Antifungal Susceptibility Testing: Guidelines to Practical approach

Dr Deepti Rawat
Introduction

• Increase incidence of fungal infections
• Increase in immunosuppressive states
• Increasing evidence of invasive mycosis and life threatening infections
• Increased use of antifungal drugs
• Emergence and recognition of antifungal resistance
Objectives

At the end of the session, the participant should be able to

• Understand the need for and principles of antifungal susceptibility testing (AFST)

• Become familiar with terminology used in AFST

• Become aware of the various modalities available for AFST and their limitations
Antifungal Agents

• Allylamines and Benzylamines: Terbinafine and Naftifine
• **Azoles:** Fluconazole, Itraconazole, Voriconazole, Posaconazole and Isavuconazole
• **Polyenes:** Systemic infections: Amphotericin B
  Topical use: Nystatin, Natamycin and Mepartricin
• **Echinocandins:** Anidulafungin, Caspofungin and Micafungin
• Flucytosine
• Griseofulvin
Resistance to Antifungal Agents

• The increased use of antifungals has induced a higher selective pressure on fungal strains and resistance has emerged in two main ways

1. Several species have acquired secondary resistance

1. Susceptible species have been replaced by resistant ones changing the global epidemiology of fungal infections eg nonalbicans candida like C. krusei and C. glabarata increasingly isolated from patients on azole treatment or prophylaxis
Resistance to Antifungal Agents

• Two types of Resistance - Intrinsic & acquired
• **Intrinsic resistance**: inherited characteristic of a species or strain
• Candida krusei is intrinsically resistant to azoles

• **Acquired**: fungi develop resistance over time during antifungal treatment
• Emergence of yeast isolates resistant to azoles (e.g. fluconazole)

• How in-vitro resistance correlates with clinical response- an unresolved issue
### Species with high resistance to antifungal agents

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Drugs</th>
<th>Class of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus terreus</td>
<td>Amphotericin B</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>Azoles</td>
<td>Intrinsic &amp; acquired</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>Azoles</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Candida lusitaniae</td>
<td>Amphotericin B</td>
<td>Intrinsic &amp; acquired</td>
</tr>
<tr>
<td>Histoplasma capsulatum</td>
<td>Fluconazole</td>
<td>Acquired</td>
</tr>
<tr>
<td>Scedosporium apiospermum</td>
<td>Amphotericin B</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Scedosporium prolificans</td>
<td>Amphotericin B</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Trichosporon beigeli</td>
<td>Amphotericin B</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Zygomycetes</td>
<td>Echinocandins</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Fusarium</td>
<td>Echinocandins</td>
<td>Intrinsic</td>
</tr>
</tbody>
</table>
Why do we need antifungal susceptibility testing?

• To detect antifungal resistance and determine best treatment option for a specific fungus

• Correlate with in vivo activity and predict the likely outcome of therapy

• Predict the therapeutic potential of newly discovered antifungal agents

• To provide reliable measures of relative activities of two or more antifungal agents

• To monitor the development of resistance among a normally susceptible population of organisms

• To know about local and global epidemiology of antifungal Resistance
**When do we need to perform AFST??**

- Performance of antifungal susceptibility testing not required for every clinical fungal isolate in routine practice.

- The decision to perform an AFST for a particular clinical strain in routine practice requires the presence of specified indications
  - Isolation of an established pathogen from any sterile site
  - Pure culture, repeated cultures, multiple cultures
  - Febrile neutropenia or immunocompromised
  - Patient not improving on long term antibiotic
Table 3  Well-defined indications for application of routine antifungal susceptibility testing for clinical isolates* [65–67,84]

<table>
<thead>
<tr>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive infection; <em>Candida</em> strain isolated from a sterile body site</td>
</tr>
<tr>
<td><em>Any Candida</em> species for which high rate of resistance to an antifungal drug or a class antifungal drug is well-known to be possible (e.g., <em>C. glabrata</em>-fluconazole and other triazoles)</td>
</tr>
<tr>
<td>Unexpected clinical failure during standard therapy of a <em>Candida</em> infection</td>
</tr>
<tr>
<td>Surveillance antifungal susceptibility testing to detect/rule out emergence of secondary resistance following prior antifungal therapy</td>
</tr>
</tbody>
</table>
Key principles of antifungal susceptibility testing

- In order to be useful clinically, in vitro susceptibility testing should reliably predict the in vivo response to therapy in human infections.

