Factors influencing the results of metronidazole resistance testing

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Postgraduate Technical Workshop on Clostridium difficile (Maribor, September 2-4, 2015)
Reduced susceptibility to MTZ: an emerging phenotype in *C. difficile*

- Metronidazole is the drug of choice in the treatment of mild-to-moderate CDI.

- Treatment failure is higher with MTZ than VAN (22.4% vs 14.2% p = 0.002)
  Recurrence rate is similar (27.1% vs 24.0%, p = 0.26)
  *Vardacas et al. IJAA 2012, 40:1-8*

- Recently, reduced susceptibility (>2 μg/ml) to MTZ and heteroresistance (have raised) have been reported more frequently in *C. difficile* population
  *Barbut et al.: AAC 1999, 43: 2607-2611*
  *Brazier et al.: JAC  2001, 48: 741-742*
  *Pelaez et al.: AAC 2002, 46:1647-1650*
  *Baines et al.: JAC 2008, 62: 1046-1052*
  *Moura et al: JAC 2013, 68: 362-365*

* Taken over from the presentation of Patrizia Spigaglia 5th ICDS **© by author**
Contradictory data in the EUCAST database:

Metronidazole / Clostridium difficile
International MIC Distribution - Reference Database 2015-08-13

MIC distributions include collated data from multiple sources, geographical areas and time periods and can never be used to infer rates of resistance.

MIC:
Epidemiological cut-off (ECOFF): 2 mg/L
Wildtype (WT) organisms: ≤ 2 mg/L

7842 observations (17 data sources)
Antibiotic susceptibility testing (for routine and research)

- In some cases it is more important for the clinicians than identification
- In *C. difficile* not tested in routine labs
- Quantitative (MIC) - semiquantitative (DD)
- Several factors influence the result
  - Media, inoculum, the age of the culture, incubation time, etc.
  - Inhibition zone breakpoints are set in comparison with the MICs
  - To get comparable results standardization is mandatory
  - In the case of anaerobic bacteria (*C. difficile*) the level of anaerobiosis may be important factor
Antibiotic susceptibility testing methods for *C. difficile*

- **MIC determination**
  - *agar dilution method* (ADM) according to CLSI (M11-A6). BBA supplemented with vitamin K and hemine. Inoculum freshly made $10^5$ cfu/spot *(Hecht et al. AAC 2007, 51: 2716-2719)*
  - *agar incorporation method* (AIM) according to Leeds. Inoculum Schaedlers broth (48 h), Willkins-Chalgren agar $(10^4$ cfu/spot *) *(Freeman et al. JAC 2005, 56: 988-989)*
  - *E-test method* (changed rule how to read the result!!) *(Barbut et al. AAC 1999, 43: 2607-261; Moura et al. JAC 2013, 68: 362-365)*
    - (Spiral-gradient, macro- and micro-broth dilution: have many disadvantages)
- **Disc diffusion**
  - Debated for a long time – now new EUCAST approach
The same anaerobic isolates in the presence of the same amount of antibiotic, but different media (agar dilution method – ADM)

Imipenem 0.125 ug/ml

Different MICs will be seen if the media is not supporting the growth of the isolates.
The principle of the agar dilution method (ADM)

- Antibiotics are incorporated in the solid media in different concentration (doubling dilution). After inoculation, incubation in proper environment.

MIC: 0.5

MIC: 1

0.25 μg/ml  0.5 μg/ml  1 μg/ml
MIC determination by E-test methodology

The amount of the inoculum is not influencing the MIC

The diffusion time before incubation is not influencing the MIC

Not completely true for anaerobes!!!

(AB Biodisc)
C. difficile testing require proper oxygen free environment

- (“Fortner” and roll-tube methods) not used any more
- Anaerobic chambers
- Others: GasPacks, plastic boxes and bags, Anoxomat
C. difficile testing require proper oxygen free environment

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**C. difficile** testing require proper oxygen free environment

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C. perfringens (ATCC13124) spore suspension was used
- Metronidazole 5µg/disc (Oxoid)
- Incubation for 24 h
- The level of the oxygen was measured by the Anoxomat jar system

In conclusion we present an inexpensive, simple, sensitive and reproducible quality control method of the anaerobic atmosphere. With the described method a metronidazole zone diameter above 27 mm would indicate acceptable anaerobic conditions for the culture of anaerobic bacteria.
How can the reduced activity of metronidazole be used to test the level of oxygen?


- The killing action of MTZ requires the reduction of the nitro group, which is needed for the drug to enter the susceptible cell involving ferredoxin-linked reaction. The reduced agent causes strand breakage of DNA and inhibit DNA repair mechanisms.

- In the presence of oxygen in the cultures the redox potential raises to positive values, drug reduction does not occur and MIC values will increase erroneously, indicating the presence of resistant organism.

- This was proven not only for *C. perfringens* but for other anaerobic bacteria
Antibiotic susceptibility testing of *C. difficile* by disc diffusion

- EUCAST aproche to use DD

- 217 *C. difficile* recent isolates
- E-test MIC versus inhibition zone
- 4 isolates with "elevated" MICs (1.5-3 μg/ml)
- BB agar (supplemented) with 1.0 McFarland inc.
- 5 μg metronidazole disc
- Incubation in anaerobic chamber for 48 h
Antibiotic susceptibility testing of *C. difficile* by disc diffusion

- EUCAST aproche to use DD


Antimicrobial susceptibility testing of *Clostridium difficile* using EUCAST epidemiological cut-off values and disk diffusion correlates.

