Antifungal drug resistance: towards the most reliable detection method?

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Faculty disclosure

- Invited speaker: Pfizer, Gilead, MSD, Schering-Plough
- Consultant: Pfizer, Gilead, Schering-Plough
- Research Grants: Pfizer, Gilead, Schering-Plough
Outline

Current status of susceptibility testing for yeast and moulds
- Pro and cons

Antifungal breakpoints and susceptibility
- Clinical breakpoints
- Relationship with clinical outcome?
A brief history of antifungal susceptibility testing standardization

- 1982: Established subcommittee
- 1986: Developed reproducible method
- 1992: M27-P method introduced
- 1997: M27-A method introduced

20% hospitals performing testing for yeast; intra/inter-laboratory agreement poor

Synthetic medium (RPMI)
Broth-based method
0.5-2.5 x10^3

Conidia forming filamentous fungi
Disk-diffusion method-yeast
Disk-diffusion method-moulds

Breakpoints

Higher glucose; 24 hour endpoint; spectrophotometer

Why Susceptibility Testing?

• To provide information for therapy
  • To provide early warning
  • To get epidemiological data
  • To get information for species ID
Facts: Susceptibility Testing

• We lack clinically derived breakpoint for most drug/bug combinations
  • Technical factors influence MICs
  • Which *in vitro* method reflects best outcome
• Host factors are more important for survival
Methods: Susceptibility testing

- Standards: CLSI, EUCAST
- E-test, Agar-diffusion
- Ready to use test assays
- Others (FACS)
# Reference Methods

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CLSI M27-A3</th>
<th>EUCAST Def</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Suitability</strong></td>
<td>Yeasts</td>
<td>Ferramentative Yeast</td>
</tr>
<tr>
<td><strong>Inoculum</strong></td>
<td>0.5-2.5x10^3 CFU/ml</td>
<td>0.5-2.5x10^5 CFU/ml</td>
</tr>
<tr>
<td><strong>Test medium</strong></td>
<td>RPMI 1640 0,2%G</td>
<td>RPMI 1640 G2%</td>
</tr>
<tr>
<td><strong>Format</strong></td>
<td>Microdilution</td>
<td>Microdilution</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>35°C</td>
<td>35°C</td>
</tr>
<tr>
<td><strong>Duration of incubation</strong></td>
<td>46-50h</td>
<td>24h</td>
</tr>
<tr>
<td></td>
<td>24 h for yeasts</td>
<td></td>
</tr>
<tr>
<td><strong>Endpoint</strong></td>
<td>80% inhibition M27-A2</td>
<td>80% amphotericin B</td>
</tr>
<tr>
<td></td>
<td>50% inhibition M27-A3 (azole)</td>
<td>50% inhibition azole</td>
</tr>
<tr>
<td><strong>Reading</strong></td>
<td>Visually</td>
<td>Plate reader</td>
</tr>
</tbody>
</table>
Overall exists good inter/intralaboratory agreement!
Common Problems with Microdilution Methodology

| Visual determination of azole MIC endpoint (trailing growth) “prominent reduction” |
| MEC: Aspergillus and Candins |
| Dramatic increase in azole MIC from 24 to 48 hours |
| Poor growth of some isolates in tray |
| Time consuming |
| “Clustering” of AMB MICs |
| Candins: Drug solubility/stability |
| Batch to batch variations |

Lass-Flörl et al., Mycoses 2009
Agar-based MIC determination

• Often more friendly to the workflow of the clinical laboratory (fast and easy)
• Better visualization of organisms response?
• Agar-based methods
  - (Agar dilution)
  - Disk diffusion (M44-P)
  - Etest (AB Biodisk/BioMereaux)

http://www.mycology.adelaide.edu.au/
MICs defined via E-test

Growth of fungus

MIC = zone of inhibition
Development of Fluconazole Resistance in *C. albicans*
Some problems with caspofungin

