Perspectives on antifungal susceptibility testing in 
*Candida*

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Received: 28.06.14; Accepted: 11.09.14; Published: 11.09.14

ABSTRACT

**Background:** Along with the increase in the incidence of candidiasis, there has been an important shift away from *Candida albicans* towards non *albicans* spp. Many species from non *albicans* *Candida* group exhibit varying degrees of resistance, either intrinsic or acquired or both, to commonly used antifungal drugs. The emergence of antifungal resistance and a gradual increase in the number of new and broad spectrum antifungal drugs has complicated the choice of antifungal drug for treatment of candidiasis. Accurate, reproducible and reliable antifungal susceptibility testing is necessary for the selection of an appropriate and accurate antifungal therapeutic agent. The standard reference method of *in vitro* antifungal susceptibility testing is complex and labor intensive. **Aim:** This paper reviews various methodologies commonly used for antifungal susceptibility testing of *Candida* spp is reviewed. **Method:** Relevant research articles on Pubmed and Google Scholar were collected and reviewed. **Results:** A total of 51 research articles from various journals were reviewed for various methodologies commonly used for antifungal susceptibility testing of *Candida* spp. **Conclusion:** The *in vitro* antifungal susceptibility of an infecting species of *Candida* is one of the several factors that influence the successful therapy. In developed countries, antifungal susceptibility testing is now increasingly used to supplement the treatment of mycotic infection. In resource-constrained laboratories of developing countries where determination of antifungal susceptibility testing by the Clinical and Laboratory Standards Institute (CLSI) standardized broth method is not always feasible, simple, user friendly methods like disc diffusion, Etest can be easily incorporated as alternative reliable techniques.

**Key words:** Antifungal resistance, *Candida albicans*, broth dilution, disc diffusion, drug resistance, non-albicans *Candida* species.

INTRODUCTION

In the field of Medical Microbiology, two events are considered seminal: a change in the epidemiology of infections, and the emergence of drug resistance. Once considered “microbiological curiosities” with little or no clinical significance, fungi have emerged as an important cause of infections.[1] Immunodeficiencies either induced or acquired, and more intensive and aggressive clinical procedures are among...
the important risk factors for fungal infections.[2]

Among various pathogenic fungi, Candida spp. are pervasive pathogens causing a wide range of infections in humans.[3] Although Candida infections are common in seriously ill or otherwise immunocompromised patients, due to its versatility and ability to survive in various anatomical sites it can provoke infections in otherwise healthy persons as well.[3]

In recent years, along with an increase in the incidence of candidiasis, there has been an important shift away from Candida albicans towards non albicans spp.[4] The change in the epidemiology of Candida infections can be attributed to various factors like severe immunocompromised status of the host, exposure to broad spectrum antibacterial agents and empirical use of antifungals.[5] However, the clinical manifestations of infections caused by different non albicans Candida (NAC) spp. are usually indistinguishable from those by C. albicans.[6]

Many species from the NAC group exhibit varying degrees of resistance, either intrinsic or acquired or both to commonly used antifungal drugs.[7]

The emergence of antifungal resistance and gradual increase in the number of new and broad spectrum antifungal drugs has complicated the choice of antifungal drug for treatment of candidiasis.[8,9] Therefore, species identification and antifungal susceptibility testing of Candida isolates is a must for selection of an appropriate and accurate antifungal therapeutic agent.

In vitro antifungal susceptibility testing is now standardized internationally and is becoming essential in patient management and resistance surveillance.[9] it remains less utilized than antibacterial testing. The standard reference method of antifungal testing of yeast is performed routinely in few specialised laboratories. However, complex and labor intensive methodology restricts its utility.[10,11] Here, we present an overview of various methodologies commonly used for antifungal susceptibility testing of Candida spp.

METHODOLOGY

Literature was collected by searching on Pubmed and Google Scholar with key words.

REVIEW

Methods of antifungal susceptibility testing in Candida spp.

The Clinical Laboratory Standards Institute (CLSI), formerly the National Committee on Clinical Laboratory Standards has approved methods for testing of both yeast and filamentous fungi. For yeast, two standard methods for antifungal testing have been released. These include, M27-A3 for macrobroth and microbroth dilution testing[12] and M44-A for disc diffusion susceptibility testing.[13] In addition to CLSI methods, a variety of commercial (both manual and automated) systems are available for antifungal susceptibility of Candida spp.

