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Laboratories and Professor of Laboratory Medicine and Pathology in the Division of Clinical Microbiology at Mayo Clinic in Rochester, Minnesota. Dr. Wengenack provides an overview of the current standards for antifungal drug susceptibility testing of yeast and the development of species-specific breakpoints and epidemiological cutoff values.

Thank you. Today’s Hot Topic presentation will focus on the introduction of species-specific breakpoints for yeast and will highlight a relatively new concept in antifungal drug susceptibility testing, which is the use of epidemiological cutoff values.
Disclosures

• None

I have no disclosures that are relevant to this Hot Topic.
In today’s presentation, I will describe the test methodology used for antifungal drug susceptibility testing of yeast. In addition, I will review the process by which antifungal drug breakpoints and interpretive criteria are established. Then we will examine the species-specific breakpoints that are currently available for selected Candida species. Finally, I will introduce the concept of epidemiological cutoff values, or ECVS, and we will then review the ECVs that are available for selected Candida species.
Antifungal drug susceptibility testing of yeast isolates is important to help guide clinicians on the choice of antifungals that may be useful in treating clinically significant disease. There are at least 2 major international organizations that provide guidance to clinical laboratories on how to perform antifungal drug susceptibility testing of yeast. Those 2 organizations are the European Committee on Antimicrobial Susceptibility Testing (or EUCAST) and the Clinical and Laboratory Standards Institute (also known as CLSI). Many of the recommendations of the 2 groups are harmonized, but some differences do exist. In this Hot Topic, I will focus on the CLSI method and interpretive criteria, but I would encourage you to also visit the EUCAST website at www.eucast.org for more information on their standards.
CLSI document M27 provides the methodologic framework for performing the antifungal testing in clinical laboratories. Previously, interpretive tables were provided in a Supplement to M27, which was called M27-S4, but later this year the tables will be transitioned to a stand-alone document that will be CLSI document M60. The stand-alone document will allow breakpoints and interpretive criteria to be updated much more rapidly than in the past and updated breakpoints and ECVs should now be available annually rather than on the previous 3-year cycle.
The recognized reference method for antifungal susceptibility testing of yeast is broth dilution. The method was developed and agreed upon through an international consensus process that tapped the knowledge and experience of mycology experts in clinical microbiology laboratories, government entities such as the CDC, the FDA, and the Canadian Public Health Services, as well as those from the pharmaceutical industry and device manufacturers. The use of a reference method greatly facilitates interlaboratory agreement when testing yeast for resistance to antifungal agents and enhances patient care by providing robust susceptibility results regardless of the geographic location of the patient. As I mentioned, the reference method is broth dilution, but either macrobroth or microbroth dilution can be performed and equivalent results can be achieved regardless of which format is performed. The CLSI M27 document is intended to provide methodology for testing of yeast such as Candida species and Cryptococcus neoformans, but it is not intended for use with the yeast forms of the endemic dimorphic pathogens such as Histoplasma capsulatum or Blastomyces dermatitidis. The method is also not intended for use with filamentous molds such as Aspergillus species and there is a separate CLSI standard for mold susceptibility testing, which is M38.
In order to perform the microbroth dilution method, a pure isolate of the yeast to be tested should be selected from an agar plate culture of the organism. Care must be taken to ensure that mixtures of yeast or yeast mixed with bacteria are not tested as results may be inaccurate. A suspension of the yeast is prepared in sterile saline or sterile water to a concentration equivalent in transmittance at 530 nm to a 0.5 McFarland standard. From this, a working inoculum is prepared by diluting the yeast suspension into RPMI broth to a final concentration of approximately 10^3 cells/mL. The inoculum is added to each well of the microtiter plate which contains the drugs to be tested in a dilution series. The plate is sealed and incubated at 35°C in ambient air for 24-48 hours for *Candida* species; incubate for up to 72 hours for *Cryptococcus neoformans*. Cryptococcus neoformans grows more slowly so incubation for up to 72 hours may be needed for this organism.
Following incubation, the panel can be read either manually using a light box or using a semiautomated plate reader. The endpoint is determined by comparison of the growth in each well compared with the control well. The minimal inhibitory concentration (or MIC) is the lowest drug concentration that prevents growth of the organism or it is the lowest drug concentration that produces a 50% reduction in growth depending on the particular drug or drug class being examined. Some panels have colorimetric indicators that assist with endpoint determination and an example of that is shown in the figure on this slide where the color blue indicates a lack of growth and pink or purple indicates growth of the yeast isolate in that well. The well in position A1 is the positive growth control well and that well is pinkish-purple in color indicating sufficient growth so the plate is ready for reading. The echinocandin drugs (anidulafungin, micafungin, and caspofungin) are shown in the top 3 rows with the endpoint for each drug circled. For anidulafungin, the endpoint is 0.015 mcg/mL, for micafungin the endpoint is 0.03 mcg/mL, and for caspofungin some skipping of wells is seen with the alternating blue and purple colors so the endpoint is read as 0.12 mcg/mL. For the echinocandins, there is currently a discussion occurring in the AST community suggesting that in vitro susceptibility testing of caspofungin may be technically challenging for reasons that are not completely understood yet and which do not correlate with in vivo activity of the drug. Therefore, some experts are recommending using anidulafungin or micafungin AST results as the class-agents for the echinocandins. This is still controversial and does not mean that caspofungin should not be used clinically if indicated; it simply means that in vitro laboratory testing of caspofungin may not be representative of its utility in vivo.

