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REVIEW ARTICLE

In vitro Antifungal Susceptibility Testing: A Review

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ABSTRACT

Candida albicans is a diploid fungus and a causal agent of opportunistic oral and genital infections in humans. Systemic fungal infections have emerged as important causes of morbidity and mortality in immunocompromised patients. In addition, hospital-related infections in patients not previously considered at risk have become a cause of major health concern. *C. albicans* is commensal and is among the gut flora, the many organisms which live in the human mouth and gastrointestinal tract. Under normal circumstances, *C. albicans* lives in 80% of the human population with no harmful effects, although overgrowth results in candidiasis. candidiasis is often observed in immunocompromised individuals such as HIV-positive patients. Candidiasis also may occur in the blood and in the genital tract. candidiasis, also known as "thrush", is a common condition which is usually easily cured in people who are not immunocompromised. To infect host tissue, the usual unicellular yeast-like form of *C. albicans* reacts to environmental cues and switches into an invasive, multicellular filamentous forms. In a process which superficially resembles dimorphism, *C. albicans* undergoes a process called phenotypic switching, in which different cellular morphologies are generated spontaneously. One of the classically studied strains which undergoes phenotypic switching is WO-1 which consists of two phases - one which grows as smooth white colonies and one which is rod-like and grows as flat gray colonies. The switching is reversible, and colony type can be inherited from one generation to another. While several genes which are expressed differently in different colony morphologies have been identified, some recent efforts have focused on what might be controlling these changes.

Key words: *Candida*, Antifungal agents, Anticandidal effect and Susceptibility.

1. INTRODUCTION

Candida albicans and related species live as benign commensals in one or more body locations in a majority of healthy individuals and it is responsible for the two different types of the fungal infections both the superficial as well the deep/ systemic fungal infections and affecting both immunocompromised and immunocompetent hosts. Available typing methods for *Candida* include serotyping, morphotyping, resistotyping, biotyping, and killer yeast typing. The potential impact of the phenomenon of phenotypic switching on the reproducibility of these typing methods is discussed. It is concluded that many of the available typing methods have not been adequately assessed by their developers and that several have only poor discriminatory power or reproducibility [1].

During the 1980s, a large number of typing methods for the strain differentiation of *Candida albicans* were described in the literature. Although these methods have been based on a variety of physiological and genetic markers, none is ideal. Until recently, morphotyping, a method evaluating fringe and surface characteristics of streak colonies grown on malt agar, has been recommended as a simple and unexpensive typing method for *Candida albicans* isolates [2]. Morphotypes observed in *C. albicans* strains isolated from the mouth of children with and without Down's syndrome, was analyzed by Ribeiro *et al.* [3] and 9 different *C. albicans*

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morphotypes had been isolated form children with Down's syndrome and children without Down's syndrome.

Phenotypic switching (a.k.a. phenotypic dimorphism) is switching between two cell -types. An example is *Candida albicans,* which, when it infects host tissue, switches from the usual unicellular yeast-like form of into an invasive, multicellular filamentous form [4]. This switching between two cell - types is known as dimorphism. Phenotypic switching in *C. albicans* includes the switch from white cells to opaque cells in need for sexual mating. Several typing schemes have been developed in recent years for the sub-species differentiation of *Candida albicans.* These include biotyping [5], resist O - typing [6], sensitivity to "killer yeasts" [7], extracellular enzyme production [8], immunoblotting [9] and DNA fingerprinting [10]. So far none of these methods has been shown to differentiate between strains of high and low virulence. A new typing scheme for *C. albicans*, which is based on colonial morphology (morphotyping), has recently been described [11].

Morphological features of streak colonies allowed distinguishing 11 different morphotypes while RAPD fingerprinting yielded 25 different patterns and CHEF electrophoresis recognized 9 karyotypes. The discriminatory power calculated with the formula of Hunter and Gaston was 0.780 for morphotyping, 0.984 for RAPD fingerprinting, and 0.630 for karyotyping. A variety of typing system has been adopted. A variety of typing methods for *C. albicans* have been described, but none of them have particularly good discriminatory power and some suffer from poor reproducibility. To compare the discriminatory capacity of several different phenotyping methods to distinguish 94 strains of *C. albicans* isolated from a homogeneous population, that is, the genital tract of female prostitutes with vulvovaginal infection, and the practical application of such methods in clinical microbiology laboratories were studied by the [12].

2. *In vitro* ANTIFUNGAL SUSCEPTIBILITY TESTING: METHODS OF ANTIFUNGAL SUSCEPTIBILITY TESTING

2.1. Broth Dilution

Macrodilution broth testing was established as the reference standard for antifungal susceptibility testing, serving as the basis for comparison with all alternative methods. Detailed procedures for performing macrodilution and microdilution tests of *Candida* sp. and *C. neoformans* are available elsewhere. The methods were developed to facilitate conformity among laboratories and to be used as references for development of other methods. Adherence to NCCLS methods provides greater than 90% intra-laboratory and inter-laboratory reproducibility.