1. Broth-based methodology

The first standard method for antifungal susceptibility was the macrobroth dilution method.[14] This methodology became an ‘approved’ level document (M27-A) in 1997 after passing through the stages of ‘proposed’ document (M27-P) in 1992 and ‘tentative’ document (M27-T) in 1995.[15] It was developed by CLSI after approximately 15 years of collaborative work.[15] The susceptibility testing by this method was carried in 1 ml test tubes and RPMI 1640, a totally defined medium used to prevent drug-medium interaction.[16] Inoculum size (0.5-2.5x10^3 CFU/ml) and preparation, test medium (RPMI-1640), incubation temperature (35°C) and time (24-72 h) were specified. The end point reading for 5-flucytosine (5 FC), amphotericin B and azoles was also specified. For 5 FC and triazoles the endpoint was referred to as 80% reduction in growth relative to the growth control (turbidity as compared to the drug-free control tubes), whereas for amphotericin B it was defined as the lowest dilution that resulted in zero visible growth.[17]

As this method is cumbersome and labor intensive it proved unwieldy for routine application in laboratories with a high daily workload.[11] This cumbersome and labor intensive macrodilution method was further modified in to relatively easy microdilution broth technique. CLSI standardized broth microdilution methodology remains the reference for antifungal susceptibility testing.[15] It became the method of choice for antifungal testing of Candida spp and other yeasts and yeast like fungi. This technique is performed in microtiter plate and hence a large number of isolates can be tested at a
time. As microtiter plates can be easily prepared and frozen well in advance, the time required is less as compared to the macrodilution method.\[^{15}\]

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has suggested modifications to the CLSI microdilution method. This method resembles the CLSI microdilution broth technique with respect to principle, medium, incubation temperature and prominent inhibition minimum inhibitory concentration (MIC) endpoints for both the triazoles and echinocandins, but differs in certain aspects.\[^{18}\] The difference between CLSI and EUCAST microdilution broth methods is tabulated in Table 1.

**Table 1: Difference between CLSI and EUCAST microdilution broth methods.\[^{18}\]**

<table>
<thead>
<tr>
<th>Character</th>
<th>CLSI method</th>
<th>EUCAST method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum concentration (cfu/ml)</td>
<td>0.5x10(^3) to 2.5x10(^3)</td>
<td>0.5x10(^3) to 2.5x10(^5)</td>
</tr>
<tr>
<td>Concentration glucose (%)</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Microdilution well</td>
<td>Round bottom</td>
<td>Flat bottom</td>
</tr>
<tr>
<td>Method of endpoint reading</td>
<td>Visual</td>
<td>Spectro-photometric</td>
</tr>
</tbody>
</table>

Both CLSI and EUCAST broth are recognised standard methods for the performance of antifungal susceptibility testing of *Candida* spp.\[^{18}\] These methods provide MIC data for azole and echinocandin antifungal agents that are both quantitatively and qualitatively similar.\[^{9}\] However, these two reference methods are difficult to implement in a clinical laboratory as routine since they involve rather complex methods not suited to use on a case-per case basis.\[^{19, 20}\]

The CLSI microbroth dilution method continues to be augmented. In addition to standard interpretive breakpoints (resistant and susceptible), CLSI has also proposed the novel category “susceptible-dose dependent” (SDD) for the azole group of antifungal agents. This category indicates the need to maximize drug dosage and delivery.\[^{21}\] The SDD category is unique to yeast testing and is not interchangeable with the intermediate category associated with bacterial and 5-fluorocytosine (5FC) breakpoints. Interpretive categories for 5FC include susceptible, intermediate, and resistant whereas, for candins it is susceptible or nonsusceptible. The term nonsusceptible is used for this group instead of resistant as there is insufficient data to create a resistance category.\[^{17}\]

Important new developments include validation of 24h MIC determination, the establishment of epidemiological cutoff values (ECVs) for systemically active antifungal drugs and different *Candida* spp. and development of specific clinical breakpoints (CBPs) for fluconazole, voriconazole and the echinocandins.\[^{9}\]

As compared to other fungi, the growth of *Candida* is rapid. The majority of *Candida* spp. (except *C. glabrata*) achieve sufficient growth within 24 h. Therefore MIC can often be read at 24 h of incubation. This development is important specifically in *Candida* isolates demonstrating trailing phenomenon (partial inhibition of growth over extended range of antifungal concentration).\[^{16}\] Shortening the incubation period helps clarify trailing end points.\[^{22}\] This development is more efficient and practical for use in the clinical laboratory.\[^{9}\] Due to a reduction in time for reading MIC endpoints, the results of antifungal susceptibility testing of *Candida* is available within the same time frame as antibacterial susceptibility tests.

