Chapter 13

Microbroth Dilution Susceptibility Testing of *Candida* species

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Abstract

Antifungal susceptibility testing for *Candida* species is now widely accepted as a methodology to predict the success or failure of antifungal therapy for some antifungal/*Candida* species combinations. There are many different ways to perform susceptibility testing of antifungal agents, but broth microdilution has become the most popular over the last 10 years. This chapter describes in detail two methods for antifungal susceptibility testing of *Candida* species using the commercially available microbroth dilution tray (YeastOne®) and a commercially available gradient agar diffusion technique (Etest®) for isolates that appear resistant.

Key words Susceptibility testing, Antifungal, Broth microdilution, Etest, MIC

1 Introduction

Antifungal susceptibility testing is a useful protocol for mutation discovery, for epidemiology of clinical failure, and for drug discovery. However, the major purpose of antifungal susceptibility testing is to determine the minimum inhibitory concentration (MIC) of antifungal drugs against clinical isolates in order to predict clinical outcome. This latter use is the basis for a difficult determination because an MIC is an in vitro measurement of susceptibility and clinical outcome is dependent upon the health and immune status of the host, the pharmacodynamics/pharmacokinetics of the antimicrobial at the particular dosage/site of infection, and the overall sensitivity of the infecting organism to the particular antifungal. Nevertheless, methodologies for antimicrobial susceptibility testing have been developed that allow the rapid and relatively reliable determination of MIC values.

There are four major methods for manual susceptibility testing: broth macrodilution, broth microdilution, disk diffusion, and microgradient diffusion. Broth macrodilution involves inoculation of the test organism into a set of test tubes containing growth

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medium and increasing concentrations of an antimicrobial agent. The endpoint is read as the concentration in the tube in which microbial growth is decreased to a prespecified degree. Broth microdilution is similar, but instead of using test tubes, microbial growth takes place in a 96-well microtiter plate. Again, the endpoint is read as the concentration in the well in which microbial growth is decreased to a prespecified degree. Disk diffusion is performed by placing a paper disk saturated with an antimicrobial onto an agar plate inoculated with a test organism. The antimicrobial diffuses into the agar and forms a gradient around the disk. The endpoint is read as the diameter of the clearing around the disk in which there is no microbial growth. This methodology requires that the zone diameter is related to susceptibility or resistance, so that the diameter obtained with the test organism can be put into context. Microgradient diffusion uses a paper or plastic strip impregnated with various amounts of antimicrobial in a continuous concentration gradient. The strip is placed on an agar plate inoculated with a test organism. The antimicrobial diffuses into the agar and forms an elliptical gradient centered on the strip. The endpoint is read as the concentration where the microbial growth intersects the strip. Each of these mechanisms has their good and bad points but broth microdilution has become the standard for susceptibility testing in Europe and the United States. In Europe, a standardized protocol for testing yeasts by broth microdilution has been established by the European Committee for Antimicrobial Susceptibilitytesting (EUCAST) as document EDef 7.1 [1]. In the United States, the standardized protocol for yeasts has been established by the Clinical and Laboratory Standards Institute (CLSI) in document M27-A3 [2].

In this chapter, the CLSI methodology for broth microdilution testing of yeasts will be described. As preparation of the antifungal dilutions and the 96-well microtiter plates is described quite succinctly in the CLSI document M27-A3, this chapter will concentrate on using a commercially available microbroth dilution tray (YeastOne®) and a commercially available gradient agar diffusion technique (Etest®). Both of these methodologies compare favorably to the CLSI M-27 and EUCAST methodologies [3–6].

2 Materials

Sabouraud's Dextrose agar or other fungal growth medium.

Inoculating loops.

Sterile water—2 ml in a tube that will fit the spectrophotometer.

Vortex.

Spectrophotometer or nephelometer.

RPMI 1640 powder (with glutamic acid and phenol red, without bicarbonate).

MOPS (3-[*N*-morpholino] propanesulfonic acid) buffer.