Erikstrup LT¹, Danielsen TK, Hall V, Olsen KE, Kristensen B, Kahlmeter G, Fuurstved K, Justesen US.

Zone diameter breakpoint for metronidazole susceptible strains (wild-type strains) was suggested to be ≥23 mm with these standard method.

Major error (false resistance) with DD method was <1.4%!!
Our aim was to evaluate the DD method for *C. difficile* isolates including more isolates with elevated MICs (ESGAI & ESGCD)

- 64 *C. difficile* clinical isolates from a Europe-wide study (Leeds) belonging to 28 different ribotypes including 24 strains with elevated MICs for MTZ (4 and 8 μg/ml)
- 188 recent Hungarian isolates, which were tested for MICs of 5 antibiotics (Eitel et al. Anaerobe 2015, 31: 47-49)
- The strains were kept in -80°C till testing.
- MIC determination of MTZ was carried out in Leeds (AIM) for the 64 strains (Baines et al. JAC 2008; Moura et al. 2012)
- DD method (+ E test) in Szeged and in Vaxjö (Erikstrup et al. CMI 2012, 18: E266-272)
<table>
<thead>
<tr>
<th>Zone diameter (mm)</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibition zone &lt;27 mm</td>
<td>35</td>
</tr>
<tr>
<td>(cut off for reduced susceptibility for MTZ)</td>
<td></td>
</tr>
</tbody>
</table>

- 188 recent clinical isolates from Szeged and 64 selected strains from Leeds
- Media: BBA supplemented with vitamin K1 and haemin
- 1 McFarland inoculum from a 48 h subculture
- 5 μg MTZ disc, incubation for 24 h anaerobic Chamber
Distribution of zone diameters of MTZ for 252 *C. difficile* isolates

- 188 recent clinical isolates from Szeged and 64 selected strains from Leeds
- Media: BBA supplemented with vitamin K1 and haemin
- 1 McFarland inoculum from a 48 h subculture
- 5 μg MTZ disc, incubation for 24 h anaerobic Chamber
Comparison of MIC data (Leeds) and zone diameters (Szeged) for 64 \textit{C. difficile} strains obtained from Leeds

Many discrepant results during first testing with very major error (false susceptible with DD method) !!!
<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>PCR-ribotype</th>
<th>Zone diameter MET 5 ug/ disc</th>
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<td></td>
<td></td>
<td>1st measurement</td>
<td>2nd measurement</td>
<td>Metronidazole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MIC Leeds</td>
</tr>
<tr>
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<td>32</td>
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<td>H61</td>
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<td>8</td>
</tr>
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</table>
Metronidazole *C. difficile* H60 (Ribotype 106)

- Leeds: AIM MIC: 8 μg/ml
- Szeged: Etest MIC: 0.25 μg/ml
- DD (2 separate measurements)
  - 35 mm / 35 mm
Metronidazole *C. difficile* H44 (Ribotype 027)

- **Leeds:** AIM MIC: 4 μg/ml
- **Szeged:** Etest MIC: 0.25 μg/ml
- **DD (2 separate measurements):** 36 mm / 37 mm
Metronidazole *C. difficile* H44 (Ribotype 027) Wilkins-Chalgreen agar

Leeds: AIM MIC: 4 μg/ml  
Szeged: Etest MIC: 0.25 μg/ml 
Etest MIC WC agar: 0.125 μg/ml

DD (2 separate measurements)  
36 mm / 37 mm 
BB WC agar: 42 mm
## Comparative MTZ data with different methods

<table>
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<th>Number of the strains</th>
<th>Ribotype</th>
<th>MTZ DD, Szeged</th>
<th>MTZ MIC Leeds</th>
<th>MTZ MIC AIM</th>
<th>MTZ MIC Szeged</th>
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<td>(ug/ml)</td>
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<td>4</td>
<td>1</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* very week growth on the control plates
**C. difficile**

Strain 44

MIC 4/0.25

Anaerobic chamber

Gaspack system

*Erika Matuschek Vaxjö*
Contradictory effect of presence of oxygen for the metronidazol sensitivity testing of *C. difficile*

- Presence of small amount of oxygen influences zone diameters (MICs) of metronidazole (smaller inhibition zone, higher MIC) in the case of *C. difficile* (false resistance)

- Presence of small amount of oxygen (or incomplete media) influences growth of *C. difficile* (lower MIC, bigger inhibition zone) showing higher susceptibility
Possible explanations for these discrepant results

- Deep freezing the *C. difficile* strains may lead to losing the elevated metronidazole MICs??? (Lynch T et al. PLOSone 2013)

- Heteroresistance of clinical isolates exposed to low level metronidazole in the gut, will be lost during passages on drug free media. (Paláez et al. JCM 2008)

- *Take care on the level of anaerobiosis if you want to test MTZ susceptibility*
To summaries

- Factors influencing the results of metronidazole resistance testing of anaerobic bacteria (C. difficile)
  - Composition of media for proper growth
  - Provide the lowest oxygen level as possible
  - Agar dilution method (CLSI) should be used for mass testing (research)
  - E-test or disc diffusion (EUCAST) for routine single strain testing
Using many different criteria to assess antimicrobial resistant diseases *C. difficile* is ranked in the „High Priority Group“ in Canada together with ESBL-producing and carbapenem resistant Enterobacteriaceae and MRSA.