- However, the following factors are often more important than susceptibility test results in determining clinical outcome:
  - drug
  - host immune response
  - status of current underlying disease
  - proper patient management
  - the infecting organism
  - the interaction of the organism with the host and therapeutic agent
Key principles of antifungal susceptibility testing

• Susceptibility of a microorganism in vitro does not predict successful therapy
• Resistance in vitro may be able to predict therapeutic failure
• There is considerable evidence that the standardised antifungal susceptibility testing for candida spp and some triazoles provide results that are consistent with the 90/60 rule
The “90-60” Rule

• This rule is applicable to most of the microbial infections and can predict the outcome of treatment

• It states that-
  Infections respond to therapy about 90% of the time if the infectious agent is judged to be susceptible in vitro, while infections respond to therapy about 60% of the time if the infectious agent is judged to be resistant in vitro, even with correct interpretation of clinical breakpoints
Factors that contribute to clinical antifungal drug resistance

<table>
<thead>
<tr>
<th>Host factors</th>
<th>Drug factors</th>
<th>Fungal factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune status</td>
<td>Fungistatic nature of drug</td>
<td>Species and strain</td>
</tr>
<tr>
<td>Site of infection</td>
<td>Dosing</td>
<td>Cell type</td>
</tr>
<tr>
<td>Severity of infection</td>
<td>Frequency</td>
<td>Morphology</td>
</tr>
<tr>
<td>Presence of foreign materials</td>
<td>Quantity</td>
<td>Cell states</td>
</tr>
<tr>
<td>Poor adherence to drug regimen</td>
<td>Cumulative dose</td>
<td>Serotypes</td>
</tr>
<tr>
<td></td>
<td>Pharmacokinetics</td>
<td>Biofilms</td>
</tr>
<tr>
<td></td>
<td>Absorption</td>
<td>Genomic stability of strain</td>
</tr>
<tr>
<td></td>
<td>Distribution</td>
<td>Size of population</td>
</tr>
<tr>
<td></td>
<td>Metabolism</td>
<td>Population “bottlenecks”</td>
</tr>
<tr>
<td></td>
<td>Drug-drug interactions</td>
<td>Strain MIC</td>
</tr>
</tbody>
</table>
Terminology

• **Minimal inhibitory concentration (MIC)** – the lowest concentration of an antimicrobial agent that causes a specified reduction in visible growth.

• **Minimal effective concentration (MEC)** – the lowest concentration of an antimicrobial agent that leads to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well.

• A **breakpoint** is a chosen concentration (mg/L) of an antibiotic which defines whether an organism is susceptible or resistant to the antibiotic.

• If the **MIC** is less than or equal to the susceptibility **breakpoint** the organism is considered susceptible to the antibiotic.
Breakpoints

These breakpoints categorise fungal isolates into

- **Susceptible**: The drug is an appropriate treatment

- **Resistant**: The drug is not recommended as treatment

- **Intermediate**: The drug may be appropriate treatment under certain circumstances

Implies clinical efficacy in body sites where

- drugs are physiologically concentrated (fluconazole in urinary infection)
- higher than normal dosage of drug can be used
Breakpoints

- **Susceptible Dose Dependent (SDD)**
  - Only applies when multiple approved dosing options are available
  - same clinical response as Susceptible if higher or more frequent dosing is used

- **Nonsusceptible (NS)**
  - The “nonsusceptible” category is used for isolates for which only a susceptible interpretive criterion has been designated because of the absence or rare occurrence of resistant strains.
  - Isolates for which the antimicrobial agent MICs are above or zone diameters below the value indicated for the susceptible breakpoint should be reported as nonsusceptible.
Trailing end Point

- Standard susceptibility testing monitors growth at 24 or 48 hrs and MIC is defined as the concentration that inhibits growth by 50% or 80%

- For most isolates, the difference between reading at 24 hours versus 48 hours is minimal and will not alter the interpretative category

- However some isolates show a dramatic rise in MIC over time (e.g. for fluconazole from 0.5 ug/ml at 24 hours to 256 ug/ml at 48 hours).