NaOH (1 mol/l).

Bacto Agar.

D-Glucose.

15 ml screw cap test tubes.

15 × 100 mm plastic petri dishes.

Inoculum reservoir.

12 channel pipette.

Manual mirror viewer.

CLSI documents M27-A3 and M27-S4.

Quality control isolates *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258.

Seed trough.

YeastOne® susceptibility plate (Thermo Fisher Scientific, Oakwood Village, OH).

Etest strips:

Amphotericin B (BioMerieux, cat# 526348).

Fluconazole (BioMerieux, cat# 510858).

Itraconazole (BioMerieux, cat# 525858).

Voriconazole (BioMerieux, cat# 532840).

3 Methods

3.1 Media

RPMI 1640 broth

RPMI 1640 powder (with glutamic acid and phenol red, without bicarbonate) 10.4 g/l (Sigma, #1383).
MOPS (3-[*N*-morpholino] propanesulfonic acid) buffer 34.5 g/l.
NaOH (1 mol/l).
15 ml screw cap test tubes.

1. Dissolve RPMI, MOPS, and glucose into 900 ml of distilled H_2O. Adjust to pH 7.0 at 25 °C using 1 mol/l NaOH. Adjust the final volume to 1 l.
2. Filter sterilize using a 0.2 μm filter.
3. Dispense into 11 ml aliquots in test tubes and store at 4 °C.

RPMI Agar Plates

RPMI 1640 powder (with glutamic acid and phenol red, without bicarbonate) 10.4 g/l (Sigma, #1383).

MOPS (3-[*N*-morpholino] propanesulfonic acid) buffer 34.5 g/l.

NaOH (1 mol/l).

Bacto Agar 15 g/l.

D-Glucose 20 g/l.

15 × 100 mm plastic petri dishes.

1. Dissolve RPMI, MOPS, and glucose into 450 ml of distilled H_2O. Adjust to pH 7.0 at 25 °C using 1 mol/l NaOH.
2. Filter sterilize using a 0.2 μm filter.
3. Sterile filter (0.2 μM) and place in water bath to increase temperature to 50 °C.
4. Add 15 g of agar to 400 ml of H_2O. Autoclave for 20 min to sterilize.
5. Add the sterile agar to the RPMI broth, adjust the final volume to 1 l with sterile distilled H_2O and stir to mix.
6. Aseptically dispense 25 ml per petri dish and store at 4 °C for up to 6 months.

3.2 Sample Preparation and Inoculation

1. Starting from a frozen stock or a fresh culture, streak the test isolate on a SAB agar plate or another fungal growth medium and allow it to grow for 24 h at 37 °C. If taken from a frozen stock, re-streak on a SAB agar plate and allow additional growth for 24 h. Include a fresh culture of the two quality control isolates ATCC 22019 (*C. parapsilosis*) and ATCC 6258 (*C. krusei*). Streak all of the isolates for colony isolation.
2. Prepare a stock solution from each isolate by picking two to three small isolated colonies from the fresh culture and suspending them in 2 ml of sterile water, vortexing well. The amount picked will depend on the size of the colony and can be adjusted according to the subsequent density readings.
3. Vortex resulting suspension for 15 s, then adjust cell density with sterile water to a spectrophotometer transmittance reading of 80–82 % at a wavelength of 530 nm. This will yield a yeast stock suspension of $1–5 \times 10^6$ cells per ml. Alternatively, use a nephelometer and adjust the turbidity to 0.5 McFarland units.
4. Transfer 20 μl of the cell suspension to the 11 ml RPMI broth tubes and vortex well. Pour this suspension in the seed trough and use for primary inoculation of the plates.

