PROVIDENCE
Sacred Heart
Medical Center &
Children's Hospital

Candida Susceptibility by Disk Diffusion

Department of Microbiology

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Table of Contents

1.0 Clinical Significance 2
2.0 Purpose or Principle 2
3.0 Scope 2
4.0 Safety - Personal Protective Equipment 2
5.0 Specimen Requirements 2
6.0 Materials 2
6.1 Equipment 2
6.2 Consumables 2
6.3 Media & Disks 2
6.4 Control Organisms 3
7.0 Procedure 3
7.1 Glucose-Methylene Blue Supplementation of Mueller-Hinton Agar 3
7.2 Inoculum Preparation: Direct Colony Suspension Method 3
7.3 Inoculation of Test Plates 3
7.4 Application of Disks 3
7.5 Reading the Plates 4
8.0 Interpretation & Reporting of Results 4
8.1 Susceptible (S) 4
8.2 Susceptible-Dose Dependent (S-DD) 4
8.3 Resistant (R) 4
9.0 Quality Control & Quality Assurance 5
10.0 Limitations 5
11.0 Verification Information 5
12.0 References 6
13.0 Document Control History 6

1.0 Clinical Significance

Due to the increased incidence of systemic fungal infections and number of antifungal agents available for systemic administration, antifungal susceptibility testing can help guide clinicians in the selection of antifungal therapy.

2.0 Purpose or Principle

Disk diffusion testing of *Candida* species against the triazoles (fluconazole and voriconazole) typically provides qualitative results within 24 h. While the majority of clinical isolates test susceptible to both fluconazole and voriconazole, the disk diffusion method provides the means to screen for resistant strains. The use of Mueller-Hinton agar supplemented with 2% Glucose and 0.5 µg/mL Methylene Blue Dye enhances yeast growth and improves definition of the zones of inhibition.

3.0 Scope

This procedure is classified under CLIA as highly complex. It should be carried out by technical personnel familiarized and trained to perform susceptibility testing and interpret results.

4.0 Safety - Personal Protective Equipment

Performance of this procedure may expose testing personnel to biohazardous material. All specimens must be handled as potentially infectious material as outlined in the Providence Sacred Heart Microbiology Safety Guidelines.

To perform this procedure, you must use:

- Laboratory Coat – must be worn when handling cultures.

Disinfectant following procedure:

- Bleach dilution sprayers can be used for on demand disinfectant.

5.0 Specimen Requirements

This method is intended for testing *Candida* species. This method does not currently encompass other genera. A 24 - 48 h culture on blood agar, Sabouraud dextrose agar, CHROMagar Candida, or 10% Blood BHI agar with gentamicin and chloramphenicol is needed to prepare inoculum. Fluconazole and voriconazole testing should be performed on all clinically significant isolates. Significant isolates include those from sterile body sites, wounds (unless part of mixed flora), tissues, BAL, bronchial brushings, transtracheal aspirates, mouth, throat, and urine (unless listed with mixed flora). Isolates that do not warrant susceptibility testing include those from stool, female genital (unless requested), and sputum specimens. Sputum isolates will be tested when isolated on fungal cultures. If there is any question about testing isolates, consult on Rounds.

6.0 Materials

6.1 Equipment

- Incubator set at 35 °C $\pm$ 2 °C with ambient air

6.2 Consumables

- BBL Prompt Inoculation System
- Sterile cotton swabs

6.3 Media & Disks

- Mueller-Hinton Agar 100-mm plates
- Glucose
- Methylene Blue dye

- BD BBL™ Sensi-Disc™ antimicrobial susceptibility disks. Disks should be kept frozen at -14°C or below until needed. Once a cartridge of disks has been removed from its sealed package, it should be placed in a tightly sealed, desiccated container
    - 25-µg fluconazole disks
    - 1-µg voriconazole disks

6.4 Control Organisms

- *Candida albicans* ATCC 90028
- *Candida parapsilosis* ATCC 22019

7.0 Procedure

7.1 Glucose-Methylene Blue Supplementation of Mueller-Hinton Agar

1. Mueller-Hinton agar plates should be unpackaged and held at room temperature for 1-2 days ahead of time to allow excess moisture to evaporate. This facilitates better absorption of the supplement.
2. Dissolve 0.1 g of methylene blue dye in 20 mL of distilled water and warm gently to dissolve.
3. Prepare a 0.4 g/mL stock solution of glucose by dissolving 40 g of glucose in 100 mL of distilled water. Heat gently and mix to dissolve.
4. Add 200 µL of the methylene blue dye stock solution to 100 mL of the glucose stock solution to make GMB stock with a final concentration of 40% glucose and 10 µg/mL of methylene blue dye.
5. Autoclave for 15 min at 121°C followed by slow exhaust.
6. Store at room temperature and handle aseptically. Do not refrigerate because this may cause precipitation. A one-year shelf life is generally assumed.
7. Prepare plates by adding 1.5 mL to the surface of a 100-mm plate.
8. Tilt the plate to spread the supplement evenly.
9. Allow the GMB solution to completely absorb before inoculating the plate. This requires the plates to be held from 4 to 24 h before use. The plates can be dried at room temperature or incubator temperature. Plates should be stored refrigerated and should be used within 7 d unless adequate precautions such as wrapping in plastic have been taken to minimize drying of the agar.