- The difference between the 24 and 48 hour growth is known as trailing
Trailing end Point

- Trailing occurs when the turbidity continually decreases as the drug concentration increases but the suspension fails to become optically clear (partial inhibition of growth over an extended range of antifungal concentrations).

- To help resolve this issue the M27-A2 methodology for Candida has provided both 24 hour and 48 hour microdilution MIC ranges for the two QC strains and eight systemic antifungal agents.

- Ideally plates should be read at 24 hours whenever there is sufficient growth.

- Occurs mainly in Azoles, particularly fluconazole since these drugs are fungistatic.

- More common in Candida (5-20% cases).
Paradoxical (Eagle) effect

- Occurs mainly with echinocandins with candida & Aspergillus

- Echinocandins inhibited growth of C. albicans at low concentrations but at concentrations from 8–32 μg/ml some growth was observed and at even higher concentrations growth inhibition was achieved again.

- This paradoxical growth at supra MICs differs from the trailing growth phenomena observed in some clinical isolates that are capable of persistent but reduced growth at higher drug concentrations.

- ↓ in β 1-3 & 1-6 glucan & a rapid shift of fungal cell wall polymer to chitin.

- *In vivo* the response is not consistent & clinical significance unclear.

- To date there is no clinical evidence that paradoxical growth contributes to resistance or breakthrough infections.
EPIDEMIOLOGICAL CUTOFF VALUES

• A value used to assist with interpretation of MIC data where sufficient clinical data is not available for the purpose of establishing clinical breakpoints

• Chosen by taking into account the MIC distribution of a species, the modal distribution, and the inherent variability in MIC testing methods

• Ideally, test sets contain in excess of 1000 strains with wild-type strains comprising 95-99%

• Strains with MICs above the ECV are considered to have potentially acquired mechanisms of resistance

• MIC below /above the ECV dose not tell you that the isolate is susceptible/resistant
Antifungal Susceptibility Testing Methods
Methods used for antifungal susceptibility testing

<table>
<thead>
<tr>
<th>Test method</th>
<th>Means of endpoint determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth macrodilution (yeasts)</td>
<td>Visual comparison of turbidity (≥50% inhibition) with that of growth control (M27-A3 document)</td>
</tr>
<tr>
<td>Broth microdilution (yeasts)</td>
<td>Visual comparison of turbidity (≥50% inhibition) with that of growth control (M27-A3 document)</td>
</tr>
<tr>
<td>Colorimetric microdilution (yeasts [YeastOne] and molds)</td>
<td>Visual observation of color change</td>
</tr>
<tr>
<td>Spectrophotometric microdilution (yeasts)</td>
<td>Turbidimetric MIC determination by spectrophotometer (EUCAST)</td>
</tr>
<tr>
<td>Macro- and microdilution (filamentous fungi)</td>
<td>Visual comparison of growth (50% inhibition or more [nondermatophytes] or 80% or more [dermatophytes] or MEC) with that of growth control (M38-A2 document)</td>
</tr>
<tr>
<td>Agar macrodilution (yeasts and molds, standard dishes)</td>
<td>Visual</td>
</tr>
<tr>
<td>Agar diffusion (yeasts and molds)</td>
<td>Zone diameter (visual)</td>
</tr>
<tr>
<td>Disk</td>
<td>Zone diameter (visual)</td>
</tr>
<tr>
<td>Antifungal strip (Etest)</td>
<td>Ellipse of inhibition (visual)</td>
</tr>
</tbody>
</table>
In vitro susceptibility testing is influenced by a number of variables

<table>
<thead>
<tr>
<th>Technical</th>
<th>Fungi related</th>
<th>Drug related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innoculum size and preparation</td>
<td>Slow growth rates</td>
<td>solubility</td>
</tr>
<tr>
<td>Medium formulation and pH</td>
<td>Ability of certain dimorphic fungi to grow either in yeast form that produce blastoconidia or filamentous form that may produce conidia or sporangiospores</td>
<td>Chemical stability</td>
</tr>
<tr>
<td>Duration and temperature of incubation</td>
<td></td>
<td>Mode of action</td>
</tr>
<tr>
<td>Criteria used for MIC endpoint determination</td>
<td></td>
<td>Tendency to produce partial inhibition of growth over a wide range of concentration above the MIC: trailing</td>
</tr>
</tbody>
</table>
What are the standardized methods available?

