Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution

European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)

INTRODUCTION

Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing against which other methods, such as disk diffusion, are calibrated. MIC methods are widely used in the comparative testing of new agents. In clinical laboratories they are used to establish the susceptibility of organisms that give equivocal results in disk tests, for tests on organisms where disk tests may be unreliable, and when a more accurate 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 microplate wells of broth (broth microdilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is known as the MIC.

The agar dilution method described in this document is based on that described in the report of an international collaborative study of antimicrobial susceptibility testing [1] and is very similar to those described and recommended in many countries, including France [2], Germany [3], Norway [4], Sweden [5], the UK [6] and the USA [7].

MEDIUM

Although several susceptibility testing media are available in Europe, a clear choice for a reference medium remains to be determined. Mueller–Hinton (MH) agar shows no performance advantages over some other media but is probably the most widely used medium internationally, and there is a USA National Committee for Clinical Laboratory Standards (NCCLS) document which describes procedures for evaluating MH agar [8]. MH agar which meets the requirements of the NCCLS standard [8] is considered the reference medium.

Supplements should not be used unless necessary for growth of the organisms. Five per cent defibrinated blood is added for fastidious organisms such as streptococci and *Moraxella catarrhalis*. Several supplements for *Haemophilus* spp. and *Neisseria* spp. have been suggested but evidence of performance is needed. Five per cent defibrinated blood with 20 mg/L NAD, 5% chocolated blood, and supplemented GC agar have been suggested.

ANTIMICROBIAL AGENTS

Obtain antimicrobial powders directly from the manufacturer or from commercial sources. The agent must be supplied with a stated potency (mg or International Units per g powder, or as a percentage potency), 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, and one aliquot 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 an analytical balance when weighing agents. Allowance for the potency of the powder can be made by use of the following formula:

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

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

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

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. Manufacturers' recommendations for solvents and diluents should be followed, but where possible agents should be dissolved and diluted in sterile distilled water. Some agents that require alternative solvents are listed in Table 1. Sterilization of solutions is not usually necessary. If required, sterilization should

Table1 Solvents and diluents for 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
Ceftazidime	Saturated sodium bicarbonate solution	Water
Chloramphenicol	Ethanol 95%	Water
Clavulanic acid	Phosphate buffer 0.1 M, pH 6.0	Phosphate buffer 0.1 M, pH 6.0
Erythromycin	Ethanol 95%	Water
Fluoroquinolones	Half volume water, a minimum volume of 0.1 M NaOH to dissolve, then make up to total volume with water	Water
Fusidic acid	Ethanol 95%	Water
Imipenem	Phosphate buffer 0.01 M, pH 7.2	Phosphate buffer 0.01 M, pH 7.2
Mezlocillin	Methanol	Water
Meropenem	Phosphate buffer 0.01 M, pH 7.2	Phosphate buffer 0.01 M, pH 7.2
Nalidixic acid	Half volume water, a minimum volume of 0.1 M NaOH to dissolve, then make up to total volume with water	Water
Nitrofurantoin	Dimethylformamide	Phosphate buffer 0.1 M, pH 8.0
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 of 0.1 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 of 0.1 M lactic acid or 0.1 M HCl to dissolve, then make up to total volume with water	Water

be by membrane filtration, and samples before and after sterilization must be compared by assay to ensure that adsorption to the membrane has not occurred. Unless otherwise instructed by the manufacturer, store stock solutions frozen in aliquots at −20 °C or below. Most agents will keep at −60 °C for at least 6 months. Stock solutions must be frozen as soon as possible after preparation, used promptly on defrosting and not re-frozen.

Preparation of working solutions

The range of concentrations tested will depend on the organisms and antimicrobial agent being tested, but a two-fold dilution series based on 1 mg/L is conventionally used. Twenty-milliliter volumes of agar are commonly used in 9-cm Petri dishes for agar dilution MICs. Two alternative dilution schemes are given in Tables 3 and 4. Both schemes involve adding 19-mL volumes of molten agar to 1-mL volumes of antimicrobial solution. The more conventional method is based on diluting a 10 240 mg/L stock solution, always measuring 1-mL volumes of antimicrobial solution (Table 2). The other method (Table 3) is based on diluting a 10 000 mg/L stock solution and involves measuring various volumes of antimicrobial solution by the use of high-precision variable-volume micropipettes, which are now widely available. An alternative to the method in Table 3 is to omit the distilled water added to make antimicrobial volumes up to 1 mL and instead to add a variable volume of molten agar to make the total volume 20 mL.

