



Antifungal susceptibility testing of candida – Methods and issues in current practice

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ABSTRACT

Despite the availability of many antifungal drugs in clinical practice, the occurrence of antifungal drug resistance is on the rise. Since the antifungal susceptibility testing (AFST) is not done routinely in many of the microbiology laboratories, it is very difficult to determine which antifungal agent is very effective for a particular infection. There is a real need for precise, reproducible and extrapolative antifungal susceptibility testing methods to aid the therapeutic management. The practice of empirical treatment for fungal infections further promotes the emergence of resistant strains. The AFST practice would essentially help the clinicians in appropriate decision making. Although conventional AFST methods are somewhat cumbersome, many novel AFST methods are currently available in many laboratory settings which would provide a quicker result many times. In essence, the application of AFST along with identification of the fungus up to species level would definitely be very helpful in selecting the primary antifungal agents for treatment especially in difficult to manage and invasive fungal infections. This review will throw light on the various AFST methods available and their issues in the current practice.

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INTRODUCTION

Invasive fungal infections, especially those caused by *Candida* species is on the rise. Antifungal drug resistance makes the management of such infections more difficult. However, other than the inher-

ently resistant species majority of the fungal infections are clinically treated by the common antifungal drugs which include azoles, echinocandins and polyenes group of drugs. There is a broad unanimity in the clinical practice that the prognosis of the fungal infections would be better when the treatment is started early (Karthaus *et al.*, 2011).

Although many classes of antifungal drugs are currently available, such as azoles, polyenes, flucytosine, echinocandins, etc. the emergence of antifungal drug resistance is on the rise. This antifungal resistance is a great concern especially for fungal strains exhibiting resistance to the routinely prescribed antifungal drugs (Pfaller, 2012).

In the recent decade, there is a significant rise in the incidence of fungal infections (Pfaller and Wenzel, 1992). The availability of antifungal susceptibility testing is limited due to cumbersome techniques

and lack of reproducibility. As a result, the clinical applications of AFST is also limited to tertiary centres. The AFST basically produces data on the susceptibility, intermediate susceptibility or resistance towards the antifungal drug by the organism. The results of AFST could influence the selection of therapy, facilitating the clinician in the management of challenging cases.

Antifungal susceptibility testing is one of the dynamic and challenging fields in medical microbiology. Though the standardisations of the testing methods by Clinical Laboratory Standards Institute (CLSI) and European Committee on Antibiotic Susceptibility testing (EUCAST) had made a remarkable change in the AFST, MIC breakpoints and the clinical significance of these tests in the management of cases still remains unclear in many at times. Depending upon the established MIC breakpoints, it is promising to determine the antifungal susceptibility of *Candida* strains. However, MIC breakpoints and clinical significance of antifungal susceptibility testing for other fungi remain unclear. Though many other methods like E-tests, agar dilution methods, colorimetric microdilution methods, flow cytometry, etc. are under investigations, they are yet to become standardised and acceptable. Ergosterol quantitation is one of the novel methods, is yet to be studied further. This paper will focus on various methods of AFST of *Candida* species and their challenges in routine practice.

Broth-Based AFST:

Microdilution methods are the gold standard methods for AFST. This is one of the optimized and standardized methods developed by Clinical Laboratory Standards Institute (CLSI) for routine antifungal susceptibility testing. Standardized microdilution based methods by CLSI and EUCAST are the widely accepted procedures for AFST in laboratory practice (Arendrup *et al.*, 2012). However, the broth-based AFT methods are time-consuming and cumbersome for routine diagnostic use (Alastruey-Izquierdo and Cuenca-Estrella, 2012).

In 1985, the CLSI formed a subcommittee on Antifungal susceptibility testing which published the document M27A "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard" in the year 1997. This M27A document clearly defines the reference strains with Minimum inhibitory concentrations (MICs) ranges and breakpoints for antifungal drugs against *Candida* and other yeasts. EUCAST formed a subcommittee in 1977, which published a standard on Antifungal susceptibility testing in 2008. Though there are differences in these microdilution broth based

methods in inoculation size, time and the medium composition, the results by both the methods are usually comparable (Chryssanthou and Cuenca-Estrella, 2006; Rodriguez-Tudela *et al.*, 2007).