- CLSI (Clinical and Laboratory Standards Institute), formerly NCCLS

- EUCAST (European Committee on antifungal Susceptibility testing)
Antifungal susceptibility testing methods (CLSI)

- Standardization of antifungal susceptibility testing assays were initiated by CLSI.
- CLSI has released standard methods for antifungal susceptibility testing including
  - selection & preparation of antifungal agents
  - implementation and interpretation of test procedures
  - Interpretation of quality control procedures
CLSI Antifungal Susceptibility Testing of Yeasts by Broth dilution – CLSI, M-27 A3

- CLSI, M-27 A3 documents guidelines for broth dilution AST of yeasts (Candida, C. neoformans)

- First published in 1997

- Current document is 2008

- Protocol for amphotericin B, flucytosine, ketoconazole, fluconazole, itraconazole, posaconazole, ravuconazole, voriconazole, & echinocandins
CLSI Antifungal Susceptibility Testing of Yeasts by Disc Diffusion – CLSI, M44 A2

• CLSI, M44 A2 document has been developed and validated only for AST by disc diffusion for Candida spp vs azoles and echinocandins

• Classifies isolates into one of the following category: susceptible, resistant, susceptible dose dependent and Nonsusceptible

• Good agreement with CLSI broth dilution technique's
Quality Control strains

• C.parapsilosis  ATCC 22019
• C.krusei  ATCC 6258
CLSI Antifungal Susceptibility Testing of Filamentous Fungi by Broth Dilution – M 38A2

• CLSI subcommittee has developed both broth micro and macrodilution methods for testing moulds that are frequently associated with invasive infections

• CLSI, M-38A2 document guidelines for broth dilution AST of filamentous fungi

• Published in 2002, updated in 2008
CLSI Antifungal Susceptibility Testing of Filamentous Fungi by Broth Dilution – M 38A2

Scope

• Aspergillus spp., Fusarium spp., Rhizopus spp., P. boydii (S. apiospermum), S. prolificans
• The mycelial form of S. schenckii
• Other Zygomyctes and opportunistic monilaceous and dematiaceous moulds
• Trichophyton, Microsporum, and Epidermophyton spp

Not used

• for yeast or mould form of dimorphic fungi,
  – Blastomyces dermatitidis, Coccidioides immitis, Coccidioides posadasii, Histoplasma capsulatum variety capsulatum, Penicillium marneffei
CLSI Antifungal Susceptibility Testing of Filamentous Fungi by Disc Diffusion – M 51 A

- CLSI, M 51A and supplement M51-S1 document guidelines for AST by disc diffusion for nondermatophyte filamentous fungi
Quality control for mould testing

- C.parapsilosis  ATCC 22019
- C.krusei  ATCC 6258
- Paecilomyces variotti  ATCC MYA-3630
Antifungal susceptibility testing methods (EUCAST)

- Developed by the subcommittee on antifungal susceptibility testing of EUCAST (European Committee for Antimicrobial Susceptibility Testing)

- Reference method for determination of minimum inhibitory concentration (MIC, µg/ml) by broth dilution for yeasts and molds

- Standard for yeasts including *Cryptococcus* was published in 2008 and updated in 2012

- Standard for molds was published in 2008
Antifungal susceptibility testing methods (EUCAST)

• Good intralaboratory reproducibility and good agreement between CLSI and EUCAST methods documented