7.2 Inoculum Preparation: Direct Colony Suspension Method

1. All organisms need to be cultured on blood agar, Sabouraud dextrose agar, or CHROMagar Candida to ensure purity and viability. The incubation temperature throughout must be 35°C ± 2°C or 30°C if BHI blood agar with gentamicin and chloramphenicol is used.
2. Inoculum is prepared by using a BBL Prompt wand to pick 5 distinct colonies of approximately 1 mm diameter from a 24-h culture. If colonies are small (0.5 to 1 mm in diameter) pick 10 colonies.
3. Place the inoculation wand into the tube of saline suspending solution. Vortex the tube for 10 sec to release cells from the wand. This inoculum should produce semi-confluent growth for most *Candida* species isolates.

7.3 Inoculation of Test Plates

1. Optimally, within 15 min of inoculum preparation, a sterile cotton swab is dipped into the suspension. The swab should be rotated several times and pressed firmly against the inside wall of the tube above the fluid level to remove excess fluid from the swab.
2. The dried surface of a sterile Mueller-Hinton + GMB agar plate is inoculated by evenly streaking the swab over the entire agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum.

7.4 Application of Disks

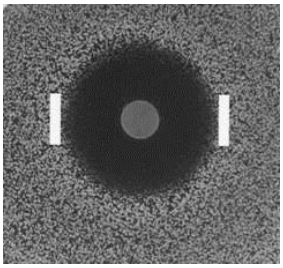
1. Antimicrobial disks are dispensed onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure its complete contact with the agar surface. The disks

should be distributed no closer than 24 mm from each other (center to center). Because the drug diffuses almost instantaneously, a disk should not be moved once it has come into contact with the agar surface.

2. The plates are inverted and placed in an ambient incubator set to 35°C.

7.5 Reading the Plates

1. Examine each plate after 20 to 24 h of incubation. If the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a semi-confluent lawn of growth.
2. The plate is held a few inches above a black, nonreflecting background illuminated with reflected light. Measure the zone diameter to the nearest whole mm at the point at which there is a prominent reduction in growth. This is highly subjective. Pinpoint microcolonies at the zone edge or large colonies within a zone are frequently encountered and should be ignored. If these colonies are subcultured and retested, identical results are usually obtained. Read at 48 h only when insufficient growth is observed after 24 h incubation.



A 25-µg fluconazole disk on a lawn of 10^4 CFU/mL of *C. albicans* after 24 h of incubation. The inhibitory zone diameter is measured at the transitional point where growth abruptly decreased (interior edges of bars), as determined by a marked reduction in colony sizes.[2]

8.0 Interpretation & Reporting of Results

Table 1 provides CLSI zone diameter interpretive criteria to categorize the levels of susceptibility to, fluconazole, and voriconazole.

Table 1

Antifungal Agent	Disk Content	Zone Diameter, Nearest Whole (mm)		
		R	**S-DD**	**S**
Fluconazole	25 µg	≤ 14	15 – 18	≥ 19
Voriconazole	1 µg	≤ 13	14 – 16	≥ 17

8.1 Susceptible (S)

The susceptible category implies that an infection due to the strain may be appropriately treated with the dose of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated.

8.2 Susceptible-Dose Dependent (S-DD)

The susceptible-dose dependent category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. Susceptibility is dependent on achieving the maximal possible blood level. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

8.3 Resistant (R)

Resistant strains are those that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or when zone diameters have been in a range where clinical efficacy has not been reliable in treatment studies.

Confirm all resistant results by staining growth around the disks. Bacterial contamination can produce false resistant results. Isolates that yield a resistant result should be brought up for

Rounds consultation. When entering a resistant result in LIS, a prompt will appear to add the following comment, **This yeast is presumptively resistant to one or more anti-fungal agents. If indicated, confirmatory susceptibility testing should be ordered. The organism will be saved for 5 days. [YSTSR]**

Isolates of *Candida krusei* are assumed to be intrinsically resistant to fluconazole and should be reported as such regardless of the zone size. *Candida* species that are fluconazole resistant should be identified to the species level. The resistance comment should only be used for *Candida krusei* if the voriconazole zone is also interpreted as resistant.

9.0 Quality Control & Quality Assurance

Quality control should initially be performed on each new batch of Mueller-Hinton + GMB agar and on each new lot of fluconazole and voriconazole, and then tested weekly thereafter. The quality control strains include *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019. Table 2 provides the acceptable ranges for testing these two strains.

