ANTIMICROBIAL SUSCEPTIBILITY TESTING: DILUTION METHODS

Luis Martínez Martínez
Department of Molecular Biology
University of Cantabria
Service of Microbiology
Hosp. Univ. Marqués de Valdecilla
Santander, Spain
Antibiogram: Methods

1. Phenotypic assays
   (Direct bacteria-antimicrobial agent interaction)
   Diffusion methods: Disk assay
   Gradient diffusion
   Dilution methods:
   Broth (macro- & microdilution)
   Solid medium (agar)
   (Semi)-automatic methods

2. Detection of biochemical mechanisms of resistance

3. Detection of resistance genes
MIC
The lowest concentration OF ANTIMICROBIAL AGENT (in mg/l) inhibiting visible growth of the tested microorganism under standard conditions.

MBC
The lowest concentration OF ANTIMICROBIAL AGENT (in mg/l) killing 99.9%* of the initial inoculum of the tested microorganism under standard conditions.

* [99%?]
MIC:

Translated into CLINICAL CATEGORIES by using BREAKPOINTS

S, I, R
The European Committee on Antimicrobial Susceptibility Testing - EUCAST

Clinical breakpoints

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is a standing committee jointly organized by ESCMID, ECDC and European national breakpoint committees. EUCAST deals with breakpoints and technical aspects of phenotypic in vitro antimicrobial susceptibility testing and functions as the breakpoint committee of EMA and ECDC. EUCAST does not deal with antibiotic policies, surveillance or containment of resistance or infection control. The Steering Committee is the decision making body. It is supported by a General Committee with representatives from European and other countries, FESC and ISC. The Steering Committee also consults on EUCAST proposals with experts within the fields of infectious diseases and microbiology, pharmaceutical companies and susceptibility testing device manufacturers.

EUCAST has a subcommittee on antifungal susceptibility testing and on methods for detection of resistance mechanisms of clinical and/or epidemiological importance.

Subcommittees on expert rules for antimicrobial susceptibility testing and antimicrobial susceptibility testing of anaerobes have completed their tasks and
Clinical breakpoints

Clinical breakpoints are for everyday use in the clinical laboratory to advise on patient therapy. In EUCAST tables, the I-category is not listed. It is implied as the values between the S-breakpoint and the R-breakpoint.

For a breakpoint listed as $S \geq 1$ mg/L and $R > 8$ mg/L, the intermediate category is 2 - 8 (technically $>1 - 8$) mg/L.

For a breakpoint listed as $S \geq 22$ mm and $R < 18$ mm, the intermediate category is 18 - 21 mm.

- Clinical breakpoints - bacteria (v 2.0) - pdf file for printing (2012-01-01)
- Clinical breakpoints - bacteria (v 2.0) - Excel file for screen (2012-01-01)

A new set of tables for antibacterials were uploaded on Feb 23, 2012, only because all links to MIC distributions were updated because the database was moved.

- Ceftaroline breakpoints, Addendum Sept 11, 2012
# Breakpoint tables for interpretation of MICs and zone diameters

Version 2.0, valid from 2012-01-01

<table>
<thead>
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<tbody>
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<td>Gram-negative anaerobes</td>
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<td><em>Helicobacter pylori</em></td>
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<td><em>Listeria monocytogenes</em></td>
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<td>Non-species related breakpoints</td>
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</table>
Enterobacteriaceae

**EUCAST Clinical Breakpoint Table v. 2.0, valid from 2012-01-01**

**Disk diffusion (EUCAST standardised disk diffusion method)**
- **Medium:** Mueller-Hinton agar
- **Incubation:** 35±1°C, 18-24h
- **Reading:** Read zone edges as one point showing no growth viewed from the back of the plate against a black background illuminated with reflected light.
- **Quality control:** Escherichia coli ATCC 25922

### Penicillins¹

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<th>MIC breakpoint (mg/L)</th>
<th>Disk content (µg)</th>
<th>Zone diameter breakpoint (mm)</th>
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<td>S ≤ R &gt;</td>
<td>S ≥ R &lt;</td>
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#### Benzylpenicillin

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#### Ampicillin

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#### Amoxicillin

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#### Amoxicillin-clavulanate

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#### Piperacillin

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#### Piperacillin-tazobactam

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#### Ticarillin

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</tbody>
</table>

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¹ Wild type Enterobacteriaceae are categorised as susceptible to amoxicillin. Some countries prefer to categorise wild type isolates of *E. coli* and *P. mirabilis* as intermediate. When this is the case, use the MIC breakpoint $S \leq 0.5$ mg/L and the corresponding zone diameter breakpoint $S \geq 50$ mm.

² Tangent growth that may appear as a thin inner zone on some batches of Mueller-Hinton agar.