• However CLSI breakpoints should not be used to interpret EUCAST MICs since EUCAST MICs are considerably lower and can result in false susceptible results
<table>
<thead>
<tr>
<th></th>
<th>CLSI M27-A3</th>
<th>EUCAST Edef 7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Format</strong></td>
<td>Microdilution</td>
<td>Microdilution 7.2</td>
</tr>
<tr>
<td><strong>Well shape (bottom)</strong></td>
<td>round</td>
<td>flat</td>
</tr>
<tr>
<td><strong>Media</strong></td>
<td>RPMI 1640</td>
<td>RPMI 1640</td>
</tr>
<tr>
<td><strong>Glucose content</strong></td>
<td>0.2%</td>
<td>2%</td>
</tr>
<tr>
<td><strong>Inoculum size</strong></td>
<td>0.5-2.5 x 10^3</td>
<td>0.5-2.5 x 10^5</td>
</tr>
<tr>
<td><strong>Incubation temperature</strong></td>
<td>35 °C</td>
<td>35 °C</td>
</tr>
<tr>
<td><strong>Incubation time</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMB, FCZ, Candins</td>
<td>24h</td>
<td>24h</td>
</tr>
<tr>
<td>Azoles</td>
<td>48h</td>
<td>24h</td>
</tr>
<tr>
<td>Cryptococcus</td>
<td>72h</td>
<td>48h</td>
</tr>
<tr>
<td><strong>Reading</strong></td>
<td>Visual</td>
<td>Spectrophotometric</td>
</tr>
<tr>
<td><strong>Endpoint</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMB</td>
<td>100% inhibition</td>
<td>90% inhibition</td>
</tr>
<tr>
<td>Azoles and Candins</td>
<td>50% inhibition</td>
<td>50% inhibition</td>
</tr>
</tbody>
</table>

Differences between two methods are in bold. AMB = amphotericin B; FCZ = fluconazole; Candins = anidulafungin, caspofungin, micafungin.
Susceptibility Testing for Yeasts
**TABLE 2  CLSI M27-A3 document broth dilution guidelines for antifungal susceptibility testing of yeasts**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth medium</td>
<td>RPMI 1640 broth buffered with MOPS buffer (0.165 M) and 0.2% dextrose to a pH of 7.0 at 25°C</td>
</tr>
<tr>
<td>Medium modifications</td>
<td>(i) Yeast nitrogen base broth (pH 7.0) with MOPS provides better growth for C. neoformans and (ii) RPMI 1640 with 2% dextrose</td>
</tr>
<tr>
<td>Inoculum preparation</td>
<td>Five colonies from 24-h (Candida spp.) or 48-h (C. neoformans) cultures on Sabouraud dextrose agar or potato dextrose agar</td>
</tr>
<tr>
<td>Stock inoculum suspension</td>
<td>Adjusted by spectrophotometer at 530 nm to match the turbidity of a 0.5 McFarland standard (1 × 10⁶ to 5 × 10⁶ CFU/ml)</td>
</tr>
<tr>
<td>Test inoculum</td>
<td>1:2,000 (macrodilution) or 1:1,000 (microdilution) dilutions with medium of the stock inoculum suspension; inoculum size after inoculation, 0.5 × 10³ to 2.5 × 10³ CFU/ml (both methods)</td>
</tr>
<tr>
<td>Drug dilutions</td>
<td>Additive 10× (macrodilution) or 2× (microdilution) twofold drug dilutions with medium (fluconazole, caspofungin, micafungin, and flucytosine) or 100× with solvent (amphotericin B, other azoles, and anidulafungin)</td>
</tr>
</tbody>
</table>
## Broth dilution

<table>
<thead>
<tr>
<th>Drug dilution ranges</th>
<th>Flucytosine and fluconazole</th>
<th>Other drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.12–64 μg/ml</td>
<td>0.03–16 μg/ml</td>
</tr>
</tbody>
</table>

### Methods

<table>
<thead>
<tr>
<th></th>
<th>Macrodilution</th>
<th>Microdilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.9 ml of diluted test inoculum plus 0.1 ml of 10× drug concn</td>
<td>100 μl of diluted test inoculum plus 100 μl of 2× drug concn</td>
</tr>
</tbody>
</table>

