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)

INTRODUCTION

In vitro susceptibility tests are performed on microorganisms suspected of causing disease, particularly if the organism is thought to belong to a species that may exhibit resistance to frequently used antimicrobial agents. The tests are also important in resistance surveillance, epidemiological studies of susceptibility and in comparisons of new and existing agents.

Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing. MIC methods are used in resistance surveillance, the comparative testing of new agents, to establish the susceptibility of organisms that give equivocal results in disc tests, for tests on organisms where disc tests may be unreliable and when a quantitative result is required for clinical management.

In dilution tests microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) or in broth (broth dilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent (in mg/L) that, under defined in vitro conditions, prevents the appearance of visible growth of a microorganism within a defined period of time, is known as the MIC. The MIC is a guide for the clinician to the susceptibility of the organism to the antimicrobial agent and aids treatment decisions. Careful control and standardization is required for intra- and interlaboratory reproducibility as results may be significantly influenced by the method used. It is generally accepted that broth MIC tests are reproducible to within one doubling dilution of the real end point (i.e. ± one well or tube in a dilution series).

Broth dilution is a technique in which containers holding identical volumes of broth with antimicrobial solution in incrementally (usually geometrically) increasing concentrations are inoculated with a known number of bacteria.

Broth microdilution denotes the performance of the broth dilution test in microdilution plates with a capacity of ≤500 μL per well.

The methods described in this document are intended mainly for the testing of pure cultures of aerobic bacteria that are easily grown by overnight incubation on agar and which grow well in Mueller-Hinton broth with minimal supplementation. The broth dilution methods described in this document are essentially the same as those used in many countries including France [1], Germany [2], Sweden [3], the UK [4], and the United States [5]. All these methods are based on those described by Ericsson and Sherris [6].

MEDIUM

Cation-supplemented Mueller-Hinton broth is the most widely used medium internationally for broth dilution methods. It allows good growth of most nonfastidious pathogens and is generally low in antagonists. Mueller-Hinton broth which meets the requirements of the NCCLS standard [5] is considered the reference medium.

Supplements should not be used unless necessary for growth of the test organism. Different supplements have been used for fastidious organisms and comparative data on performance are needed. Defibrinated or lyzed blood (LB) is commonly added at 3–5% v/v for fastidious streptococci. LB at 3–5% and 20 mg/L β-NAD are commonly added for *Haemophilus* spp.

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ANTIMICROBIAL AGENTS

Pure antimicrobial powders must be obtained directly from the manufacturer or from commercial sources. The agent must be supplied with a lot number, potency (μg or International Units (IU) per mg powder, or as a percentage active ingredient), an expiry date and details of recommended storage conditions. Store powders in sealed containers in the dark at 4 °C with a desiccant unless otherwise recommended by the manufacturer. Ideally, hygroscopic agents should be dispensed into aliquots, one of which is used on each test occasion. Allow containers to warm to room temperature before opening them to avoid condensation of water on the powder.

Preparation of stock solutions

Use a calibrated analytical balance to weigh antimicrobial agents. Allowance for the potency of the powder can be made by use of the following formula:

$$\text{Weight of powder (mg)} = \frac{\text{Volume of solution (mL)} \times \text{Concentration (mg/L)}}{\text{Potency of powder (mg/g)}}$$

Alternatively, given a weighed amount of antimicrobial powder, the volume of diluent needed may be calculated from the formula:

$$\text{Volume of diluent (mL)} = \frac{\text{Weight (mg)} \times \text{Potency (mg/g)}}{\text{Concentration (mg/L)}}$$

Concentrations of stock solutions should be 1000 mg/L or greater, although the solubility of some agents will be limiting. The actual concentrations of stock solutions will depend on the method of preparing working solutions. Agents should be dissolved and diluted in sterile distilled water unless the manufacturer states otherwise. Some agents require alternative solvents (Table 1).

Sterilization of solutions is not usually necessary. If required, sterilization should be by membrane filtration, and samples before and after sterilization should be compared by assay to ensure that adsorption has not occurred. Unless otherwise instructed by the manufacturer, stock solutions may be stored in aliquots at −20 °C or below. Most agents will keep at −60 °C for at least 6 months. Use stock solutions promptly on defrosting and discard unused solutions.