Broth dilution testing of antifungal can be performed by micro dilution or macro dilution. Both methods are prepared similarly, except the volume for macrodilution is 1 ml and the test is conducted in test tubes, whereas the microdilution volume is 200 µl/well and the test is done in a 96-multiwell microdilution plate. Serial 2-fold dilutions of the drug in RPMI (Roswell Park Memorial Institute) 1640 test medium are prepared and dispensed into the tubes or wells as appropriate. Both methods adjust the starting inoculum to 0.5-2.5 x 10^3 colony-forming units (cfu)/ml (1 x 10^4 cfu/ml for *C. neoformans*) in RPMI 1640 test medium buffered to a pH of 7.0 with morpholinepropane-sulfonic acid (MOPS) 0.165 M. Medium, drug, and inoculum suspension are added to the test tubes or wells and are incubated at 35°C for 48 hours (72 hrs for *C. neoformans*). The MIC for amphotericin B is defined as the lowest concentration with no visible growth. The MIC for azoles and flucytosine are read as the lowest concentration with either 80% growth reduction (macrodilution) or 50% growth reduction (microdilution) compared with control (drug-free) tubes/well. The macrodilution method is cumbersome and impractical for most clinical laboratories to perform. Although, microdilution is more convenient, improvements are necessary to ensure optimal performance.

2.2. Colorimetric Testing

An alternative method uses colorimetric growth indicators. One colorimetric antifungal panel (Trek Diagnostics, Westlake, OH) consists of a microtiter plate with dried antifungal drugs (amphotericin B 0.008-16 µg/ml, fluconazole 0.125-256 µg/ml, itraconazole 0.008-16 µg/ml, ketoconazole 0.008-16 µg/ml, 5-flucytosine 0.03-64 µg/ml). Each well incorporates an oxidation-reduction indicator, Alamar Blue, that changes from blue to pink in the presence of microbial growth. The first well to show a change from pink (growth) to purple or blue (growth inhibition) was recorded as the MIC. Several multicenter studies found good correlation between NCCLS microbroth dilution and Alamar Blue colorimetric susceptibility tests among *Candida* sp. and *C. neoformans.*

Another preliminary colorimetric test used for filamentous fungi and yeast isolates incorporates a dye indicator MTT [3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide], which is added with the initial inoculum. Preliminary reports suggest this may be an alternative to Broth micro dilution. Another new approach may provide MIC readings in less than 8 hours, compared with 48 hours required by Broth micro dilution. The rapid susceptibility assay (RSA) is based on a colorimetric reaction. It assumes that the uptake of exogenous substrates, such as glucose, will be suppressed in susceptible fungi in the presence of antifungal drugs. The relative residual glucose concentration, represented by optical density, can be plotted against the increasing concentration of the drug. The susceptibility of an isolate can be determined based on the relationship between drug concentration and optical density. The RSA has been tested with amphotericin B, flucytosine, fluconazole, and itraconazole against *Candida* sp. Among 106 yeast isolates, 100% and 98% agreement was observed for MICs with amphotericin B and flucytosine, respectively, compared with broth microdilution. Agreement was lower, 61-63%, with fluconazole and itraconazole. These preliminary results suggest that RSA may be useful clinically for rapid MIC determination.

2.3. Spectrophotometric Testing

Spectrophotometric methods are alternatives to visual inspection of MIC end points. The MIC end points are determined at the lowest drug concentration resulting in 50% or greater or 90% or greater inhibition of turbidity observed in control wells for azoles and flucytosine or amphotericin B, respectively. Spectrophotometric end point readings of MICs may eliminate subjectivity associated with the trailing effect of azoles. Agitation of the microtiter plate before reading MIC end points may improve the quality of visual as well as spectro-photometric MIC end point determination.

2.4. E-Test

The E-test stable agar gradient method (AB BIODISK, Solna, Sweden) has been evaluated for antifungal susceptibility testing of *Candida* species, *C. neoformans* and filamentous fungi and appears to be a good alternative to broth dilution reference methods. The E-test involves placing a plastic strip containing a gradient of an antifungal agent on the surface of an inoculated agar plate. The drug diffuses into the agar and establishes a stable concentration gradient. Inhibition of fungal growth produces an ellipse, and the MIC is read where the ellipse intersects the test strip. Plates are inoculated by streaking a standard suspension of yeast or mold, turbidity equal to 0.5 McFarland standard (1 McFarland standard for *C. neoformans*), across the entire surface of agar in three directions. Plates are incubated at 35°C for 48 hours for *Candida* sp, 72 hours for *C. neoformans,* and 48-72 hours for molds. The RPMI 1640 supplemented with 2% glucose and buffered with MOPS at pH 7.0 agar is used as the test medium for *Candida* sp., *C. neoformans,* and molds.