### Growth control(s)

<table>
<thead>
<tr>
<th></th>
<th>Macrodilution</th>
<th>Microdilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.9 ml of diluted inoculum plus 0.1 ml of drug-free medium (or plus 2% of solvent)</td>
<td>100 μl of diluted inoculum plus 100 μl of drug-free medium (or plus 2% of solvent)</td>
</tr>
</tbody>
</table>

### Time of reading

- Amphotericin B, 24 or 48 h; fluconazole, 24 or 48 h; echinocandins, 24 h only; 5-FC and other azoles, 48 h

### MIC by visual examination

- Amphotericin B, macro- and microdilution: Lowest drug concn that prevents any discernible growth (100% inhibition)
- Flucytosine, azoles, caspofungin, and other echinocandins: Lowest drug concn that shows prominent (~50%) decrease in turbidity
Limitations of reference dilution-based methods

- Laborious & time consuming (24-72 h)
- Cannot discriminate amphotericin B-resistant isolates from the susceptible very clearly
- MIC breakpoints for several fungus-antifungal drug combinations are yet NOT established
- Azole and flucytosine MICs hard to read and-interpret particularly for heavy trailing isolates
- Poor growth of some isolates in micro-titre plate
Broth-based alternative approaches for Yeast

• To improve interlaboratory reproducibility
• To better serve clinical laboratory needs

- Spectrophotometric method (EUCAST)
- Colorimetric methods
- Flow cytometry
- VITEK 2
Spectrophotometric method

- Microdilution methods allow the determination of endpoints with automated plate reading and yield MIC values that are more accurate and objective
- This method allows the determination of different levels (percentages) of growth inhibition

- **GI50**: Turbidimetric growth inhibition of 50% in case of azoles and flucytosine
- **GI90**: Turbidimetric growth inhibition of 90% in case of Amphotericin B
Colorimetric method

• Colorimetric indicators or dyes can facilitate determination of MIC endpoints

• The Sensititre Yeast One (Trek Diagnostics) panel follows the same microdilution format as the CLSI reference method and is FDA approved for Fluconazole, itraconazole and flucytosine.

• Reading of the endpoints is enhanced by the inclusion of Alamar blue as the oxidation reduction colorimetric indicator

• This method minimises the trailing effect of azoles
SENSITITRE® YeastOne
Microbroth dilution MIC test

AmB 0.008-16
Flu 0.125-256
Itra 0.008-16
Keto 0.008-16
5Fc 0.03-64

MIC
0.125
1.0
0.125
0.125
0.18

C. albicans

AccuMed International Ltd [Dutec Diagnostics in Australia]
Colorimetric method

- Another commercially available system is the SensiQuattro Candida EU (bestibiondex, Germany)

- Amp B, Flu, Vori, Posa, Flucytosine, Ecchinocandins

- This correlates well with the antifungal clinical breakpoints established by EUCAST for Azoles and Amp B
Vitek 2 (Biomeriux Inc.)

- Automated commercial system based on spectrophotometric analysis

- This has been shown to produce reproducible, rapid and accurate results consistent with those produced by CLSI broth microdilution for Amphotericin B, Flucytosine, fluconazole and voriconazole with candida

- FDA has cleared it only for testing fluconazole as part of patient care
Commercial kits

- VITEK 2 (BioMerieux)
  fully automated system
Flowcytometry

- Adapted for antifungal susceptibility testing by introducing DNA binding vital dyes into the culture to detect fungal cell damage after exposure to an antifungal agent

- Results faster (4-6hrs)

- Not FDA approved
Agar based approaches for Yeasts

- Disc diffusion
- E strip
- Fungicidal Activity
TABLE 5  CLSI M44-A2 document guidelines for antifungal disk diffusion susceptibility testing of Candida spp.ª