Preparation of working solutions

The range of concentrations selected for testing will depend on the organisms and antimicrobial agent. For full-range MIC testing around 12 dilutions are commonly tested. This may be reduced to 5–8 dilutions to cover a therapeutically achievable range for each agent. Breakpoint MIC testing represents a modification of the method where the concentrations of the agent tested are restricted to the breakpoint concentrations distinguishing the different categories of susceptibility (i.e. Susceptible, Intermediate or Resistant). A two-fold dilution series up and/or down from 1 mg/L is conventionally used, with the dilutions prepared in the broth.

Broth dilution

Table 2 illustrates one method of preparing these dilutions. A minimum of 1 mL of each dilution per tube or vial is required for the test. Since the antimicrobial agent is usually diluted in sterile broth then mixed with broth inoculated with microorganisms, dilutions are prepared at twice the desired final concentration.

Broth microdilution

Small volumes (≤500 μL) are dispensed into the wells of microwell plates. Antimicrobial solutions may be prepared as described for broth dilution and dispensed into the plates or produced by dilution with broth in the microwell plates themselves. Plates are immediately sealed in plastic bags and frozen at or below −20 °C. A number of commercial manufacturers offer microdilution plates with agents dried into the wells which, after reconstitution with broth (usually 50 to 200 μL), provide selected concentrations of agents. These plates must be stored as recommended by the manufacturer.

PREPARATION OF INOCULUM

Standardization of inoculum is vital for accurate and reproducible susceptibility testing. The inoculum may be prepared by diluting a broth culture or by emulsifying overnight colonies from an agar medium in broth or saline. For either method four or five colonies of a pure culture on agar are chosen to avoid selecting an atypical variant.

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Table 1 Solvents and diluents for making stock solutions of antimicrobial agents requiring solvents other than water

Antimicrobial agent	Solvent	Diluent
Amoxycillin	Phosphate buffer 0.1 M, pH 6.0	Phosphate buffer 0.1 M, pH 6.0
Ampicillin	Phosphate buffer 0.1 M, pH 8.0	Phosphate buffer 0.1 M, pH 6.0
Azithromycin	Ethanol 95%	Water
Aztreonam	Saturated sodium bicarbonate solution	Water
Cefepime	Phosphate buffer 0.1 M, pH 6.0	Phosphate buffer 0.1 M, pH 6.0
Cefpodoxime	0.1% sodium bicarbonate solution	Water
Ceftazidime	Saturated sodium bicarbonate solution	Water
Cephalothin	Phosphate buffer 0.1 M, pH 6.0	Water
Chloramphenicol	Ethanol 95%	Water
Clarithromycin	Methanol	0.1 M phosphate buffer, pH 6.5
Clavulanic acid	Phosphate buffer 0.1 M, pH 6.0	Phosphate buffer 0.1 M, pH 6.0
Erythromycin	Ethanol 95%	Water
Fusidic acid	Ethanol 95%	Water
Imipenem	Phosphate buffer 0.01 M, pH 7.2	Phosphate buffer 0.01 M, pH 7.2
Levofloxacin	Half volume water, a minimum volume 1 M NaOH to dissolve, then make up to total volume with water	Water
Meropenem	Phosphate buffer 0.01 M, pH 7.2	Phosphate buffer 0.01 M, pH 7.2
Naladixic acid	Half volume water, a minimum volume 1 M NaOH to dissolve then make up to total volume with water	Water
Nitrofurantoin	Minimum volume dimethylformamide to dissolve, then make up to total volume with phosphate buffer 0.1 M, pH 8.0	Phosphate buffer 0.1 M, pH 8.0
Norfloxacin	Half volume of water, a minimum volume 1 M NaOH to dissolve, then make up to total volume with water	Water
Ofloxacin	Half volume water, a minimum volume 1 M NaOH to dissolve, then make up to total volume with water	Water
Rifampicin	Methanol	Water
Sulbactam	Phosphate buffer 0.1 M, pH 6.0	Phosphate buffer 0.1 M, pH 6.0
Sulfonamides	Half volume water, a minimum volume 1 M NaOH to dissolve, then make up to total volume with water	Water
Ticarcillin	Phosphate buffer 0.1 M, pH 6.0	Phosphate buffer 0.1 M, pH 6.0
Trimethoprim	Half volume water, a minimum volume 0.1 M lactic acid or 0.1 M HCl to dissolve, then make up to a total volume with water.	Water