Looking at the other drugs on the plate, the endpoint for 5-flucytosine in row D is 0.06 mcg/mL, posaconazole in row E has an endpoint of 0.25 mcg/mL, voriconazole
This slide presents a sample report for an isolate of Candida glabrata. Interpretive criteria are available from CLSI for the echinocandins, which are anidulafungin, caspofungin, and micafungin, and as you can see, all are reported as susceptible on this report. Typically for the echinocandins, if 1 agent is susceptible, the others 2 agents will be susceptible as well, but this is not always the case so testing of all 3 echinocandins is recommended when possible. As can be the case for Candida glabrata, the MIC for fluconazole is very high at 128 mcg/mL, which puts this isolate firmly in the resistant category. No CLSI interpretive criteria are available for the other antifungals including amphotericin, 5-flucytosine, itraconazole, posaconazole, and voriconazole and, therefore, only the MIC is reported.
One question that Clinical Microbiology laboratories receive often from physicians treating patients with fungal infections is something like “since there are no interpretive criteria available for several of the drugs on the panel, can I just use the drug with the lowest MIC to treat my patient? Does a lower MIC mean the isolate is more susceptible to the drug than to other drugs with a higher MIC?” The answer to that question is no, not always. A low MIC is generally a good thing, but in order to select the best drug for use, one also needs to understand the pharmacokinetic (or PK) and pharmacodynamic (or PD) characteristics for the antifungal agent. Two measures that are often used in PK determinations are the Cmax or the peak drug concentration achieved and the AUC24, which is the area under the “concentration verses time curve” for a 24-hour period. The PK describes what happens to the drug in the patient’s body. How well is it absorbed? How is the drug distributed to various sites in the body? How is it metabolized and how quickly? How is it excreted? The PD describes what happens to the drug when it meets up with the infecting pathogen and often the MIC is used to help define the PD. Putting the PK and PD information together, the Cmax over the MIC, or the AUC over the MIC, or the time above the MIC, can help predict potential outcomes for a particular antifungal and yeast species combination.
So the antifungal drug that has the lowest MIC value may not always be the best choice for treating the infection. For example, if a yeast isolate has an MIC = 1 mcg/mL for antifungal “X,” it may be categorized by the microbiology laboratory as “S” susceptible to drug X.

- If drug X can’t achieve an adequate concentration at the site of infection, it should not be used as a single agent to treat the infection.

On the other hand, if the same yeast isolate has an MIC = 4 mcg/mL for antifungal “Y,” it may also be categorized as “S” by the microbiology lab.

- If drug Y can achieve an adequate concentration at the site of infection and can remain above the MIC for the time necessary to achieve an effect on the yeast, it may be a good choice despite its higher MIC in the laboratory.

So the antifungal drug that has the lowest MIC value may not always be the best choice for treating the infection. For example, if a yeast isolate has an MIC=1 mcg/mL for antifungal “X,” it may be categorized by the microbiology laboratory as “S” susceptible to drug X. However, if drug X can’t achieve an adequate concentration at the site of infection, then it should not be used as a single agent to treat the infection.