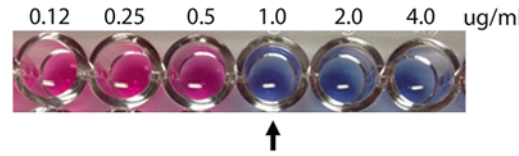

Fig. 1 Sample wells of *Candida albicans* and fluconazole following 24 h of incubation. The arrow indicates the well at which the MIC should be read. The MIC of this isolate against fluconazole is 1.0 μg/ml

5. Using the 12 channel pipette, dispense 100 μl of the suspension into each well of the YeastOne plates (*see* **Note 1**).
6. A colony count to ensure correct inoculum should be performed by taking 10 μl out of the positive control growth well and plating it on Sabouraud's dextrose agar. If the inoculum is correct, and depending on how the density was adjusted, 50–500 colonies should grow on the plate.
7. Seal the YeastOne plates and incubate at 35 °C for 24 h in a non-CO2 incubator.
8. Plates are read after 24 h of incubation.

3.3 Reading the Plates and Assigning an Interpretation

1. Plates are read visually using normal room lighting. Set the plate on top of the manual mirror viewer. You will be looking at the bottom of the plate in the mirror.
2. With the YeastOne colorimetric system, all of the wells start out as blue. As growth appears, the wells turn red due to a change in the colorimetric indicator dye. Make sure that the positive control well has turned red. If not, the plate can be reincubated for up to another 24 h.
3. The MIC is read as the lowest concentration where the well has remained blue (indicating a lack of growth) (Fig. 1) (*see* **Notes 2** and **3**).
4. If there is no color change in any of the wells for a given antifungal agent, the MIC is read as ≤ the lowest concentration of the antifungal tested. If all of the wells have turned red, the MIC is read as > the highest concentration of the antifungal tested.
5. Before interpretation of the breakpoints is made, the MIC values for the two quality control isolates should be determined to make sure that they are in range (Table 1). If the quality control isolates are not in the established quality control range, the test is invalid.
6. Species-specific interpretive breakpoints are available for *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei* against fluconazole, micafungin, caspofungin, and anidulafungin; for *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C.*

Table 1
Quality control ranges for the two quality control isolates

	MIC in μg/ml	
Antifungal	*C. parapsilosis* ATCC 22019	*C. krusei* ATCC 6258
Fluconazole	0.5–4	8–64
Voriconazole	0.015–0.12	0.06–0.5
Itraconazole	0.12–0.5	0.12–1
Posaconazole	0.06–0.25	0.06–0.5
Micafungin	0.5–2	0.06–0.25
Caspofungin	0.25–1	0.12–1
Anidulafungin	0.25–2	0.03–0.12
Flucytosine	0.5–4	4–16
Amphotericin B	0.25–2	0.5–2

krusei against voriconazole; and *C. guilliermondii* against micafungin, caspofungin, and anidulafungin [7]. There are no breakpoints for any other species of *Candida* for any other antifungal agents and there are no breakpoints for any *Candida* species for itraconazole, posaconazole, isavuconazole, amphotericin B, or flucytosine. As the breakpoints are not static, the latest antifungal document from the Clinical and Laboratory Standards Institute (http://clsi.org) should be checked to determine the most recent breakpoints.

7. True resistance to the azole antifungals among *C. albicans* and *C. tropicalis* isolates is rare. Quite often it is the result of a misinterpretation of a trailing isolate (*see* **Note 3**). These isolates should have their MIC values confirmed by Etest to determine whether they are truly resistant or just show an in vitro trailing effect as described in **Note 3**.

3.4 Etest Susceptibility Testing

The Etest is based upon a continuous concentration gradient infused on a plastic non-porous strip. The antifungal agent diffuses into an agar plate and allows an accurate determination of MIC values based on elliptical growth around the antifungal gradient.