PREPARATION OF PLATES

Prepare agar as recommended by the manufacturer. Allow the sterilized agar to cool to 50 °C in a water-bath. Prepare a dilution series of antimicrobial agents, as above, in 25–30-mL containers. Include a drug-free control. Add 19 mL of molten agar to each container, mix thoroughly, and pour the agar into prelabeled sterile Petri dishes on a level surface. Allow the plates to set at room temperature and dry the plates so that no drops of moisture remain on the surface of the agar. Do not overdry plates.

Plates should not be stored unless the agents have been shown to be stable on storage. Clavulanic acid and carbapenems are particularly unstable.

Table 2 Preparation of dilutions of agents for use in agar dilution susceptibility tests

Antimicrobial concentration (mg/L) in stock solution	Volume stock solution (mL)	Volume distilled water (mL)	Antimicrobial concentration obtained (mg/L)	Final concentration in medium after addition of 19 mL of agar
10 240	1	0	10 240	512
10 240	1	1	5120	256
10 240	1	3	2560	128
2560	1	1	1280	64
2560	1	3	640	32
2560	1	7	320	16
320	1	1	160	8
320	1	3	80	4
320	1	7	40	2
40	1	1	20	1
40	1	3	10	0.5
40	1	7	5	0.25
5	1	1	2.5	0.125
5	1	3	1.25	0.06
5	1	7	0.625	0.03
0.625	1	1	0.3125	0.015
0.625	1	3	0.1562	0.008
0.625	1	7	0.0781	0.004

Table 3 Alternative method of preparation of dilutions of antimicrobial agents for use in agar dilution susceptibility tests

Antimicrobial concentration (mg/L) in stock	Volume stock solution (mL)	Volume distilled water (mL)	Antimicrobial concentration obtained (mg/L)	Final concentration in medium after addition of 19 mL of agar
10 000	1	9	1000	–
10 000	0.1	9.9	100	–
100	1	9	10	–
100	0.1	9.9	1	–
10 000	1.024	0	10 240	512
10 000	0.512	0.488	5120	256
10 000	0.256	0.744	2560	128
10 000	0.128	0.872	1280	64
1000	0.64	0.36	640	32
1000	0.32	0.68	320	16
1000	0.16	0.84	160	8
100	0.8	0.2	80	4
100	0.4	0.6	40	2
100	0.2	0.8	20	1
10	1	0	10	0.5
10	0.5	0.5	5	0.25
10	0.25	0.75	2.5	0.125
10	0.125	0.875	1.25	0.06
1	0.625	0.375	0.625	0.03
1	0.313	0.687	0.3125	0.015
1	0.156	0.844	0.1562	0.008
1	0.078	0.922	0.0781	0.004

PREPARATION OF INOCULUM

Standardize the density of inoculum to give 10^4 colony-forming units (CFU) per spot on the agar. Use four or five colonies of a pure culture to avoid selecting an atypical variant. The inoculum may be prepared by emulsifying overnight colonies from an agar medium or by diluting a broth culture. The broth used must not be antagonistic to the agent tested. A 0.5 McFarland standard may be used for visual comparison to adjust the suspension to a density equivalent to approximately 10^8 CFU/mL. Alternatively, inocula can be adjusted photometrically. Dilute the suspensions of organisms in 0.85% saline or broth to give 10^7 CFU/mL. Plates must be inoculated within 30 min of standardizing the inoculum, to avoid changes in inoculum density.

INOCULATION OF PLATES

Mark the plates so that the orientation is obvious. Transfer diluted bacterial suspensions to the wells of an inoculum-replicating apparatus. Use the apparatus to transfer the inocula to the series of agar plates, including a control plate without antimicrobial agent. Replicator pins 2.5 mm in diameter will transfer about 1 μL, i.e. an inoculum of 10^4 CFU/spot. Alternatively, a micropipette or standard loop may be used to inoculate plates. Allow the inoculum spots to dry at room temperature before inverting the plates for incubation.