In macrobroth dilution method, in order to avoid drug medium interaction, Roswell Park Memorial Institute Medium (RPMI-1640) with glutamine and phenol red as a PH indicator, without bicarbonate, was used with an optimum incubation 35°C for 48-72hrs. The endpoint was defined as the lowest dilution that resulted in zero visible growth for Amphotericin B (or) an 80% reduction in turbidity as compared to the control tube (without drug) for the other drugs. Microtiter dilution method is similar to macro broth dilution method. However, the endpoints were defined as zero visible growth for Amphotericin B (or) 50% reduction in turbidity as compared to the control well for other drugs (CLSI, 2004).

Fungitest:

This is a simple, rapid commercial micro method for broth dilution breakpoint testing of *Candida* isolates and yeasts against antifungal drugs such as amphotericin B, flucytosine, fluconazole, itraconazole, ketoconazole, and miconazole. The Fungitest method is a commercial testing kit provided with 16 well microtitre plate with 2 negative and 2 positive control wells and 12 drug-containing wells. The antifungal drugs will be in dried form, which can be reconstituted by adding the isolate suspension in RPMI medium. A colorimetric indicator is usually included in the medium to find the endpoint. This test is comparable to CLSI broth based AFST tests in azole-susceptible strains, however, this method fails to detect azole-resistant strains effectively (Davey *et al.*, 1998; Witthuhn *et al.*, 1999).

E test:

E-test is one of the routinely followed commercially available antifungal susceptibility tests. It is one of the simple, reliable agar based gradient tests for quantitative analysis of AFST for yeasts and moulds. E test quantifies the AFST in terms of discrete MIC values. The e test strip is an inert, thin strip containing the gradient of the antifungal agent in a dried and immobilised form with maximum concentration in one end and minimum at the other end. This method requires surface inoculation of the fungi on the agar plate and application of the E-strip containing the antifungal drug in a gradient form over the inoculum and incubated. After incubation, in case of susceptible organisms, a zone of inhibition can be seen and the point at which the zone intersects the strip is taken as the MIC value. The results are believed to correlate well with the CLSI values (Warnock *et al.*,

1998; Szekely *et al.*, 1999). The inhibition ellipses in E test are sharper and can be easily interpreted.

E test strips are commercially available for various antifungal drugs like fluconazole, amphotericin B, itraconazole, ketoconazole, voriconazole, caspofungin and posaconazole. E test is found to be more efficient in detecting amphotericin B resistance in *Candida* isolates. However, the correlation between the clinical outcome of the patient and the diagnosis of the antifungal susceptibility by the Etest is yet to be validated effectively. (Park *et al.*, 2006)

colorimetric microdilution method :

In order to make the visual reading easy and accurate, colorimetric indicators are incorporated in the assays, Sensititre Yeast One is now widely used. This is a commercially available colorimetric microdilution method based on CLSI. In this method, Alamar blue is used as an oxidation-reduction colorimetric indicator, in RPMI medium supplemented with 2% glucose. Red colour indicates the growth of the fungus, whereas purple colour denotes growth inhibition and blue colour conveys no growth. This method has been widely used in the antifungal susceptibility testing of *Candida* species as well as few filamentous fungi against fluconazole, amphotericin B, itraconazole and flucytosine. (Carrillo-Munoz *et al.*, 2006)

ASTY colorimetric microdilution panel is another method, which is under investigation. (Pfaller *et al.*, 1998). Further, there is another non-commercial method which is based on mitochondrial dehydrogenase enzymes reducing tetrazolium salts 2,3-bis {2-methoxy-4-nitro-5-[(sulfonyl amino) carbonyl]-2H-tetrazolium-hydroxide} (XTT). XTT is being studied for susceptibility testing of yeast species. There will be a colour change from yellow to purple indicating the conversion of XTT to a formazan derivative.

Agar dilution methods:

These methods are carried out by using doubling dilutions of the antifungal drugs which are going to be incorporated into the molten agar. The agar plates containing the drugs are inoculated with suspensions ranging from 100 000 – 10000000 cells/ ml. AM3, RPMI, and YNB are the commonly used solidified medium for agar dilution method of testing antifungal susceptibility.

Disc diffusion tests:

Disc diffusion testing is one of the simple methods for in-vitro antifungal susceptibility testing for antifungal drugs such as voriconazole, fluconazole, flucytosine, echinocandins, etc. CLSI has approved the disc diffusion method for testing the susceptibil-

ity of yeast isolates to antifungal drugs (CLSI M44A2 document) and susceptibility breakpoints have also been established for various antifungal drugs.

The zone of inhibition can be measured and correlated with the MIC values. This method uses the paper discs impregnated with antifungal drugs. The critical concentration (CC) value represents the concentration of the antifungal agent at the edge of the inhibition zone after 24 hours of incubation at 35°C. (Bartizal *et al.*, 1997).