Table 2 Preparation of dilutions of agents for use in broth dilution susceptibility tests. Modified from Ericsson and Sherris [6]

Antimicrobial concentration (mg/L) in stock solution	Volume stock solution (mL)	Volume broth* (mL)	Antimicrobial concentration obtained (mg/L)
5120	1	9	512
512	1	1	256
512	1	3	128
512	1	7	64
64	1	1	32
64	1	3	16
64	1	7	8
8	1	1	4
8	1	3	2
8	1	7	1
1	1	1	0.5
1	1	3	0.25
1	1	7	0.125

*Broth used for dilution is that used in the susceptibility test. Any supplementation must take place before diluting the antimicrobial agent to maintain the required concentrations.

Broth culture method

The colonies are touched with a loop and the growth transferred to broth such as tryptic soy broth or brain heart infusion. The broth used must not be antagonistic to the agent tested. The broth is incubated at 35–37 °C until the growth reaches a turbidity equal to or greater than that of a 0.5 McFarland standard. The culture is adjusted with sterile distilled water, saline or broth or to give a turbidity equivalent to the McFarland 0.5 standard. This can be done photometrically (using 625 nm and a 1-cm path the absorbance will be 0.08–0.10) or, using good light, by visually comparing the appearance of black lines through the inoculum and McFarland standard suspensions (the inoculum and McFarland standard must be in the same sized tubes). Alternatively, one of the commercially available nephelometers may be used.

Colony suspension method

Cultures must be less than 30 h old. The colonies are touched with a loop and the growth transferred to sterile broth or saline. The suspension is adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard, as described above for the broth culture method.

For all organisms the precise concentration of cells in the final inoculum will depend on the state of the culture. This effect is most pronounced for fastidious organisms such as *Haemophilus* spp. and *Streptococcus pneumoniae* where use of older cultures can significantly reduce the number of viable cells in the suspension. A correctly adjusted suspension prepared by either method will contain approximately 1.5×10^8 cfu/mL for the most commonly isolated organisms. Alternative methods that reliably produce the correct inoculum are also acceptable. Laboratories should ensure the accuracy of the method chosen. Plates or tubes must be inoculated within 30 min of standardizing the inoculum to maintain viable cell density.

The inoculum prepared above is diluted in broth to give a final organism density of 5×10^5 cfu/mL (range $3–7 \times 10^5$ cfu/mL). The dilution required depends on the method being used for testing, e.g. transfer of 0.1 mL of organism suspension to a tube containing 9.9 mL of broth will give an inoculum density of 1×10^6 cfu/mL which, when mixed with an equal volume of antimicrobial solution in tubes or wells will result in a final inoculum of 5×10^5 cfu/mL. To produce an inoculum containing 5×10^5 cfu/mL for direct inoculation of commercially prepared dried panels, transfer 50 μL of the 0.5 McFarland organism suspension to 10 mL of broth. *S. pneumoniae* has been found to require transfer of 100 μL of the 0.5 McFarland equivalent suspension to 10 mL of broth to achieve a final inoculum of 5×10^5 cfu/mL.

INOCULATION OF TUBES OR MICRODILUTION PLATES

A volume of bacterial suspension (see Preparation of inoculum) equal to the volume of diluted antimicrobial solution (see Antimicrobial agents) is added to each tube or well of antimicrobial agent. Commercial inoculators are available for inoculation of microdilution plates. Commercially available dried panels usually require reconstitution with 50 μL to 200 μL per well of the organism in suitable broth. If using one of these systems, follow the manufacturer's instructions.

Periodically viable counts should be performed on inoculum suspensions to ensure that inocula contain approximately 5×10^5 cfu/mL. This may be done by removing 10 μL from the growth control well or tube immediately after inoculation and diluting it in 10 mL of broth or saline. 100 μL of this dilution is spread over the surface of a suitable agar plate which is then incubated overnight. Fifty colonies would be expected from an original inoculum of 5×10^5 cfu/mL.

It is recommended that a purity check is performed on the inoculum by plating a sample on to a nonselective agar plate and incubating overnight.