A. flavus  C. tropicalis  A. fumigatus
Caspofungin 2 µg-A. fumigatus

<table>
<thead>
<tr>
<th>Antifungal Agent</th>
<th>Disk Content</th>
<th>Zone Diameter, Nearest Whole (mm)</th>
<th>Equivalent MIC Breakpoints (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R*</td>
<td>S-DD*</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>25 µg</td>
<td>≤14</td>
<td>15 - 18</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>1 µg</td>
<td>≤13</td>
<td>14 - 16</td>
</tr>
</tbody>
</table>

In 2002, the CLSI issued formal fluconazole disk interpretive criteria of
19 mm = S, 15-18 mm = SDD, and 14 mm = R
In 2005, the tentative Voriconazole breakpoints are
17 mm = S, 14-16 mm = SDD, and 13 mm = R

The disk diffusion test using the MH agar plate can be performed quickly, simply, and cost-effectively, and is practicable method for the initial testing of the susceptibility of Candida spp. to voriconazole and fluconazole.

(Lee SC. et al, JMII 2009)
Common Problems with Agar Based Methods, Endpoint reading!

| Visual determination of azole MIC endpoint (growth within the ellipse) “prominent reduction” |
| Similar is for Aspergillus and Candids |
| Dramatic increase in azole MIC from 24 to 48 hours |
| Which medium is best? G2%, RPMI, HR? |
| “Clustering” of AMB MICs |
| Medium: Batch to batch variations |
| Reading: Zone of inhibition for molds? 24 vs 48 h? |

Lass-Flörl et al., Mycoses 2009
**Candida krusei**

+ve growth control

5Fc = I 4-32 ug/ml

Amb = S <2 ug/ml

Micon = I 1-8 ug/ml

Keto = S <0.5 ug/ml

Itra = S <0.5 ug/ml

Flu = SDD 16-32ug/ml

-ve growth control
The Sensititre YeastOne colorimetric microdilution panel appears to be a viable alternative to the CLSI reference method for testing yeasts against a variety of antifungal agents (Hollis RJ, 2007).
Species identification and in vitro susceptibility: up to 18 hours

The Vitek 2 system was able to identify all but 2 of 59 investigated fluconazole-resistant organisms. (Posteraro et al. 2009 JCM)

The VITEK 2 system reliably detected fluconazole resistance among Candida spp. and demonstrated excellent quantitative and qualitative agreement with the reference BMD method (Pfaller et al. 2007, JCM)
ATB FUNGUS 2 (bio-Merieux, France)

- ATBF 2(3) is an objective, reproducible and simple method for the accurate determination of MICs of the most common antifungal drugs in yeasts (Torres-Rodríguez, 2007 JAC; Quindos, 2008)
- 24 hour incubation
Among non-\textit{C. albicans} strains, the percentages of susceptible isolates were as follows:

- \textbf{CLSI M27-A}, 74.0%
- Etest, 83.8%
- Sensititre YeastOne, 64.1%
- Disk, 80.6%
- Fungitest, 76.6%
- Integral System Yeasts, 28.3%
- Candifast, 27.4%

All methods except Candifast and Integral System Yeasts showed good agreement with \textbf{CLSI M27-A} results for both \textit{C. albicans} and non-\textit{C. albicans} isolates (Morace et al., JCM, 2002)
## Interpretive Guidelines for In Vitro Susceptibility Testing of Candida spp. Breakpoints according CLSI and EUCAST