CBPs aid to indicate those clinical isolates that are most likely to respond to treatment with a given antifungal agent administered using the approved dosing regimen for that agent.\[^{23}\] On the other hand, ECVs may be used as a means of tracking the emergence of reduced susceptibility to antifungal agents among *Candida* spp. It is also used to identify isolates that are less likely to respond to antimicrobial therapy due to acquired resistance mechanisms.\[^{19}\] Over the past few years, ECVs have been established for antifungal drugs like amphotericin B, fluconazole, the triazoles and the echinocandins and 11 species of *Candida*.\[^{9}\]

Following are the modifications that are potentially useful but not included in CLSI methodology.

- Supplementation of the RPMI 1640 medium with 18 g glucose per litre enhances the growth isolates without altering the observed MICs of amphotericin B, fluconazole, ketoconazole and fluconazole.\[^{15, 24}\]
Agitation of microdilution plates prior to reading simplifies visual reading of end points.\textsuperscript{[25]}

Shortening the incubation period to 24 h.\textsuperscript{[26]}

Reducing the pH of the incubation medium clarifies trailing end points.\textsuperscript{[27]}

**Commercial system based on microbroth dilution methodology**

Candifast (International Microbio/Stago Group, Milan, Italy), Integral Systems Yeasts (Liofilchem Diagnostics, L'Aquila, Italy), and Fungitest (Bio-Rad SDP [formerly Sanofi Diagnostics Pasteur], Paris, France), ATB Fungus (API-biomeriux, Marcy l'Etoile France), Mycostandard (Institute Pasteur, Paris, France) Mycototal (Behring Diagnostic, Rueil-Malmaison, France) and are examples of broth-based commercial systems. However, studies show that these methods have limited correlation with the M27-A reference method.\textsuperscript{[9,26,29]}

A commercial system that includes a colorimetric response based on the Alamar Blue redox marker is marketed as Sensititre Yeast One Colorimetric Antifungal Panel (Trek Diagnostic Systems, Inc., Westlake, Ohio).\textsuperscript{[9]} This kit is based on microbroth dilution methodology and has proved easy to interpret. A full range of drug concentrations is tested by this kit and results correlate closely with those obtained by the CLSI M27-A reference method.\textsuperscript{[30]} In this test, a chromogenic substrate facilitates endpoint interpretation.\textsuperscript{[14]} Inclusion of voriconazole, capsofungin and posaconazole makes this method useful for all the systemically active antifungal agents.\textsuperscript{[10]} Pfaller et al.\textsuperscript{[31]} reported ASTY colorimetric panel (Kyokuyo Pharmaceutical Industrial, Ltd., Tokyo, Japan) to have a similarly good correlation with the CLSI M27-A reference method.

VITEK 2 (bioMerieux, Inc., Hazelwood, MO, USA), a fully automated commercial antifungal susceptibility testing system when compared with the CLSI reference broth microdilution method was found to be a reliable, excellent reproducible and practical standardised method. By this method, identification of infecting species of *Candida* along with antifungal susceptibility profile is obtained within 24 h.\textsuperscript{[32]}

2. Agar based methods

As compared to broth dilution methodology, agar based techniques are convenient, economical and easier to perform on large numbers of isolates.\textsuperscript{[8]} These methods can be carried by either inoculating *Candida* isolates in the agar medium and placing the antifungal drug on the agar surface, e.g. disc diffusion and well diffusion techniques or vice versa, such as agar dilution method.\textsuperscript{[8,33]} The size of inoculum, incubation temperature and time greatly alter the results of agar based methods.\textsuperscript{[8]} Partial growth inhibition and the presence of “persistor” or “sic” colonies within inhibition zone affects the estimation of zone size. Relatively insoluble azoles like miconazole, ketoconazole and itraconazole poorly diffuse in an agar diffusion system.\textsuperscript{[8]} The Growth of some NAC spp. may be inhibited in agar containing medium.\textsuperscript{[15]} 