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar medium</td>
<td>Mueller-Hinton agar + 2% dextrose and 0.5 µg of methylene blue dye/ml</td>
</tr>
<tr>
<td>Inoculum preparation</td>
<td>From 24-h cultures on Sabouraud dextrose agar as described in Table 2 for broth micro- and macrodilution methods</td>
</tr>
<tr>
<td>Test inoculum</td>
<td>Stock inoculum suspension, adjusted by spectrophotometer at 530 nm to match the turbidity of a 0.5 McFarland standard: 1 × 10⁶ to 5 × 10⁶ CFU/ml</td>
</tr>
<tr>
<td>Disk contents</td>
<td>Caspofungin, 5 µg; fluconazole, 25 µg; posaconazole, 5 µg; voriconazole, 1 µg</td>
</tr>
<tr>
<td>Incubation conditions</td>
<td>20–24 h at 35°C</td>
</tr>
<tr>
<td>Reading zone diameter</td>
<td>To the nearest whole millimeter at the point at which there is prominent reduction in growth. Pinpoint microcolonies at the zone edge or large colonies within the zone should be ignored.</td>
</tr>
</tbody>
</table>

ªData from CLSI M44-A2 (17)
E-Test

- E-test is a commercially available method (AB Biodisk, Sweden)
- MICs determined from the point of intersection of a growth inhibition zone with a calibrated strip impregnated with a gradient of antimicrobial concentration and placed on an agar plate streaked with the microbial isolate under test
- RPMI 1640 supplemented to 2% glucose is the most commonly used medium
- Other media including Casitone agar (particularly for azoles), YNB (particularly for testing C. neoformans) and MHA supplemented with 2% glucose and methylene blue have also been used
MHA - C. albicans (flu: MIC-0.38 μg/ml)
C. glabrata (flu: MIC >256 μg/ml) & C. lusitanea (AMB)
E-Test

• For clinical use FDA has approved only fluconazole, Itraconazole and

• It is a reliable & reproducible method correlating well with CLSI methodology

• Better than reference method for AMB- resistant isolates as produces results over a greater concentration range that more readily distinguishes susceptible and resistant strains
Fungicidal activity

• Minimum fungicidal concentration (MFC) is defined as the lowest drug concentration resulting in either no growth or 3-5 colonies

• The determination of MFC requires the subculturing onto an agar medium of fixed volume from each MIC tube or well that shows complete inhibition of growth

• However standard testing parameters are not available for evaluating MFC of antifungals
Molecular methods

• There are multiple mechanisms that lead to reduced susceptibility or overt resistance to azole antifungal drugs in candida spp.
  - changes in cell wall composition leading to reduced uptake
  - increased efflux
  - mutation in target enzymes
Molecular methods

• Thus any method for molecular determination of resistance must be a multiplex system and must be capable of determining not just the presence but also the upregulation of housekeeping genes

• Resistance to echinocandins is usually due to mutations of the FKS genes
Susceptibility Testing for Filamentous Fungi
# Broth dilution for filamentous Fungi

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium for conidial growth</strong></td>
<td>Potato dextrose, 35°C (7 days); <em>Fusarium</em> spp. may need 25–28°C incubation for the last 4 days</td>
</tr>
<tr>
<td><strong>Inoculum morphology</strong></td>
<td>Conidia or sporangiospores</td>
</tr>
<tr>
<td><strong>Recommended OD ranges</strong></td>
<td>For <em>Aspergillus</em> and for <em>R. arrhizus</em>, 0.09–0.13; for <em>Fusarium</em> and <em>S. apiospermum</em>, 0.15–0.17; stock inoculum suspensions, $0.4 \times 10^6$–$5 \times 10^6$ CFU/ml</td>
</tr>
<tr>
<td><strong>Inoculum concn (final)</strong></td>
<td>$0.4 \times 10^4$ to $5 \times 10^4$ CFU/ml or 1:50 dilution of stock suspension (<em>S. apiospermum</em>, 2:50)</td>
</tr>
<tr>
<td><strong>Test medium</strong></td>
<td>RPMI 1640 as for the yeasts (pH, 7.0 ± 0.1)</td>
</tr>
<tr>
<td><strong>Format</strong></td>
<td>Microdilution assay; total volume/well, 200 µl</td>
</tr>
<tr>
<td><strong>Drug concn</strong></td>
<td>Amphotericin B, ketoconazole, itraconazole, posaconazole, ravuconazole, and voriconazole, 0.0313–16.0 µg/ml; fluconazole and fluconazole, 0.125–64.0 µg/ml; anidulafungin, caspofungin, and micafungin, 0.015–8.0 µg/ml. Different ranges should be used for testing dermatophytes: ciclopirox, 0.06–32 µg/ml; fluconazole and griseofulvin, 0.125–64 µg/ml; itraconazole, voriconazole, and terbinafine, 0.001–0.5 µg/ml; posaconazole, 0.004–8.0 µg/ml.</td>
</tr>
</tbody>
</table>
Broth dilution for filamentous Fungi