MALDI-TOF Mass Spectrometry-Based AFST Methods

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been widely used as a rapid, reliable and cost-effective method for identification of species (Fenselau and Demirev, 2001). This technology has been equally good in susceptibility testing which has revolutionised as an excellent alternative method to traditional procedures (Lange *et al.*, 2014; Sparbier *et al.*, 2012). It significantly reduces the turnaround time compared to biochemical and nucleic acid-based techniques (Arvanitis *et al.*, 2014; Posteraro *et al.*, 2015). This method is commonly employed for rapid detection of susceptible and resistant isolates to caspofungin.

NOVEL METHODS:

Flow Cytometry :

Flow cytometry (FC) (fluorescence-activated cell sorting-FACS) is one of the novel technology for antifungal susceptibility testing developed to improve the quality of the AFST in the microbiology laboratories. In this method, the alterations in the fungal cell viability is observed as changes in the cell fluorescence for various concentrations of the antifungal drugs. This is quite different from the routine conventional methods where inhibition of the growth of the organism is measured. This method has the advantage of short incubation time nearly 4 -6 hours only. FC uses various DNA binding dyes like FUN-1, propidium iodide, 3,3'- dipentylloxacarbocyanine iodide or acridine orange (Rudensky *et al.*, 2005). The results are determined by the increase or decrease in the fluorescence intensity of the stained cells on exposure to the antifungal drugs.

Ergosterol Quantification :

This newer method measures the cellular ergosterol content by spectrophotometric absorbance profile between 240 – 300 nm. Ergosterol is first isolated from yeast cells by saponification. Non-saponifiable lipids are separated with heptane. This method has been used for susceptibility testing of *Candida* strains against fluconazole and itracona-

zole ([Arthington-Skaggs et al., 1999, 2000](#)). This method is proposed to be a reliable method for prediction of the outcome of the patient in azole-resistant isolates. ([Arthington-Skaggs et al., 2000](#))

CURRENT CHALLENGES:

The antifungal susceptibility testing methods were recently standardized and are yet to come under routine laboratory practice in many centres. With the increasing emergence of fungal infections taking precedence in the clinical scenario, it becomes crucial to know the susceptibility of the fungal pathogens. This can be very helpful for both the patient and clinician in deciding the outcome of the infection. In the treatment of fungal infections, the correlation between MIC and susceptibility category to provide a desired clinical outcome is still an ongoing challenging arena, which makes the interpretation of clinical breakpoints a difficult one. The antifungal breakpoints can be established based on factors like MIC distribution curve for wild type organism, their pharmacokinetic and pharmacodynamic properties, the clinical outcome of patients with antifungal drugs ([Johnson, 2008](#)).

Clinical breakpoints can be used to differentiate strains for which there is a high likelihood of treatment success (organisms that are clinically susceptible) from those for which treatment is more likely to fail (clinically resistant) ([Eschenauer and Carver, 2013](#)). Although clinical breakpoints provide guidance and help to choose the antifungal drug, they do not confer any information about the resistance mechanism of the pathogenic isolate. This along with the variability in the host response confounded any chance to predict the in vivo outcome of the susceptibility results based on in vitro testing of antifungal agents.

For the past two decades, there have been a lot of changes in the host environment, the fungal agents and clinically used antifungal drugs. These changes include increase in the incidence of infections that cause profound immunosuppression leading to the invasion of fungal pathogens and raised mortality, newer fungal infectious agents which are becoming pathogenic, emergence of antifungal resistance in the clinical environment due to inadequate treatment as the antifungal therapy needs prolonged duration of treatment for at least near complete cure in most conditions, underdiagnosis of superadded fungal infection which slowly compromises the patients' immune status further worsening the condition ([Kontoyiannis and Lewis, 2002](#); [Bille et al., 2005](#)). The reference data is also limited and is available only for few fungal pathogens (candida, Cryptococcus, Aspergillus, and Fusarium) and anti-

fungal agents.

Disc Diffusion methods are commonly used for the antifungal susceptibility testing of Candida species and Aspergillus as the method is easy to perform, cheap and rapid.

Azoles like fluconazole and itraconazole produce partial growth inhibition of Candida species. This is shown as reduced but persistent growth over an extended range of the concentrations of the drug in susceptibility testing. This phenomenon is referred to as 'trailing' which leads to difficulty in the interpretation of visual endpoints for the zone in susceptibility testing. Trailing increases with increased duration of reading the results. This offers difficulty in interpretation to be applied for clinical outcomes.