2.5. Disk Diffusion and Agar Dilution

Disk diffusion testing is limited to fluconazole and yeasts and is performed more in Europe than in the United States. Studies reported some clinical usefulness. This is a simple and economic alternative to broth dilution tests and can be performed quickly in busy clinical laboratories. It was used to test isolates of *Candida* sp to determine fluconazole resistance among patients infected with the human immunodeficiency virus (HIV) and oropharyn-geal candidiasis. A good correlation was found between broth dilution MIC and diameter of inhibition surrounding the 25 µg fluconazole disk (r=0.85, p=0.001). *Candida albicans* isolates with decreased susceptibility to fluconazole could be identified by disk diffusion and linked to clinical resistance.

Fluconazole susceptibility was analyzed by comparing the commercially available disk diffusion test Neo-sensitabs (Rosco Diagnostica, Taastrup, Denmark) with NCCLS macrobroth dilution. Correlation was found between the tests, indicating that Neo-sensitabs may have a potential as a screening tool to identify fluconazole resistance among *Candida* isolates. A large surveillance study determined fluconazole susceptibility of 20,900 clinical *Candida* isolates using a 25 µg fluconazole disk diffusion method. Mueller-Hinton agar medium was supplemented with 2% glucose and methylene blue. Methylene blue was not used for its dye property but rather to improve zone edge definition with slow-growing *Candida* sp. Plates were inoculated with a 0.5 McFarland and were incubated at 35°C for 18 - 23 hours before reading. Zone diameters were determined electronically at 80% growth inhibition by the BIOMIC system for all clinical strains and quality control isolates. The zone size surrounding the fluconazole disk was compared with the NCCLS reference method. By using

regression analysis, tentative zone interpretive criteria were established for fluconazole susceptibility: 19 mm or greater = susceptible, 12 mm or less = resistant, and 13-18 mm = susceptible-dose dependent. This study showed that the simple and inexpensive fluconazole disk diffusion could be used for large surveillance studies in addition to routine use in the clinical laboratory. Further studies must be completed to evaluate this method.

Another simple agar screening method for fluconazole susceptibility of *C. neoformans* consists of Sabouraud's dextrose agar supplemented with 2% glucose and 8 or 16 µg/ml of fluconazole. Colonies that were resistant had similar growth size on fluconazole-containing and fluconazole-free media. In contrast, fluconazole-susceptible isolates were much smaller on drug-containing media than on drug-free media. This method may be an easy and inexpensive screening method for fluconazole resistance.

2.6. Flow Cytometry

Flow cytometry is an investigational method that can provide an MIC value in 2 and 6 hours for amphotericin B and fluconazole, respectively. It was evaluated with *Candida* sp and *C. neoformans.* The method measures permeability of the fungal cell membrane after contact with various concentrations of antifungal agent. A membrane-impermeable fluorescent DNA-intercalating dye is placed in the standardized yeast suspension 2-6 hours after exposure to the drug. The suspension then is placed in a flow cytometer that measures the intensity of the fluorescent dye binding to intracellular DNA. The MIC is determined to be the lowest concentration of antifungal agent that produces an increase of 50% in mean channel fluorescence. This method yields MIC results comparable with NCCLS broth dilution methods. Although flow cytometry requires special laboratory equipment, it provides MIC results in hours rather than days [13].

2.7. Notes on the disk diffusion and ETEST methods.

The disk agar gradient method can accurately and reproducibly determine the susceptibility of fungi to antifungal agents eg fluconazole. Improvements include the use of RPMI glucose agar, inoculum density adjustment with a 0.5 McFarland Standard, and incubation at 35C for 24 hours. These changes standardise the test to current CLSI guide-lines and make it relatively comparable to standard bacterial test methods. This method and interpretive criteria apply to rapidly growing yeast species, defined as strains producing growth within a 24 - 48 hour incubation period. Data on slower growing moulds is largely in-house at present.