INCUBATION OF TUBES AND MICRODILUTION PLATES

Macrodilution tubes must be capped and microdilution plates sealed in polythene bags or fitted with a tight lid or adhesive seal before incubation in order to prevent desiccation.

Incubate tubes or plates at 35–37 °C in air for 16–20 h for most antimicrobial agent/organism combinations. Incubation time should be extended to 24 h when testing *Haemophilus* spp. and streptococci and before interpreting results from suspected oxacillin resistant staphylococci or

Table 3 Target MICs (mg/L) for control organisms in cation adjusted Mueller-Hinton broth

Antimicrobial agent	*Escherichia coli* ATCC 25922 NCTC 12241 CIP 76.24 DSM 1103	*Escherichia coli* ATCC 35218 DSM 5564	*Pseudomonas aeruginosa* ATCC 27853 NCTC 12934 CIP 54.127 DSM 1117	*Staphylococcus aureus* ATCC 29213 NCTC 12973 CIP 103429 DSM 2569	*Enterococcus faecalis* ATCC 29212 NCTC 12697 CIP 103214 DSM 2570
Amikacin	1	–	4	2	128
Amoxycillin	8	–	–	0.5	–
Amoxycillin/ clavulanic acid	4/2	8/4	–	–	–
Ampicillin	4	–	–	0.5	1
Ampicillin/sulbactam	4/2	16/8	–	–	–
Azithromycin	–	–	–	1	–
Aztreonam	0.125	–	4	–	–
Carbenicillin	8	–	32	4	32
Cefamandole	0.5	–	–	0.5	–
Cefazolin	2	–	–	0.5	–
Cefepime	0.03	–	2	2	–
Cefixime	0.5	–	–	16	–
Cefonicid	0.5	–	–	2	–
Cefoperazone	0.25	–	4	2	16
Cefotaxime	0.125	–	8	2	–
Cefotetan	0.125	–	–	8	–
Cefoxitin	2	–	–	2	–
Cefpodoxime	0.5	–	–	–	–
Ceftazidime	–	–	–	8	–
Ceftizoxime	0.06	–	32	4	–
Ceftriaxone	0.06	–	16	2	–
Cefuroxime	4	–	–	1	–
Cephalexin	8	–	–	–	–
Cephalothin	8	–	–	0.25	16
Chloramphenicol	4	–	–	4	8
Ciprofloxacin	0.008	–	0.5	0.25	0.5
Clinafloxacin	0.004	–	0.25	0.016	0.06
Clindamycin	–	–	–	0.125	8
Doxycycline	1	–	–	–	4
Erythromycin	–	–	–	0.25	2
Fosfomycin + G6P[a]	1	–	–	1	–
Fusidic acid	–	–	–	–	2
Gentamicin	0.5	–	2	0.25	8
Imipenem	0.125	–	2	0.03	1
Kanamycin	2	–	–	2	32
Levofloxacin	0.016	–	–	0.125	0.5
Linezolid	–	–	–	2	2
Loracarbef	1	–	–	1	–
Meropenem	0.016	–	0.5	0.06	4
Moxalactam	0.25	–	16	8	–
Mupirocin	–	–	–	0.125	–
Nalidixic acid	2	–	–	–	–
Netilmicin	0.5	–	4	0.25	8
Nitrofurantoin	8	–	–	16	8
Norfloxacin	0.06	–	2	1	2
Ofloxacin	0.03	–	–	–	2
Oxacillin[b]	–	–	–	0.25	16
Penicillin	–	–	–	0.5	2
Piperacillin/ tazobactam	2/4	1/4	2/4	0.5/4	2/4
Piperacillin	2	–	2	2	2
Pipemidic acid	1	–	–	–	–
Quinupristin/ dalfopristin	–	–	–	0.5	4

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Table 3 continued

Antimicrobial agent	*Escherichia coli* ATCC 25922 NCTC 12241 CIP 76.24 DSM 1103	*Escherichia coli* ATCC 35218 DSM 5564	*Pseudomonas aeruginosa* ATCC 27853 NCTC 12934 CIP 54.127 DSM 1117	*Staphylococcus aureus* ATCC 29213 NCTC 12973 CIP 103429 DSM 2569	*Enterococcus faecalis* ATCC 29212 NCTC 12697 CIP 103214 DSM 2570
Rifampicin	16	–	32	0.016	2
Streptomycin	8	–	–	–	–
Sulfisoxazole	16	–	–	64	64
Teicoplanin	–	–	–	0.5	0.125
Tetracycline	2	–	16	0.5	16
Ticarcillin	4	–	16	4	32
Ticarcillin/clavulanic acid	4/2		16/2	1/2	32/2
Tobramycin	0.5	–	1	0.25	16
Trimethoprim	1	–	–	2	<1
Trimethoprim/ sulfamethoxazole	<0.5/9.5	–	16/304	<0.5/9.5	<0.5/9.5
Vancomycin	–	–	–	1	2

ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA.
NCTC, National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT.
CIP, Collection de l'Institut Pasteur, 25–28 Rue du Docteur Roux, 75724 Paris Cedex 15, France.
DSM, Deutsche Stammsammlung fûr Mikroorganismen und Zellkulturen, Mascheroder Weg 16, D-38124 Braunschweig, Germany.
[a]For fosfomycin, broth must be supplemented with 25 mg/L glucose-6-phosphate. Agar dilution is the method of choice for this agent.
[b]*S. aureus* ATCC 43300 may be used to quality control detection of oxacillin resistant staphylococci. The oxacillin MIC should be >2 mg/L.

glyco- peptide resistant enterococci. Incubation at 30–35 °C will increase the likelihood of detection of oxacillin resistance and should be used if testing oxacillin susceptibility.

In order to avoid uneven heating do not stack microdilution plates more than four high. Plates and tubes should not be incubated in a CO_2-enriched atmosphere.

READING RESULTS

Results must only be read when there is sufficient growth of the test organism (i.e. obvious button or definite turbidity in the positive growth control), no growth in the uninoculated or negative growth control (where present) and when a purity plate shows that the test organism was pure. The amount of growth in each tube or well is compared with that in the positive growth control and the MIC recorded as the lowest concentration of the agent that completely inhibits growth, or, for sulfonamides, the lowest concentration that inhibits 80% of growth. When testing glycopeptide susceptibility of enterococci, any faint turbidity should be classified as growth.

TESTING FASTIDIOUS ORGANISMS

Supplementation of the growth medium is necessary as described above. When testing fastidious organisms an appropriate quality control organism must also be tested.

OXACILLIN MICS ON STAPHYLOCOCCI

Resistance to oxacillin can be difficult to detect. The following conditions will aid detection of resistant strains:

1. Incorporation of NaCl at a final concentration of 2% w/v in the broth.
2. Use of the direct suspension method for preparing bacterial suspensions rather than the growth method.
3. Incubation of tests for a full 24 h.
4. Incubation temperature of not more than 35 °C.

TESTS ON β-LACTAMASE-PRODUCING ORGANISMS

When testing organisms that produce extracellular β-lactamases against penicillins and cephalospor-

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ins, the MIC may be significantly affected by the density of inoculum. The standard inoculum may result in MICs only slightly higher than obtained with susceptible strains. β-Lactamase production can be more reliably detected in *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Moraxella catarrhalis* by definitive tests such as nitrocefin-based techniques. Among Gram-negative organisms the effect of inoculum depends on the amount of enzyme produced, and the activity of the enzyme against the particular penicillin or cephalosporin under test. Effective standardization of the inoculum is necessary to avoid large variations in MIC results.

QUALITY CONTROL

The quality of test results is monitored by the use of control strains (Table 3). Working cultures of control strains may be stored on digest agar slopes and subcultured weekly. These cultures should be replaced monthly from lyophilized or frozen stocks (stored at −60 °C or below).

A relevant QC strain should be tested every day that specimen testing is carried out. All strains should be run when there is a change in the batch of broth or test panel.

Test colonies of control cultures in the same way as routine cultures. MICs of control organisms should be within one twofold dilution of the values given in Table 3. If MICs are used for routine susceptibility testing no more than 5% of quality control results for an antimicrobial agent/organism combination should fall outside the expected ranges and corrective action, such as checking the inoculum density, is required if this limit is exceeded. In addition the following are recommended:

1. Any control without antimicrobial agents must show adequate growth of test and control strains.
2. A sample of inoculum must be plated on suitable agar medium to ensure that it is pure.
3. Occasionally check the inoculum density by performing viable counts.
4. Ensure consistent reading of end points by all staff independently reading a selection of tests.

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