INCUBATION OF PLATES

Incubate plates at 35–37 °C in air for 18 h. In order to avoid uneven heating, do not stack plates more than five high. If the incubation period is extended for slow-growing organisms, the stability of the agent over the incubation period must be assessed by the inclusion of control strains with known MICs. Avoid incubation in an atmosphere containing 5% CO_2 unless necessary for growth of the organisms (e.g. *Neisseria* spp.). Incubate methicillin/oxacillin susceptibility tests on staphylococci at 30 °C.

READING RESULTS

The MIC is the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot. A trailing endpoint with a small number of colonies growing on concentrations several dilutions above that which inhibits most organisms should be investigated by subculture and retesting. Such trailing endpoints may indicate contamination, resistant variants, β-lactamase-producing organisms, or, if incubation is prolonged, regrowth of susceptible organisms following deterioration of the agent. With sulfonamides and trimethoprim, endpoints may be seen as a reduction in growth, and a haze of growth may be seen at several dilutions above the actual MIC.

FASTIDIOUS ORGANISMS

Testing of fastidious organisms may require supplementation of the medium, enrichment of the incubation atmosphere with 5% CO_2, or extension of the incubation time. The effect of any alterations in test conditions must be assessed by the inclusion of appropriate control organisms.

METHICILLIN/OXACILLIN SUSCEPTIBILITY TESTS ON STAPHYLOCOCCI

A combination of test conditions affects the expression of intrinsic resistance to β-lactam agents in staphylococci. The following apply to tests with staphylococci and all β-lactam agents:

1. Add 2% NaCl to the medium.
2. Incubate tests at 30 °C (note that some coagulase-negative strains do not grow well at 30 °C and may require incubation for 48 h).
3. Incubate tests for 24 h. Continue incubation for a further 24 h for coagulase-negative staphylococci.

Penicillinase hyperproducing strains of *Staphylococcus aureus* may have methicillin/oxacillin MICs raised by one or two two-fold dilution steps compared with other penicillinase-producing strains.

TESTS ON β-LACTAMASE-PRODUCING ORGANISMS

When testing organisms that produce extracellular β-lactamases, the MICs of penicillins and cephalosporins may be markedly affected by the density of inoculum. The standard inoculum may result in MICs only slightly higher than those obtained with susceptible strains. β-Lactamase production can be more reliably detected in staphylococci, gonococci, *Haemophilus influenzae* and *Moraxella catarrhalis* by definitive tests such as nitrocefin-based techniques. Among Gram-negative organisms, the effect of the 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

Routine quality control is achieved by the use of control strains, e.g. as shown in Table 4. Working cultures of control strains may be stored on digest agar slopes and subcultured

Table 4 Target MICs for control organisms on Mueller–Hinton (MH) and Iso-Sensitest (IS) media without blood