Incubation duration at 35°C without agitation. For R. arrhizus and other mucoraceous molds, 21–26 h; for Scedosporium spp., 70–74 h; for most other opportunistic filamentous fungi, 46–50 h. Echinocandin MECs should be read at 21–26 h or at 46–72 h for Scedosporium spp.

Endpoint determination, visual. Growth relative to that for positive growth control read with a reading mirror. The MIC is read as the lowest drug concn that substantially inhibits growth (100% for amphotericin B and most azoles, 50% or more for other drugs, 80% or more for dermatophytes). For echinocandins an MEC is determined as the lowest concentration that leads to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the control well.

Colorimetric (Alamar Blue). For amphotericin B, first blue well or without color change; for azoles, first blue well or slightly purple well.
Broth Microdilution methods for dermatophytes

- M38-A2 Broth microdilution method has been successfully adapted with minor modifications for the testing of dermatophytes

- The use of oatmeal agar for inoculum preparation when testing *T. rubrum*

- The time and temperature of incubation are 30°C for 4–5 days

- The MIC is defined as the lowest drug concentration that produces at least 80% growth inhibition as compared with growth in the growth control

- *Trichophyton mentagrophytes* MRL 1957, and *Trichophyton rubrum* MRL 666 are used as QC strains
Broth based Alternative Approaches for molds

• Evaluation of morphological changes used for testing echinocandins for some molds such as aspergillus

  - Minimum Effective Concentration (MEC): Concentration of drug producing significant morphological changes

• Colorimetric methods using dyes such as alamar blue
Agar based approaches for filamentous fungi

Disc Diffusion

- CLSI, M51A document guidelines for AST by disk diffusion for non-dermatophyte filamentous fungi

- Similar to that for yeasts but employs Mueller Hinton agar without the methylene blue and increased dextrose

- No breakpoints available: Epidemiological cut off values can be proposed to identify non wild type strains
Agar based approaches for filamentous fungi

E test

• Evaluated for filamentous fungi with good correlation with the M38 A2

• The interpretation of MIC results is easier than for yeasts as ellipses are usually sharper

• Etest could be a good choice
  – it is easy to perform
  – inhibition ellipse is very sharp
  – there is no need of a microscope for determining the MEC of echinocandins
  – *Trailing* effect not a problem for azole testing against most molds but not yet FDA approved
Fungicidal activity

• Minimum fungicidal Concentration testing may be performed for azoles since they demonstrate fungicidal activity for a variety of common and rare opportunistic mold pathogens

• However standardisation of this procedure is needed to reliably assess the potential value of the MFC endpoint in patient management
Molecular Tests

• Application to molds easier as resistance mechanisms appear to be fewer
Summary

• Despite standardization of susceptibility testing, MIC values do not associate with response to antifungal therapy

• Most important factors that make correlation in vitro-in-vivo data difficult are:
  ➢ Disease heterogeneity and bias of host immunity
  ➢ Inadequate concentration of the drug at the infection site
  ➢ Infections associated with prosthetic devices/catheters that act as substrates of biofilm growth
Summary contd..

• Local epidemiology of antifungal resistance aids to select empirical treatment

• Mortality due to IFIs are high despite recent advances and the emphasis must be given to:
  ➢ High index of suspicion and early diagnosis
  ➢ Restoration of host immunity
  ➢ Appropriate guided antifungal therapy for optimum management of the patient
THANK YOU