control tubes [130].

In vitro time-kill studies enable to investigate the presence of PAFE as well. A point of interest related to fungicidal activity is the relatively longer (5.3–7.5 h vs. 0.5 h) PAFE with the fungicidal drugs (amphotericin B, caspofungin, and micafungin against *C. albicans*; amphotericin B against *A. fumigatus*) as compared to that with fungistatic ones [148]. Absolute clinical significance of this finding is less clear and clinical outcome depends on several factors.

Flow cytometry

Flow cytometry (fluorescence-activated cell sorting-FACS) is one of the currently investigated techniques for antifungal susceptibility testing of yeasts and moulds. The most remarkable advantage of flow cytometry over the currently used antifungal susceptibility testing methods is its short incubation time of 4–6 h in average [149]. The technique uses various membrane potential sensitive or DNA binding vital dyes, such as FUN-1, propidium iodide, 3,3′- dipentyloxacarbocyanine iodide or acridine orange [150–153]. Depending on the dye used in the test, the results are determined by evaluation of the decrease or increase in fluorescence intensity of the cells that are stained with the dye following exposure to the drug. Flow cytometry has been investigated for testing amphotericin B, fluconazole, echinocandins (caspofungin) and flucytosine against *Candida* [150,152,154–157], and amphotericin B, itraconazole, and voriconazole against *A. fumigatus* [151,158]. The results were in general found to be in very good agreement (96–99% for amphotericin B and fluconazole against *Candida*) with the reference methods [100,150,151,153–155,158]. However, widespread use of flow cytometry in antifungal susceptibility testing currently seems unlikely due to the unavailability of the required flow cytometry equipment in several centers.

Ergosterol quantitation

This novel investigational method is based on measurement of cellular ergosterol content rather than the growth inhibition. For ergosterol quantitation, ergosterol is isolated from whole yeast cells by saponification and the nonsaponifiable lipids are extracted with heptane. Ergosterol is finally identified by its spectrophotometric absorbance profile between 240 and 300 nm. Sterol quantitation has so far been investigated for assessment of activity of fluconazole and itraconazole against *Candida* [53,159,160]. It has particularly been used for testing heavy trailer *Candida* isolates, which tend to give unclear visual endpoints for fluconazole and/or itraconazole at 48 h. The results of one of these studies showed that the 24-h visual or the spectrophotometric end point of 50% reduction in turbidity

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were the reading parameters that were best correlated with the results of sterol quantitation [53]. Based on its ability to accurately discriminate virtual azole-resistant isolates from those that tend to trail, ergosterol quantitation has been proposed as a more reliable method for prediction of *in vivo* outcome as compared to the reference microdilution assay [160]. However, the method yet remains investigational.

Testing antifungal drugs in combination

Introduction of new drugs with distinctive targets of antifungal activity and the presence of several other compounds being under investigation led to a remarkable increase in studies which explored the *in vitro* interaction of antifungal drugs in combination. Development of new echinocandins (caspofungin, micafungin, anidulafungin) which exert antifungal activity via a different target, the fungal cell wall, seems to play the major role in this respect [161,162]. Several *in vitro* studies and animal models have focused on interaction of antifungal drugs that act via different targets. Only some of these studies are cited here for exemplification [163–180].

While the accumulated *in vitro* data for *in vitro* antifungal drug combinations are now plenty and diverse, a standard and optimal method for testing the interaction of antifungal drugs *in vitro* is still lacking. Checkerboard method that is based on interpretation of fractional inhibitory concentration index (FICI) has been one of the most commonly used assays for this purpose [162,181,182]. Time-kill assay, which is less commonly applied so far is a more cumbersome method. However, it offers the advantage of measuring the effect of antifungal interaction on the rate and extent of fungal killing and provides pharmacodynamic information regarding the combination tested [183,184]. Etest has also been used by several investigators as a practical method for combination studies and the method yielded reproducible results in general [184,185]. Modern concentration-effect response surface models, the fully parametric model developed by Greco *et al*. and the 3-D analysis developed by Prichard *et al*. have drawn attention in recent years for combination studies [186,187]. Comparison of the drug interaction models appear to suggest that, analysis of checkerboard assay results might be more subjective, dependent on the MIC endpoint used, and sensitive to experimental errors, while the response surface approaches might provide more consistent results [187,188].

Not only the lack of a standard method but also the lack of knowledge about the optimal *in vitro* method that would best correlate with clinical outcome display difficulties for drawing conclusions from the generated *in vitro* combination data. Animal studies also remain poorly standardized. Furthermore, the design of clinical trials to explore the benefits of combination therapy in invasive mycoses is remarkably difficult [161,183,189,190]. The best-known synergistic antifungal drug combination with ‘clear’ advantage and adequate statistical power is that of amphotericin B and flucytosine in primary treatment of cryptococcal meningitis [191]. Apart from that, there appears to be a ‘possible’ advantage of amphotericin B and fluconazole combination in treatment of nonneutropenic candidemia, since the combination tended to provide improved success and more rapid clearance of *Candida* from the bloodstream as compared to fluconazole alone [192]. Lastly, based on the improved 3-month survival rates, salvage therapy with voriconazole and caspofungin combination in refractory invasive aspergillosis appears to be one of the ‘potential’ settings where combination therapy might be of benefit [193,194]. The readers are referred to review articles for detailed understanding of the state-of-the art and potential future applications of combination antifungal therapy [195–200].

Conclusions and future directions

Development of standard antifungal susceptibility testing assays and further refinements to overcome the limitations of the reference methods have constituted a remarkable progress. MIC breakpoints are available for fluconazole, itraconazole, voriconazole, and flucytosine against *Candida*. More importantly, antifungal susceptibility tests have proven to be a useful adjunct in direction and monitorization of fluconazole therapy in *Candida* infections. Testing other drugs against other fungal genera, on the other hand, provides information only about the extent and spectrum of *in vitro* activity in the mean time. However, clinical utility of susceptibility testing for these drug-fungus combinations other than azoles and *Candida* remains unestablished. Further studies are required to determine MIC breakpoints and the potential benefits of modifications of the available methods for these settings. Further progressions in antifungal susceptibility testing are hopefully in the horizon. Nevertheless, *in vitro* susceptibility test results will remain as only one of several factors that have impact and prediction power on clinical outcome, particularly in presence of profound immunosuppression.

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