Disc diffusion is the simplest agar based technique for antifungal susceptibility testing of yeasts. This method was developed from previously established method.\textsuperscript{[34]}

A CLSI reference method (M 44A) provides a zone of inhibition, the measurement of which can be correlated with the MIC value provided in broth dilution methods. It is a suitable method of antifungal susceptibility testing of water soluble agents such as flucytosine, fluconazole and voriconazole.\textsuperscript{[13,14,34]} In addition, it has been shown to be suitable for determining the activity of echinocandins against yeast isolates producing easy to read, sharp zones of inhibition.\textsuperscript{[35]} This method provides an enhanced detection of resistance, as in the case of amphotericin B.

The choice of growth medium appears critical; some investigators use RPMI 1640 agar supplemented with 0.2% glucose (RPG), whereas the CLSI recommends the use of Mueller-Hinton agar supplemented with 2% glucose and 0.5ug /ml methylene blue (MH-GMB).\textsuperscript{[13,36]}

In MH-GMB agar the methylene blue improves zone edge definition and enhances visualization of the zone diameters, which facilitates reading.\textsuperscript{[37]} Glucose in the medium enhances the growth of *Candida*. Trailing phenomena around the zone margin is also less frequent and minimal on MH-GMB agar.\textsuperscript{[37]} The occurrence of macrocolonies near the center of the clear zone is also less on MH-GMB agar.\textsuperscript{[37]}

At the present time, there no commercial source of MH-GMB.\textsuperscript{[34]} Mueller-Hinton (MH) agar may be prepared and supplemented with glucose and methylene blue in the
laboratory, or the surface of commercially available MHA plates may be flooded with glucose and methylene blue solution.\cite{24} When methylene blue and glucose solution is added to the surface of the MH agar, the plates should be air dried before inoculation.\cite{10} Both prepared and flooded plates should be used within 24 h of preparation. Barry et al.\cite{26} reported MH-GMB to be superior to RPG, as it supports the growth of almost all species of *Candida* of medical importance.

Chakrabarti et al.\cite{28} standardized a simple disc diffusion technique using Yeast Nitrogen Base Glucose (YNBG) agar which showed good correlation with broth dilution method amphotericin B, 5 FC and fluconazole. Deorukhkar and Saini\cite{37} compared the efficacy of YNBG agar and MH-GMB for antifungal susceptibility testing of *Candida* species isolated from BSI. MH-GMB was found to be a simple, cost effective and sufficiently accurate medium for the routine testing of antifungal susceptibility of *Candida* spp.

Being less costly and easier to perform than M27-A3, the disc diffusion technique can be easily incorporated into laboratories with limited resources. For laboratories that do not perform broth microdilution susceptibility testing of yeast species on site, sending isolates to a reference laboratory can result in delays in reporting of results, limiting the usefulness of the information.\cite{28} For these laboratories, disc diffusion susceptibility testing is often enough to guide therapy.

Etest (AB Biodisk, Solna, Sweden) is a proprietary, commercially available method for antimicrobial susceptibility testing.\cite{15} Etest is recommended as an accurate method for MIC determination of numerous bacteria, including fastidious microorganisms and also *Candida* spp.\cite{11} It is a simple method involving surface inoculation of an agar plate followed by the application of a plastic strip, which contains a concentration gradient of the antifungal under test.\cite{14}

It quantifies antifungal susceptibility in terms of discrete MIC values. MICs are determined from the point of intersection of a growth inhibition zone with a calibrated strip impregnated with a gradient of antimicrobial concentration and placed on an agar plate lawned with the microbial isolate under test.\cite{15,21} The choice of growth medium appears critical with this technique, and RPMI-based agars seem to be most useful.\cite{15} Vandenbossche et al.\cite{11} suggested the use of RPMI-agar with 2% glucose to minimise the problem of trailing endpoints due to partial inhibition by fluconazole. Many other researchers used MH-GMB, which appears to enhance the formation of inhibition ellipses with clear edges and less-elliptic growth.\cite{36}

Pfaller et al.\cite{40} evaluated the E test method for determining fluconazole susceptibility of 402 clinical yeast isolates using three different agar media: RPMI agar containing 2% glucose (RPG), casitone agar (CAS) and Mueller Hinton agar (MHA) and found that the E test method using RPG or CAS, but not MHA, appear to be viable alternative to the NCCLS reference methods for determining fluconazole susceptibility testing of yeast.