Table 2

Antifungal Agent	Disk Content	*C. albicans* ATCC 90028	*C. parapsilosis* ATCC 22019
Fluconazole	25 µg	28 – 39	22 – 33
Voriconazole	1 µg	31 – 42	28 – 37

10.0 Limitations

The disk diffusion method provides a reliable and effective means to screen for susceptibility of most clinical isolates. Some strains may be encountered that grow poorly on the MH + GMB medium. Other strains may show significant trailing within zones of inhibition making interpretation difficult. When significant trailing is encountered, the purity plate should be re-examined to verify that the results are not due to contamination. Isolates that yield equivocal results due to insufficient growth or trailing or that test resistant to either anti-fungal agent should be brought up on Rounds and will probably be sent to a reference laboratory for confirmatory testing by an alternate method, such as broth dilution MIC.

11.0 Verification Information

The testing of *Candida* species, using the method outlined above, was verified by testing isolates that were previously tested by reference laboratories by the broth dilution MIC method. There were 28 total isolates tested and compared for fluconazole, including 17 *C. albicans*, 4 *C. glabrata*, 2 *C. krusei*, 3 *C. parapsilosis*, and 2 *C. tropicalis*. The total number of isolates tested and compared for voriconazole was 21, including 10 *C. albicans*, 4 *C. glabrata*, 1 *C. krusei*, 4 *C. parapsilosis*, and 2 *C. tropicalis*. The fluconazole results from these isolates included no major discrepancies, 4 (14%) minor discrepancies (3 S-DD by MIC vs. S or R by disk diffusion and 1 S by MIC vs. S-DD by disk diffusion), and 1 indeterminate result. For voriconazole, there were 2 (10%) major discrepancies (S by MIC vs. R by disk diffusion), 1 minor discrepancy (S-DD by MIC vs. R by disk diffusion), and 1 indeterminate result. Isolates with major discrepant results were sent to a different reference laboratory for re-testing. As a result of the false-resistance detected by the disk diffusion method, it was concluded that resistant isolates will be sent out for confirmatory testing by an MIC method.

Comparison studies were also performed to evaluate the disk diffusion procedure using alternate isolation media, inoculum preparation, and isolate incubation temperatures. Ten isolates (including 3 *C. albicans*, 3 *C. glabrata*, 1 *C.* krusei, 1 *C. parapsilosis*, and 2 *C. tropicalis*) were subcultured to Sabouraud dextrose agar (SDA), BCG agar, and BHI Blood agar with gentamicin and chloramphenicol. SDA and BCG plates were incubated at 35 °C for 24 h. BHI plates were incubated at 30°C for 48 h. Testing of growth from these plates yielded identical results except for one isolate that tested R from SDA and BCG vs. S-DD from BHI. This same isolate was determined to truly be S by MIC testing at 2 separate reference labs. Five isolates (including 1 *C. albicans*, 2 *C. glabrata*, 1 *C. krusei*, and 1 *C. parapsilosis*) were also tested using different

inoculation preparation techniques. Testing was performed using suspensions that were visually adjusted to 0.5 McFarland (as outlined in CLSI M44-A). A separate set of testing was also performed using the BBL Prompt device. Both of these methods yielded identical results.

Additional comparison studies were performed to validate the use of CHROMagar Candida (CHROM) as an appropriate source of inoculum for the disk diffusion test. Test isolates that were previously obtained from BCG for susceptibility testing were subcultured to CHROM and allowed to incubate in ambient air at 35°C for 48 h. These were comprised of isolates previously used in the validation study (86%) plus 3 additional clinical isolates (14%). The test isolates included 5 *C. albicans*, 5 *C. glabrata*, 4 *C. krusei*, 5 *C. parapsilosis*, and 3 *C. tropicalis*. The test isolates contained a mixture of both susceptible and resistant strains. A standard inoculum was prepared for each isolate by using the BBL Prompt device. After 24 h of incubation of, the susceptibility test results were examined. There was 100% agreement of fluconazole and voriconazole SIR results.

12.0 References

1. Clinical and Laboratory Standards Institute (formerly NCCLS) M44-A. Volume 24, Number 15. May 2004. Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline.
2. Kirkpatrick, W. R., Turner, T. M., Fothergill, A. W., McCarthy, D. I., Redding, S. W., Rinaldi, M. G., and Patterson, T. F. 1998. Fluconazole disk diffusion susceptibility testing of *Candida* species. J. Clin. Microbiol. 36:3429-3432.

13.0 Document Control History

Reviewed by director (AR): 03/20/2007

Reviewed by Joseph Schappert: 03/10/2010

Reviewed by supervisor (JC): 03/20/2007, 06/2007, 05/2008, 07/2009, 04/01/2011, 03/2013, Jason Ammons 07/2015

Revisions and Updates: 09/29/2011 Resistant results must be verified by staining around disks to rule out bacterial contamination. 02/03/2015 Added id for fluconazole resistant isolates to rule out *C. krusei* before using the YSTSR comment. Added instructions for preparation of MH + GMB as outlined in the CLSI M44-A2 document.