2.8. Disk Method

Melt and pour RPMI-Glucose Agar plates [media may be stored in 60 ml aliquot's and then melted in a microwave to pour plates when required]. For a standard screen using disks of all the above antifungal agents we typically use 3 plates set up as follows (Additional plates may need to be poured for E-test and other investigational purposes. Note susceptibility tests for Ampohtericin may be unreliable on RPMI media; the use of Antibiotic Medium 3 may enhance the detection of resistance, but this medium is not standardized and substantial lot-to-lot variability is possible). Prepare an inoculum by suspending a single isolated colony in about 5 ml of normal saline or sterile water. Agitate to achieve a smooth suspension on the Vortex mixer. Adjust the suspension with saline or water to approximate a density of 0.5 McFarland Turbidity Standard [we use a bio Merieux Densimat]. For filamentous fungi, which do not sporulate profusely, add one drop of the wetting agent Tween 20 to the sterile distilled water and then break up the mould by shaking it with small glass beads or by grinding it into a suitable suspension. Moisten a sterile cotton (not dacron) swab in the adjusted inoculum suspension. Express excess moisture by rolling the swab on the inside of tube above fluid level. Streak the surface of an RPMI-glucose agar plate in 4 different directions (at 90 degree angles) to cover the entire surface. Let the surface of RPMI-glucose agar plates dry at 35°C with lids ajar until no droplets of moisture are on the agar surface. Using a pair of flamed sterilized forceps apply the disks containing the antifungal agent to be tested onto the surface of the inoculated agar plate and press lightly to insure complete contact with agar. Incubate plates at 35°C for 24 - 48 hours or until sufficient growth has occurred. Plates should be read as early as possible after 24 hours incubation and results recorded in the susceptibility book.

2.9. E-test method

Melt and pour one plate of RPMI-Glucose Agar for each E-test to be performed. Follow the exact method as described above for disks. Using a pair of flamed sterilized forceps apply one E-test strip and one Neo-sensitab of the antifungal agent to be tested onto the surface of the inoculated agar plate and press lightly to insure complete contact with

agar. Measure zone diameter to the nearest whole millimetre. For Amphotericin B and 5-Fluorocytosine the zone of inhibition should be determined at the point of complete (100%) or almost complete (95%) inhibition. For the azoles Ketoconazole, Fluconazole and Itraconazole the zone of inhibition should be read at the first point of significant inhibition/marked decrease in growth intensity ie (80%) inhibition. Pinpoint microcolonies at the zone edge or within the zone of inhibition should be ignored.

3.INTERPRETATION OF ZONE DIAMETERS AND MIC'S

The following zone diameter and MIC standards are for Neo Sensitabs on RPMI media [note these are in house standards and are subject to change at any time]

3.1. Multidrug resistant strain

The *Candida* strains resistant to four or more antifungal drugs were considered as multidrug resistant strains. *Candida albicans* and other *Candida* species. are opportunistic pathogens involved in an increasing number of infections of patients with immunodeficiency. Different typing methods, including biotyping [14], enzyme typing [15], morphotyping [16], serotyping [17], resistotyping [18], killer typing [19], protein typing [20, 21], and karyotyping [22], have been described previously, and attempts have been made to detect a possible correlation of some *C. albicans* types with the virulence of the isolates [23, 24].

3.2. *Candida* Morphotyping

The morphotyping technique of [25] was used to differentiate the species and the strains of Candida isolates isolated from patients groups and control group. Yeast cells from 24 hrs culture which were grown on 2% (W-V) malt agar at 25-30ºC and were suspended in 5 ml of sterile distilled water in screw capped glass bottles. That was adjusted to a Mac Farland's opacity No.4 turbidity (ie.approximate 10^7-10^8 cells/ml^{-1}). Six percent malt extract agar with the addition of 2% oxoid noble agar was autoclaved and distributed in 18-20 ml quantities and were dispensed into 9cm disposable petridishes.

A loopful of yeast suspension was inoculated onto malt agar. The plates were then incubated aerobically in a BOD incubator at 30ºC for 10 days in the dark and cultures were marphotyped according to their surface characteristics. A series of defining colony fringe characteristics were studied according to [26]. The morphotype code used was as follows.

3.2.1. Fringe characteristics

The morphotyping code used was (1) fringe characteristics of the colony streak designated as absent (0), discontinuous < 20% of streak Margin (1), discontinuous 20 - 50% of margins (2) discontinuous >50% of margins (3), continuous at periphery or strands consciously fan shaped (5), continuous filament growth (7).

3.2.2. Width characteristics

The width characteristics of the colony streak designated as absent (0), <2 mm (2), 2-5 mm (3), >5 mm (5).

3.2.3. Texture of the colony

The texture of the colony designated as absent (0), very coarse (1), coarse (2), intermediate (3), fine (4). Thus for example, a morphotype code of 532 indicates (5) a continuous peripheral fringe is present with evidence of fan shaped configurations; (3) the width of the fringe measured from the outer margin of the central streak colony to the margin of the fringe is greater than 6 mm (2) the texture of the fringe is considered coarse. To confirm the accuracy of the morphotyping method used the colony morphology was compared with the standard strain and colony photographs and morphotype code followed by [27].

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