Alexander et al.\cite{41} evaluated the Etest with Sensititre Yeast one against CLSI methodology for yeast and seven antifungals, and obtained 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 in assessing the susceptibility to amphotericin B, as this method gives much broader MIC ranges. The Etest is also highly suitable for determining the activity of echinocandins against yeast as it produces easy to read, sharp zones of inhibition. Etest has significant value for the determination of amphotericin B MICs and represents one of the more reliable ways to identify resistant isolates.

Both non-uniform growth of the fungal lawn and the frequent presence of a feathered or trailing growth edge can make end-point determination difficult. However, with experience and standardized techniques, the correlation between this method and the reference method has been acceptable for most *Candida* spp. and theazole antifungal agents.\cite{42}

Semisolid agar susceptibility technique is a simple, rapid and cost effective antifungal susceptibility testing method.\cite{43} In this method the concentration of agar in the medium is to 0.5%. As this method requires no special expertise and expensive equipment, its application in resource
 constrained clinical microbiology laboratories cannot be overstated. This technique is accurate and highly reproducible. A major advantage of this method is its potential for screening both pathogenic yeast and molds by the same protocol.[43]

3. Flow cytometry
Flow cytometry has been long recognised as a possible tool for antifungal susceptibility testing of Candida spp.[44] Staining (or lack of staining) with suitable dyes like acridine orange, FUN-1, propidium iodide permits the rapid detection of damaged fungi.

Studies by many workers have shown the potential for correlation of flow cytometry-based techniques with the reference method.[14] Favel et al.[45] suggested flow cytometry to be useful for detection of amphotericin B resistance. In conceptually related studies, fluorescent viability dyes have been used to examine the nature of drug-induced damage to yeasts.[46] Rudensky et al.[47] reported rapid flow cytometry to be a reliable, reproducible and rapid method for determining susceptibility of Candida to fluconazole and echinocandin. By this method, the results were obtained in 5 h or less and comparable with the reference method.

Other methods for antifungal susceptibility testing
Many alternative methods for antifungal susceptibility testing of Candida spp. have been proposed by many researchers. Shimokawa O and Nakayama H (1997) have shown that testing azoles against Candida species on acetate-supplemented media produces MICs that more closely approximate the critical concentration at which ergosterol biosynthesis is inhibited.[48] Incorporation of chemicals like MTT [3-(4, 5-dimethyl-2-thiazoyl)-2, 5-diphenyl-2H-tetrazolium bromide] or XTT (2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) as a colorometric marker for redox potential has been found convenient for Candida species.[49] The MIC values are comparable with CLSI reference method. Use of XTT eliminates an extraction step that is needed with MTT-based assays.[49]

Kretschmar et al.[50] proposed a rapid method for detection of fluconazole susceptibility by a bioluminesence assay measuring intracellular ATP concentration. Rieselman et al.[51] proposed a rapid method for determination of MICs by measuring glucose consumption from incubation media. Both these methods demonstrated excellent correlation with CLSI reference method.

CONCLUSION
The increased incidence of candidiasis along with emergence of drug resistance species has underlined the need of antifungal susceptibility testing. Antifungal susceptibility testing is still less utilised than antibacterial testing in many developing countries like India. The CLSI standardised broth dilution method is complex and labor intensive to use as a routine method. Alternative methods like disc diffusion and Etest have been adapted for sensitivity testing of Candida spp. by many resource constrained laboratories. These methods are simple yet comparable with CLSI standardized broth dilution method. Simple, user friendly methods like disc diffusion and Etest enables resource-constrained laboratories to determine the antifungal susceptibility in clinically useful timeframe.

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**How to cite this article**: Deorukhkar S.C, Saini S, Mathew S. Perspectives on antifungal susceptibility testing in *Candida*. Int J Infect Trop Dis 2014;1(2):51-59.

**Conflict of Interest**: None declared

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