<table>
<thead>
<tr>
<th>Antifungal Agent</th>
<th>Susceptible (S)</th>
<th>Susceptible dose dependent (S-DD)</th>
<th>Resistant (R)</th>
<th>Non-susceptible (NS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anidulafungin</td>
<td>&lt;2 n.v.</td>
<td>-</td>
<td>&gt;2 n.v.</td>
<td></td>
</tr>
<tr>
<td>Caspofungin</td>
<td>&lt;2 n.v.</td>
<td>-</td>
<td>&gt;2 n.v.</td>
<td></td>
</tr>
<tr>
<td>Micafungin</td>
<td>&lt;2 n.v.</td>
<td>-</td>
<td>&gt;2 n.v.</td>
<td></td>
</tr>
<tr>
<td>Fluconazole</td>
<td>≤8 &lt;2</td>
<td>16-32 4</td>
<td>≥64 &gt;4</td>
<td>-</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>≤0.125 n.v.</td>
<td>0.25-0.5 n.v.</td>
<td>≥1 n.v.</td>
<td>-</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>≤1 E cutoff &lt;0.125</td>
<td>2 n.v.</td>
<td>≥4 E- cutoff ≥0.125</td>
<td>-</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>n.v. n.v.</td>
<td>n.v. n.v.</td>
<td>n.v. n.v.</td>
<td></td>
</tr>
</tbody>
</table>
Echinocandin susceptibility testing of Candida species: comparison of EUCAST EDef 7.1, CLSI M27-A3, Etest, disk diffusion, and agar dilution methods with RPMI and isosensitest media

- Nine susceptibility testing methods and 12 endpoints for anidulafungin, caspofungin, and micafungin with the same collection of blinded FKS hot spot mutant (n = 29) and wild-type isolates (n = 94).
- The methods with the lowest number of errors (given as VMEs/MEs) across the three echinocandins were CLSI (12%/1%), agar dilution with RPMI-2G medium (14%/0%), and Etest with RPMI-2G medium (8%/3%). The fewest errors overall were observed for anidulafungin (4%/1% for EUCAST, 4%/3% for CLSI, and 3%/9% for Etest with RPMI-2G).
- For micafungin, VME rates of 10 to 71% were observed. For caspofungin, agar dilution with either medium was superior (VMEs/MEs of 0%/1%), while CLSI, EUCAST with IsoSensitest-2G medium, and Etest were less optimal (VMEs of 7%, 10%, and 10%, respectively).

Arendrup et al., AAC, 2010
Applying the CLSI breakpoint (S \leq 2 \mu g/ml) for CLSI results, 89.2% fks hot spot mutants were classified as anidulafungin susceptible, 60.7% as caspofungin susceptible, and 92.9% as micafungin susceptible.

In conclusion, no test was perfect, but anidulafungin susceptibility testing using the WT-UL to define susceptibility reliably identified fks hot spot mutants.

Arendrup et al., AAC, 2010
Frequency and Evolution of Azole Resistance in *Aspergillus fumigatus* Associated with Treatment Failure

Susan J. Howard, Dasa Cerar, Michael J. Anderson, Ahmed Albarrag, Matthew C. Fisher, Alessandro C. Pasqualotto, Michel Laverdiere, Maiken C. Arendrup, David S. Perlin, and David W. Denning

**Proposed EUCAST breakpoints for *A. fumigatus* and azoles**

<table>
<thead>
<tr>
<th>Azole</th>
<th>MICs (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td><em>Itraconazole</em></td>
<td>&lt; 2</td>
</tr>
<tr>
<td><em>Voriconazole</em></td>
<td>&lt; 2</td>
</tr>
<tr>
<td><em>Posaconazole</em></td>
<td>&lt; 0.25</td>
</tr>
</tbody>
</table>

Conclusions

• Echinocandins: yeasts and molds - which method?
• Amphotericin B: how to detect resistance?

• Overall agreement within azoles seems to be best!
Conclusions

Testing in the daily routine:
1. Etest, 2. Disk Diffusion, 3. others

Studies and confirmation:
EUCAST, CLSI

(Koc et al. 2000, Pfaller et 1996)
Conclusions
In vitro susceptibility testing: local epidemiology and patients' immune status

- Yeasts
  - Sterile body site plus non-C. albicans
  - Azole (?)
  - Non-responder

- Molds
  - Non A. fumigatus
  - all:
    - Non responder
  - Long treatment & azole
  - Rare species
Thank you very much for your attention!