Trailing can be overcome by the addition of methylene blue to the agar, or trailing isolates can be tested by ergosterol quantitation and spectrophotometric reading of azole MICs which eases the determination of accurate susceptibility categories ([Arthington-Skaggs et al., 2000](#)). Another limitation to agar based method is the time duration needed to identify the filamentous fungi, which can range from 24 hours to days and a further delay in the antifungal susceptibility testing time after the pathogen is isolated. Microbroth dilution method reported in NCCLS M27-A2 was widely accepted and are preferred for filamentous fungi for precise reading of antifungal susceptibility testing. (National Committee for Clinical Laboratory Standards, 2002)

The limiting factors which make it difficult for the routine testing of antifungal susceptibility in laboratories are

1. The decision of the growth media needed for the culture of the suspected pathogen.

- Growth medium broth dilution assay
- A Complex medium is needed and the various supplements that are required to be personalised for different moulds.
- Interference towards the antifungal drugs under test.
- Growth medium in agar dilution assay
- The culture medium in agar dilution assay needs to be standardised for the isolate to be tested and sometimes a complex medium like Leeming-Notman agar (LNA) is needed.
- Growth medium in disk diffusion assay

Table 1: Advantages and disadvantages of various antifungal susceptibility testing methods

Sl No.	Method of Antifungal susceptibility testing	Advantages	Disadvantages
1	Microdilution	Detects resistant strains	Usually done in specialised laboratories. Needs a longer time MIC breakpoints for antifungal drug combinations are yet to be established
2	Commercial methods like Sensititre Yeast One, E test, Vitek 2, FUNGITEST	Simple, rapid, Commonly used in routine testing of isolates.	Not advised for categorisation of resistant isolates Cautious interpretation is required for routine susceptibility testing due to the occurrence of a discrepant result as compared to CLSI reference methods.
3	Agar diffusion method	Rapid, Cheap and easy method	Not advised for categorisation of resistant isolates
4	Novel methods like MALDI TOF, FC, Ergosterol quantitation	Simple, Rapid method for detection of resistant isolates	Not advised for categorisation of resistant isolates Unavailability of the required equipment in several centres. Is under investigation

1. The medium recommended in the CLSI reference assay (document M44-A2) is Mueller-Hinton (MH) agar supplemented with 2% glucose (G) and methylene blue (M). The standardisation of inoculum preparation and additional components like tween 40 and tween 80 needed for enhanced growth of the isolate are the limiting factors.

2. Inoculum

The inoculum preparation varies between moulds and yeast which mandates technical expertise. Given the slow duration of growth of certain moulds the inoculum size needs to be standardised for each mould which adds up to the burden of the laboratories. Despite standardisation, the reproducibility for the same isolate becomes difficult owing to the changes in the host factors and the site of the sample.

3. Temperature and duration

Although most yeast and moulds grow in 48 to 72

hours, some require up to 10 days before a negative report is given. This further delays the need for susceptibility testing which could further take a few more days before the final complete report could be released.

The interlaboratory and intra laboratory result reproducibility is poor in AFST methods. The various factors for such discrepancies are the testing method, inoculum size, composition of the testing medium, temperature and incubation period, endpoint determination, etc (Rambali *et al.*, 2001; Rex and Pfaller, 2002; Johnson, 2008; Pfaller, 2012). It is also difficult to correlate the in-vitro AFT results with the clinical outcome in many cases. Interpretive breakpoints for the currently used antifungal agents need to be established and validated.

In immunosuppressed patients, antifungal susceptibility testing in vitro will remain one of the several factors that will have a profound impact in the prediction of clinical outcome.

CONCLUSION

Clinicians are facing many challenges in antifungal resistance in their day to day practice. These include increased rates of resistance to commonly used antifungal drugs and recurrence of fungal infections. Newer antifungal drugs are currently in the developmental stage. Thanks to the development of standardized reference methods for AFST, the susceptibility results are now comparable between the laboratories. Once a method is validated to provide a good comparable result, the next challenge would be to compare the result with the clinical outcome. The success and failure of the AFST methods purely depend on the prevalence of antifungal resistance in the locality as well as the rational usage of various technologies in clinical laboratory practice. Clinical utility of these AFST methods remains unestablished for several fungi. Studies are needed to establish the MIC breakpoints and the benefits of various antifungal susceptibility methods for prediction of clinical outcome.

Author contributions:

L. K and P. S prepared the manuscript. C. S reviewed and edited the manuscript.

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