On the other hand, if the same yeast isolate has an MIC=4 mcg/mL for antifungal “Y,” it may also be categorized as “S” by the microbiology lab. If drug Y can achieve an adequate concentration at the site of infection and can remain above the MIC for the time necessary to achieve the desired effect on the yeast, it may be a good choice despite its higher laboratory-determined MIC.
In the earliest versions of the Clinical and Laboratory Standards Institute Approved Standard for Broth Dilution Antifungal Susceptibility Testing of Yeast, there was a single table listing the breakpoints and interpretive criteria for all Candida species. Since that time, it is recognized that the various species of Candida respond differently to antifungals and, therefore, species-specific breakpoints have been established and published in the latest version of the document. Species-specific breakpoints and interpretive criteria are only available at this time for selected drugs, which are the echinocandins (caspofungin, micafungin, and anidulafungin) and 2 azoles (fluconazole and voriconazole). For these 5 drugs, species-specific breakpoints are available for 6 Candida species, which are Candida albicans, Candida glabrata, Candida tropicalis, Candida krusei, Candida parapsilosis, and Candida guilliermondii. Species-specific breakpoints are not available for other Candida species or for other yeast such as Cryptococcus neoformans, nevertheless laboratories must not revert to using the old, nonspecies-specific breakpoints provided in earlier versions of the M27 document. Instead, laboratories should report the MIC value without an interpretation and can consider adding reporting comments that indicate “no interpretive criteria are available for drugs without interpretive criteria reported.” Interpretive criteria for additional genera and species of yeast and for additional antifungals will be added to the M60 document as they become available from the CLSI Antifungal Susceptibility Testing Subcommittee. This group meets regularly to review new data as it becomes available and to establish additional species-specific breakpoints.
This slide contains the breakpoints and interpretive criteria established for the echinocandins and 6 Candida species. As you will note, the breakpoints are very similar for a given species between the 3 echinocandin drugs, but they can differ widely between the various species. For example, Candida albicans has a breakpoint of less than or equal to 0.25 mcg/mL for susceptibility to anidulafungin, caspofungin, and micafungin. MICs of 0.5 mcg/mL signals an intermediate level of susceptibility to all 3 antifungals, and an MIC of greater than or equal to 1 mcg/mL correlates strongly with resistance to the 3 echinocandins. For Candida glabrata, the breakpoints are slightly different than those of Candida albicans. An MIC value of less than or equal to 0.12 mcg/mL for anidulafungin and caspofungin or 0.06 mcg/mL for micafungin is considered susceptible, while greater than or equal to 0.5 mcg/mL for anidulafungin or caspofungin or 0.25 mcg/mL for micafungin is considered resistant for Candida glabrata.
This slide contains the breakpoints and interpretive criteria established for 2 azoles, fluconazole and voriconazole, across 5 Candida species. As you can see from this table, the MICs differ more widely between the 2 azoles than they did between the 3 echinocandins on the previous slide. For example, an MIC of less than or equal to 2 mcg/mL is considered susceptible for fluconazole, while the MIC for voriconazole susceptibility is less than or equal to 0.12 mcg/mL. You should also note that the interpretive categories differ for the azoles. The echinocandins had interpretive categories of S for susceptible, I for intermediate, and R for resistant to the agent. For the azoles, there is no intermediate category. Instead, an S-DD or susceptible dose dependent interpretive category is introduced. The S-DD category indicates that the susceptibility of the isolate to the azole is dependent on the ability to achieve the maximum possible blood level of the antifungal. So higher than standard dosing may be needed in adults with normal renal function and body size. You can also see from this table that Candida glabrata has only S-DD and R interpretive criteria, so susceptibility to fluconazole in this species is highly dependent upon the ability to achieve maximal blood levels of the agent. Recall that Candida glabrata is of concern for developing resistance to fluconazole over time. Also note that no breakpoints and interpretive criteria are provided for Candida krusei and fluconazole because this species is innately resistant to fluconazole. Finally, please note that no breakpoint and interpretive criteria are provided for Candida glabrata and voriconazole because the data available to CLSI at this time are not sufficient to allow a correlation between the MIC and clinical outcome to be established. Hopefully, as more data are collected...
from various laboratories around the world, breakpoints and interpretive criteria can be added for this species. In the absence of interpretive criteria, only the MIC should be reported by the laboratory.
One might ask, is there any other information available to help guide my choice of antifungal agent if my patient has another species of Candida or if the isolate is another yeast genera and species such as Cryptococcus neoformans, because there are no established breakpoints or interpretive criteria? As we discussed earlier, breakpoints are established using a collection of data including the MIC value distribution for the species and the antifungal agent as determined using a reference method, through evaluation of PK and PD data, by using clinical trial outcome data, and by using postmarketing susceptibility data. For many fungal species and antifungal drug combinations, obtaining all of this data is not practical because it could take years or even decades to complete clinical trials with enough patients enrolled who have a particular fungal infection. So, clinical outcome data obtained through well-controlled clinical trials is rarely available for many types of fungal infections. Therefore, breakpoints may never be established for many fungal species and antifungal agent combinations. But there is another piece of data that is beginning to become available for certain bacteria and fungi that can provide some insight into whether a particular isolate has a typical wild-type MIC profile or if the isolate may have acquired resistance to a particular agent. That piece of data is known as the epidemiological cutoff value.
Epidemiological cutoff values, which are sometimes referred to as ECVs or ECOFFs, represent the MIC value that separates microbial populations into those with and without acquired or mutational resistance based on their phenotypes. The ECV defines the upper limit of susceptibility for the wild-type population of the microbe.