3 For susceptibility testing purposes, the concentration of clavulanate is fixed at 2 mg/L.

4 For susceptibility testing purposes, the concentration of tazobactam is fixed at 4 mg/L.
Antimicrobial wild type distributions of microorganisms

MIC- and Inhibition zone diameter distributions of microorganisms without and with resistance mechanisms

MIC distributions
The website gives MIC distributions for individual organisms and antimicrobial agents in tables and histograms. The distributions are based on collated data from an increasing total of more than 20 000 MIC distributions from worldwide sources. Unless otherwise specifically stated, the data are representative of results obtained with a variety of MIC methods. Different methods do not give exactly the same results but the results rarely vary by more than one doubling dilution step. In this way the aggregated MIC distributions encompass the variation between different investigators and between different methods.

Inhibition zone diameter distributions
The website gives inhibition zone diameter distributions for individual organisms and antimicrobial agents in tables and histograms. The
**Antimicrobial wild type distributions of microorganisms**

### Search

**Method:**  
- [ ] MIC  
- [ ] Disk diffusion

**Antimicrobial:** Amoxicillin

**Species:** Species...

**Disk content:** Disk

**Antimicrobial:** Amoxicillin (Method: MIC)

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

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Amoxicillin / Escherichia coli
EUCAST MIC Distribution - Reference Database 2012-09-25

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

Epidemiological cut-off: WT ≤ 8 mg/L
Clinical breakpoints: S ≤ 8 mg/L, R > 8 mg/L

5277 observations (20 data sources)
CMI ≈ CMB  BACTERICIDAL DRUG
CMI < CMB  BACTERIOSTATIC DRUG
TOLERANCE

CMB>CMI (AT LEAST 16-32 TIMES)
FOR USUALLY BACTERICIDAL
ANTIMICROBIAL AGENTS

[BUT…. Difficulties to differentiate between Tolerance and Persistence!]
PARADOXICAL EFFECT

DECREASED BACTERIAL DEATH IN INCREASING CONCENTRATIONS OF ANTIMICROBIAL AGENT
DILUTION METHODS

ANTIMICROBIAL AGENTS

- Serial two-fold dilutions
- Pay attention to solvent and diluent, stability, and temperature of conservation of reference powders

INOCULUM

- $10^5$ CFU/ml (usually prepared from a suspension with a turbidity equivalent to McFarland 0.5)

MEDIUM

- Mueller Hinton agar/broth (cation-adjusted). Supplemented as necessary
- Haemophilus Test Medium; Others

INTERPRETATION

- MIC: absence of visible growth by the naked eye (usual incubation: 18h-35°C)
Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution

European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)
MIC determination of non-fastidious and fastidious organisms

The EUCAST recommendations for MIC determination for non-fastidious organisms are in complete agreement with the recommendations from the International Standards Organisation (ISO).

Media preparation

A document to summarise EUCAST methodology for MIC determination of non-fastidious (MH broth) and fastidious (MH broth with lysed horseblood and beta-NAD) organisms (streptococci including S.pneumoniae, H.influenzae, Pasteurella spp and others) is under preparation.

Quality control tables (ranges and targets of QC organisms)

Recommend page
ISO 20776-1:2006
ISO 20776-2:2007

Clinical laboratory testing and in vitro diagnostic test systems -- Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices

Part 1: Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases.

Clinical laboratory testing and in vitro diagnostic test systems — Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices —

Part 1:
Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases

Systèmes d'essais en laboratoire et de diagnostic in vitro — Essais de réceptivité d'agents infectieux et évaluation des performances des dispositifs de réceptivité antimicrobienne —

Partie 1: Méthode de référence pour la détermination de la sensibilité in vitro aux agents microbiens des bactéries aérobies à croissance rapide impliquées dans les maladies infectieuses

Reference number
ISO 20776-1:2009(E)
January 2009

Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Eighth Edition

This document addresses reference methods for the determination of minimal inhibitory concentrations (MICs) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