1. Grow the isolate to be tested and the quality control isolates as described in Subheading 3.1.
2. Remove RPMI agar plates from refrigerator and allow them to reach room temperature.
3. Following the cell dilution technique from above Subheading 3.1 [2, 3], plate approximately 100 μl of the initial

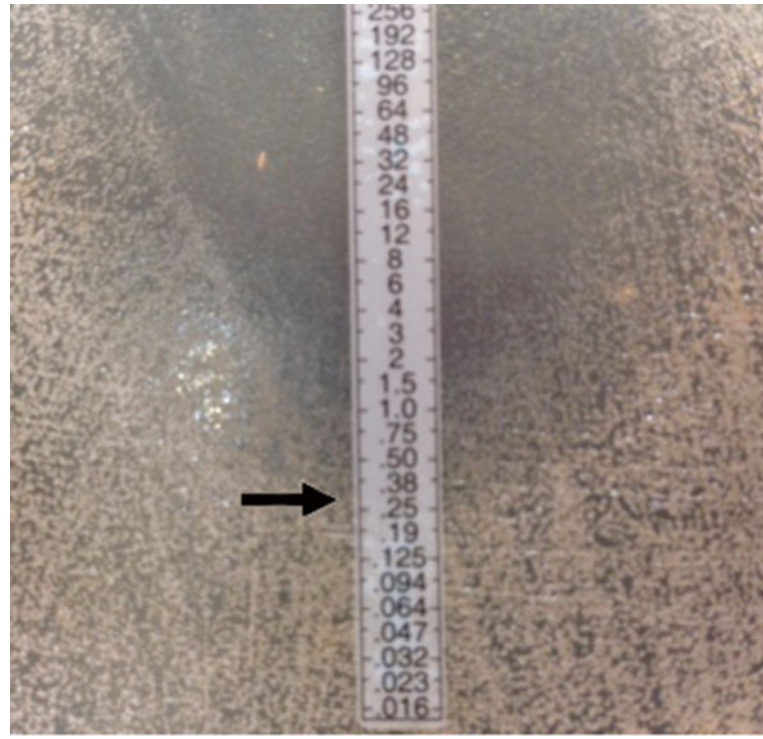

Fig. 2 Etest of an isolate with trailing growth. This is an Etest read after 20 h of growth. The trailing growth can clearly be seen inside the ellipse. The MIC is read as the first point in the ellipse without microcolonies. In this case it would be 0.38 μg/ml

sterile water cell suspension on the RPMI plate. Use a sterile cotton swab to streak the plate completely in three directions to make sure the entire plate is covered. Allow the plate to sit for at least 15 min so that the added moisture can dry.

4. Take the appropriate number of Etest strips out of the −20 °C freezer. Extract the appropriate number of test strips and allow them to come to room temperature. Using a forceps, apply the Etest strips to plates with the MIC scale facing upward, being careful to not allow bubbles under the strip. If there are bubbles, remove them by gently pressing down with the forceps, being careful not to slide the strip.
5. Incubate the plate at 35 °C for 24 h or until a confluent lawn of growth is seen.
6. The interpretation of the MIC value is dependent upon the antifungal agent. For susceptible isolates, an ellipse will be seen with the growth concentrated near the MIC endpoint. For azoles and echinocandins the MIC is read at the MIC value where the first significant decrease in the density is seen (interpreted as an 80 % decrease in growth) (*see* **Note 4**) (Fig. 2).

Always read the value on the side of the strip with the highest growth. For amphotericin B, it is interpreted as the value where there is 100 % growth inhibition. If growth is inhibited between MIC values, use the higher of the two values as the MIC.

4 Notes

1. There are alternative ways to inoculate the tray. It can be performed manually using a single pipette and inoculating all 96 wells individually. Sensititre® and a few other companies manufacture an auto inoculator that allows the user to insert the suspension tube so that each well is inoculated individually. This device ensured uniformity and avoids carpal tunnel syndrome for the laboratorian.
2. Occasionally there will be a skipped well (a blue well in between two red wells). In the case of a skipped well, if there is growth on either side, ignore the skipped well and record the highest growth. If there are two skipped wells in a row, the test is invalid. If there are two skipped wells in a single column, all of the tests on the tray are invalid.
3. Occasionally with the azole antifungals and *C. albicans*, *C. tropicalis*, and *C. glabrata*, there will be growth in the wells above what would normally be the MIC value; this is called trailing growth and the isolate is called a trailer [8]. In the case of the YeastOne plates, this is usually manifested as a purple color (in between the negative blue and the positive red). YeastOne recommends that the MIC is read as the first well that shows a less intense color change compared to the positive control well (Fig. 3). This is a very subjective interpretation. The authors recommend that trailing isolates have their MIC values confirmed by Etest.
4. For trailers, there may be fairly confluent growth on the plate but a visible ellipse can be seen when the plate is either very young or when it is held at an angle. The inside of the ellipse will be made up of microcolonies. If the isolate is resistant, no ellipse will be seen.