Antimicrobial agent	*Escherichia coli* ATCC 25922 NCTC 12241 CIP 76.24 MH (IS) agar	*Escherichia coli* ATCC 10536 NCTC 10418 CIP 54.127 IS agar	*Pseudomonas aeruginosa* ATCC 27853 NCTC 12934 CIP 76.110 (MH) IS agar	*Pseudomonas aeruginosa* ATCC 25668 NCTC 10662 CIP 103837 IS agar	*Staphylococcus aureus* ATCC 29213 NCTC 12973 CIP 103429 MH (IS) agar	*Staphylococcus aureus* ATCC 9144 NCTC 6571 CIP 53.154 IS agar	*Enterococcus faecalis* ATCC 29212 NCTC 12697 CIP 103214 MH (IS) agar
Amikacin	1 (1)	0.5	2 (2)	2	2 (2)	1	128 (128)
Ampicillin	4 (4)	2	–	–	0.5	0.06	1 (1)
Amoxycillin	4 (4)	2	–	–	–	0.125	–(0.5)
Amoxycillin/clavulanic acid	4/2 (4/2)	2/1	–	–	–(0.25/0.125)	0.125/0.06	0.5/0.25 (0.5/0.25)
Azithromycin	–	–	–	–	1 (0.125)	0.125	
Aztreonam	0.125 (0.25)	0.03	4 (2)	4	–	–	–
Carbenicillin	8	2	32	32	4	0.5	32
Cefaclor	2 (2)	1	–	–	2 (1)	1	–
Cefamandole	0.5	–	–	0.5	0.25	–	
Cefazolin	2	–	–	–	0.5	–	–
Cefepime	0.03	–	2 (2)	–	2	–	–
Cefixime	0.5 (0.25)	0.06	–	16	16 (16)	8	–
Cefonicid	0.5	–	–	–	2	–	–
Cefoperazone	0.25	–	4	–	2	–	–
Cefotaxime	0.125 (0.06)	0.03	8 (8)	8	2 (1)	0.5	–(32)
Cefotetan	0.125	0.06	–	–	8	4	–
Cefoxitin	2	4	–	–	2	2	–
Cefpirome	–(0.03)	0.03	–(1)	4	–(0.5)	–	–(16)
Cefpodoxime	0.5 (0.25)	0.25	–	128	2 (2)	0.25	–
Cefsulodin	–	–	–	2	–	–	–
Ceftazidime	0.125 (0.25)	0.06	2 (1)	1	8 (8)	1	–
Ceftizoxime	0.06	0.008	32	–	4	2	–
Ceftriaxone	0.06	0.03	16 (8)	8	2 (2)	–	–
Cefuroxime	4	2	–	–	1 (1)	0.5	–
Cephalexin	8 (8)	4	–	–	4 (4)	1	–
Cephalothin	8 (8)	4	–	–	0.25 (0.25)	0.5	–(16)
Cephradine	–	4	–	–	–	2	–
Chloramphenicol	4	2	–	128	4 (2)	2	8 (4)
Ciprofloxacin	0.008	0.015	0.5 (0.25)	0.25	0.25 (0.5)	0.125	0.5 (1)
Clarithromycin	–	–	–	–	0.25	0.12	–
Clindamycin	–	–	–	–	0.125 (0.06)	0.06	8 (8)
Colistin	1	0.5	0.5	2	–	–	–
Enoxacin	0.125	0.25	4	1	1	0.5	4
Erythromycin	–	–	–	–	0.5 (0.25)	0.125	2 (4)
Fleroxacin	0.06	0.06	2	1	0.5	0.5	4
Fusidic acid	–	–	–	–	–(0.06)	0.06	–(2)
Gatifloxacin	–	0.015	–	1	–	0.03	–

Table 4 continued

Antimicrobial agent	*Escherichia coli* ATCC 25922 NCTC 12241 CIP 76.24 MH (IS) agar	*Escherichia coli* ATCC 10536 NCTC 10418 CIP 54.127 IS agar	*Pseudomonas aeruginosa* ATCC 27853 NCTC 12934 CIP 76.110 (MH) IS agar	*Pseudomonas aeruginosa* ATCC 25668 NCTC 10662 CIP 103837 IS agar	*Staphylococcus aureus* ATCC 29213 NCTC 12973 CIP 103429 MH (IS) agar	*Staphylococcus aureus* ATCC 9144 NCTC 6571 CIP 53.154 IS agar	*Enterococcus faecalis* ATCC 29212 NCTC 12697 CIP 103214 MH (IS) agar
Gentamicin	0.5 (0.5)	0.25	1 (1)	2	0.25 (0.25)	0.125	8 (8)
Imipenem	0.125 (0.125)	0.06	2 (1)	2	0.03 (0.015)	0.015	1 (0.5)
Kanamycin	2 (1)	1	–	1	2	2	32
Levofloxacin	0.016	0.03	1 (0.5)	0.5	0.125 (0.25)	0.125	0.5
Linezolid	–	–	–	–	–	0.5	–
Loracarbef	1 (1)	0.5	–	–	1 (1)	0.5	–
Mecillinam	– (0.125)	0.125	–	8	– (64)	8	–
Methicillin	–	–	–	–	1 (2)	1	–
Meropenem	0.016 (0.008)	0.015	0.5 (0.25)	2	0.06 (0.06)	0.03	4 (2)
Mezlocillin	4	2	16	8	2	0.5	2
Moxalactam	0.25	0.03	16	8	8	8	–
Moxifloxacin	–	0.03	– (2)	2	–	0.06	– (0.25)
Mupirocin	–	–	–	–	– (0.125)	0.25	–
Nalidixic acid	2	2	–	–	– (128)	–	–
Neomycin	–	–	–	32	–	0.125	–
Netilmicin	0.5	0.5	2	1	–	0.25	8
Nitrofurantoin	8	4	–	–	16 (16)	8	8 (8)
Norfloxacin	0.06	0.06	2 (1)	1	1 (1)	0.25	2 (2)
Ofloxacin	0.03	0.06	2 (1)	1	0.25	0.25	2 (2)
Oxacillin	–	–	–	–	0.25 (0.5)	0.25	–
Pefloxacin	–	0.06	–	0.5	–	0.25	–
Penicillin	–	–	–	–	0.5 (0.125)	0.03	2 (2)
Piperacillin	2 (2)	0.5	2 (2)	4	2 (1)	0.25	2 (2)
Piperacillin/tazobactam	2/4	–	2/4	–	0.5/4	–	2/4
Quinupristin/dalfopristin	–	–	–	–	0.5 (0.25)	0.125	4 (1)
Rifampicin	16	16	32	–	0.016 (0.004)	0.004	2 (2)
Sulfisoxazole	16	–	–	–	64	–	64
Sulfamethoxazole	–	16	–	–	–	64	–
Teicoplanin	–	–	–	–	0.5 (0.5)	0.25	0.125 (0.25)
Temocillin	–	2	–	–	–	–	–
Tetracycline	2	1	16 (32)	–	0.5 (0.5)	0.125	16 (16)
Ticarcillin	4	1	16	16	4	0.5	32
Ticarcillin/clavulanic acid	4/2	–	16/2 (16/4)	32/4	1/2	–	32/2
Tobramycin	0.5 (0.5)	0.25	0.5 (0.5)	0.5	0.25 (0.5)	0.125	16
Trimethoprim	1	0.125	–	32	2 (0.5)	0.25	– (0.25)
Trimethoprim/sulfamethoxazole	–	–	–	–	–	–	–
Vancomycin	–	–	–	–	1 (1)	0.5	2 (2)