A standard for global application developed through the Clinical and Laboratory Standards Institute consensus process.
Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement
<table>
<thead>
<tr>
<th>Antibióticos</th>
<th>Concentraciones (µg/ml)</th>
<th>Enterobact.</th>
<th>P. aerug.</th>
<th>Acinet.</th>
<th>Otros</th>
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<tbody>
<tr>
<td>B-lactámicos</td>
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<td>Ac. pipémido [13]</td>
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<td>Microdilution</td>
<td>Agar dilution</td>
<td>T°</td>
<td>Incubation time</td>
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<td>Enterobacteria</td>
<td>CAMHB</td>
<td>MHA</td>
<td>35±2°C</td>
<td>16-20h</td>
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<td><em>P. Aeruginosa</em></td>
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<td><em>Vibrio cholera</em></td>
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<td>Acinetobacter spp.</td>
<td>CAMHB</td>
<td>MHA</td>
<td>35±2°C</td>
<td>24-24h</td>
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<td><em>B. cepacia</em></td>
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<td><em>S. maltophilia</em></td>
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<td>Staphylococcus</td>
<td>CAMHB</td>
<td>MHA</td>
<td>35±2°C</td>
<td>16-20h</td>
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<tr>
<td>CAMHB+2%MgCl2 (OXA)</td>
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<td>(24h, OXA 24 h, VAN)</td>
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<td>CAMHB+50mg/l (DAP)</td>
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<td>Screening: Other media</td>
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<tr>
<td>Enterococcus</td>
<td>CAMHB</td>
<td>MHA</td>
<td>35±2°C</td>
<td>16-20h</td>
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<td>CAMHB+50mg/l (DAP)</td>
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<td></td>
<td>(24h, OXA 24 h, VAN)</td>
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<td>BHIB: VAN/HLAR Screen</td>
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<td>H. influenzae; H. parainfluenzae</td>
<td>HTM broth</td>
<td></td>
<td>35±2°C</td>
<td>24-24h</td>
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Determination of the MIC: Tube Dilution Assay

Antimicrobial Agent (known concentration)

Serial Dilutions

0.1ml 0.1ml 0.1ml 0.1ml 0.1ml 0.1ml 0.1ml 0.1ml

9.9ml growth medium

Tubes are inoculated and incubated.

Growth occurs in those tubes with antibiotic concentrations below the MIC.

MIC (Minimum Inhibitory Concentration)
The lowest concentration of antimicrobial agent needed to inhibit growth.
BROTH MACRODILUTION

MIC = 0.5 mg/l
The concentration of the initial inoculum and the number and volume of samples subcultured are covariant parameters that affect the number of colonies in the sample(s) and, along with the definition of lethality, timing of subculture, and conditions of culture (i.e., temperature, atmosphere, growth phase, etc.), must be accounted for in a test procedure for MLCs.

Intrinsic sampling variability makes it impossible to use as a rejection value the number of colonies that represents precisely a 0.999 lethal effect. It was necessary to develop a test procedure that took this into account and also allowed for variability in pipetting. The Poisson distribution, as assessed by a chi-square goodness of fit test, is an acceptable probability model.
MICRODILUTION

Advantages
- MIC and MBC determination
- Easy to perform
- Plates can be prepared in advance, and be conserved at –80ºC
- Low antibiotic powder consumption
- Versatility
- Automation

Inconveniences
- Difficulties to detect contamination
- Intrinsic ± 1 dilution error

Reference Technique for most organisms
AGAR DILUTION

ANTIMICROBIAL AGENT:
- Two-fold dilutions incorporated into the agar medium before it solidifies

INOCULUM:
- Dispensed with a multi-inoculator (Steers)
- $10^4$ CFU/dot

MEDIUM:
- Similar to broth media PLUS ca. 15 g/L of agar

INTERPRETATION:
- Absence of growth on the surface of the plate ($\leq 3-5$ colonies-) (usual incubation: 18h-35°C)
AGAR DILUTION

Control (no antibiotic)

0.25 μg/ml

0.5 μg/ml
AGAR DILUTION

Advantages
Simultaneous study of >= 32 microorganisms
Contaminations are easily detected

Inconveniences
CMB “cannot” be determined
A lot of antibiotic powder is consumed
Intrinsic ± 1 dilution error

CLSI Reference method for:
- Neisseria gonorrhoeae
- Helicobacter pylori
OTHER SUSCEPTIBILITY ASSAYS

- Population analysis
- Post-antibiotic effect
- Antimicrobial combinations
  (checkerboard, killing curves)
- Minimal antibiotic concentration
- Mutant preventing concentration
- Serum bactericidal (inhibitory) assay

[Antibiotic concentration in organic fluids]
Current ESCMID courses and workshops

Hot topics on infections in the critically ill patient
31 May - 01 June 2013, Nafplio, Greece

Technical Workshop for Basic and Clinical Parasitology
17 - 21 June 2013, Porto, Portugal

Antifungal Resistance, and its Challenges in the Management of Invasive Fungal Infections
20 - 22 June 2013, Sibiu, Romania

Mechanisms of Antimicrobial Resistance: A Practical Approach
23 - 28 June 2013, Palma de Mallorca, Spain

Intracellular bacteria: From biology to clinic
26 - 30 August 2013, Villars-sur-Ollon, Switzerland

Meningitis Update 2013
2 - 4 September 2013, Izmir, Turkey

Bioinformatic Tools in Clinical Microbiology
9 - 12 September 2013, Santander, Spain