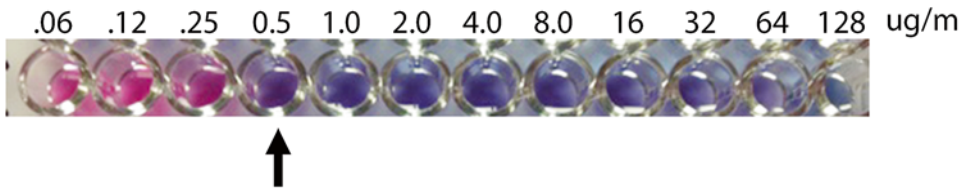

Fig. 3 Sample wells of trailing growth of *Candidaalbicans* and fluconazole following 24 h of incubation. Only in the well with 128 μg/ml of fluconazole can the authentic blue color signifying no growth be seen. The MIC is read as the first well with a less intense red color which is 0.5 μg/ml in this case

Acknowledgement

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References

1. Rodriguez-Tudela JL, Arendrup MC, Barchiesi F, Bille J, Chryssanthou E, Cuenca-Estrella M et al (2008) EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin Microbiol Infect 14:398–405
2. Clinical and Laboratory Standards Institute. 2008. Reference method for Broth dilution antifungal susceptibility testing of yeasts; approved standard—Third Edition, CLSI Document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA
3. Alexander BD, Byrne TC, Smith KL, Hanson KE, Anstrom KJ, Perfect JR et al (2007) Comparative evaluation of Etest and sensititre yeastone panels against the Clinical and Laboratory Standards Institute M27-A2 reference broth microdilution method for testing *Candida* susceptibility to seven antifungal agents. J Clin Microbiol 45:698–706
4. Bertout S, Dunyach C, Drakulovski P, Reynes J, Mallie M (2011) Comparison of the Sensititre YeastOne® dilution method with the Clinical Laboratory Standards Institute (CLSI) M27-A3 microbroth dilution reference method for determining MIC of eight antifungal agents on 102 yeast strains. Pathol Biol 59:48–51
5. Espinel-Ingroff A, Pfaller M, Messer SA, Knapp CC, Holliday N, Killian SB (2004) Multicenter comparison of the Sensititre YeastOne colorimetric antifungal panel with the NCCLS M27-A2 reference method for testing new antifungal agents against clinical isolates of *Candida* spp. J Clin Microbiol 42:718–721
6. Cuenca-Estrella M, Gomez-Lopez A, Alastruey-Izquierdo A, Bernal-Martinez L, Cuesta I, Buitrago MJ et al (2010) Comparison of the Vitek 2 antifungal susceptibility system with the clinical and laboratory standards institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Broth Microdilution Reference Methods and with the Sensititre YeastOne and Etest techniques for in vitro detection of antifungal resistance in yeast isolates. J Clin Microbiol 48:1782–1786
7. Clinical and Laboratory Standards Institute (2012) M27-S4 reference method for Broth dilution antifungal susceptibility testing of yeasts; fourth informational supplement. Clinical and Laboratory Standards Institute. Wayne, PA
8. Revankar SG, Kirkpatrick WR, McAtee RK, Fothergill AW, Redding SW, Rinaldi MG et al (1998) Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards method. J Clin Microbiol 36:153–156