- Wild-type (WT): ECV that defines isolates with no mechanisms of acquired resistance or mutational resistance for the antifungal agent being tested.
- Non-wild type (NWT): ECV that defines isolates with presumed or known mechanisms of resistance for the antifungal agent being tested.
Epidemiological cutoff values are based only on in vitro laboratory data and this data is collected from multiple laboratories. ECVs are established by pooling MIC data from at least 3 independent laboratories across at least 100 distinct isolates of the yeast species. Having more than 3 laboratories contributing MIC data from larger numbers of isolates adds additional confidence to the ECV value determination. By themselves, ECVs cannot be used to predict clinical outcome or suggest whether a particular antifungal agent should be used and an ECV should not be thought of as equivalent to a breakpoint. For example, a Candida krusei isolate with a fluconazole MIC equal to 16 mcg/mL is considered a “wild-type” isolate using the ECV, but we know that wild-type isolates of Candida krusei are resistant to fluconazole and so this agent should not be used to treat Candida krusei infections.

So what are ECVs useful for? ECVs can be useful to assist the clinician to know whether the patient’s isolate has presumed or acquired mutations that might make it less likely to respond to an antifungal agent. A wild-type ECV doesn’t mean that the drug will or won’t work against the yeast. It simply means that the isolate has no known presumed or acquired resistance mechanisms. The wide-type ECV doesn’t provide any information about PK or PD considerations or clinical outcome, which are also needed to make a prediction about the utility of the antifungal agent for this particular yeast isolate. However, a non-wild-type ECV may give a clinician pause and suggests that caution should be used when considering selection of this particular antifungal agent because the isolate has demonstrated the presence of a resistance
mechanism through an elevated MIC that is above that for the normal wild-type population of this species.
This table lists the epidemiological cutoff values currently available for antifungals and Candida species, which have no breakpoints or interpretive criteria available. We now have ECVs available for several of the less frequently encountered Candida species including Candida lusitaniae and Candida dubliniensis for the echinocandins anidulafungin and micafungin. In addition, we have ECVs for the 5 most common Candida species against amphotericin B, which is a very heavily utilized antifungal agent that has never had interpretive criteria available in the past. Now we at least have an MIC value of 2 mcg/mL, which can be used to distinguish wild-type from non-wild-type isolates, to help provide the clinician with some information about the presence of resistance mechanisms in the isolate.
To summarize the presentation today, yeast susceptibility testing against antifungal agents uses a standardized broth dilution method. There are international consensus standards provided by CLSI or EUCAST, which provide methodology and interpretive criteria. Recently, species-specific interpretive criteria have been adopted by the CLSI and are available for the most common Candida species against the echinocandins and 2 azoles. In instances where breakpoints and interpretive criteria are not available, the use of epidemiological cutoff values, or ECVs, may assist the clinician to know whether the isolate is defined as “wild type” or whether it has presumed or acquired resistance mechanisms that cause it to be outside of the MIC range normally seen for this species and antifungal combination. It is anticipated that additional breakpoints and interpretive criteria along with additional ECVs will become available over time as sufficient data is collected for larger numbers of isolates across multiple laboratories.

Conclusions

- Broth dilution is the reference method for antifungal susceptibility testing of yeast
- International consensus standards for testing and interpretation of results are provided by the CLSI or EUCAST
- Species-specific breakpoints and interpretive criteria are available for some Candida species
- When breakpoints are not available, Epidemiologic Cutoff Values (ECVs) may be available that define wild type and non-wild type MIC values for some Candida species
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