ATCC is the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA. NCTC is the National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK. CIP is Collection de l'Institut Pasteur, 25–28 Rue du Docteur Roux, 75724 Paris Cedex 15, France.

weekly. Working cultures should be replaced monthly from lyophilized or frozen cultures (at −60 °C or below).

Test colonies of control cultures as recommended for test cultures. Include control cultures with each batch of tests, and test new lots of agar before they are used routinely. In general, MICs of control organisms should be within one two-fold dilution step of the target values given in Table 4. As well as target values for tests on the reference medium, MH agar, target values for control strains tested on Iso-Sensitest agar are included for comparison. In addition:

1. The control plate without antimicrobial agents must show adequate growth of both test and control strains.
2. Plate a sample of inoculum prepared for each strain on a suitable agar medium to ensure that the inoculum is a pure culture.
3. Occasionally check that the method of producing the correct inoculum density is working by counting the number of organisms in the inocula.
4. Check that endpoints are read consistently by all staff independently reading a selection of tests.

REVISIONS

Proposals for revisions of this document should be sent to EUCAST via EUCAST Secretariat, Cornelia Hasselmann, Martin-Buber-Weg 17, D-81245 Munich, Germany (Fax: + 49 89 89712004; E-mail: Cornelia.Hasselmann@t-online.de)

REFERENCES

1. Ericsson H, Sherris JC. Antibiotic sensitivity testing. Report of an international collaborative study. *Acta Pathol Microbiol Scand Sect B* 1971; 217(suppl): 1–90.
2. Comité l'Antibiogramme de la Société Française de Microbiologie. Technical recommendations for in vitro susceptibility testing. *Clin Microbiol Infect* 1996; 2(suppl 1): S11–25.
3. Deutsches Institut für Normung. *Methods for the determination of susceptibility of pathogens (except mycobacteria) to antimicrobial agents. MIC breakpoints of antibacterial agents.* Berlin: DIN, 1998; suppl 1: 58940–4.
4. Norwegian Working Group on Antibiotics. Susceptibility testing of bacteria and fungi. *Scand J Infect Dis* 1997; 103(suppl): 1–36.
5. Olsson-Liljequist B, Larsson P, Walder M, Miörner H. Antimicrobial susceptibility testing in Sweden III. Methodology for susceptibility testing. *Scand J Infect Dis* 1997; 105(suppl): 13–23.
6. BSAC Working Party. A guide to sensitivity testing. *J Antimicrob Chemother* 1991; 27(suppl D):1–50.
7. National Committee for Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*, 5th edn. Approved Standard M7-A5. Wayne, PA: NCCLS, 2000.
8. National Committee for Clinical Laboratory Standards. *Protocols for evaluating dehydrated Mueller–Hinton agar. Approved Standard M6-A*. Wayne, PA: NCCLS, 1996.