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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
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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ISO 20776-1 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 140, In vitro *diagnostic medical devices*, in collaboration with Technical Committee ISO/TC 212, *Clinical laboratory testing and* in vitro *diagnostic test systems*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

ISO 20776 consists of the following parts, under the general title *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*

— *Part 2: Evaluation of performance of antimicrobial susceptibility test devices*

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 procedures are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference method for antimicrobial susceptibility testing. MIC methods are used in resistance surveillance, comparative testing of new agents, to establish the susceptibility of organisms that give equivocal results in routine tests, for tests on organisms where routine 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 serial 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 standardisation is required for intra- and inter-laboratory 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. $\pm$ one well or tube in a doubling dilution series).

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

Broth microdilution denotes the performance of the broth dilution test in microdilution trays.

The method described in this part of ISO 20776 is intended for the testing of pure cultures of aerobic bacteria that are easily grown by overnight incubation on agar and grow well in Mueller-Hinton broth, which may be supplemented. The broth microdilution method described in this part of ISO 20776 is essentially the same as those used in many countries, including France[1], Germany[2], Sweden[3], the United Kingdom[4], and the United States[5]. The method is also essentially the same as the broth microdilution method published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[6]. All these methods are based on those described by Ericsson and Sherris[7].

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

WARNING — The use of this part of ISO 20776 may involve hazardous materials, operations and equipment. This part of ISO 20776 does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this part of ISO 20776 to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This part of ISO 20776 describes one reference method, broth microdilution, for determination of MICs. The MIC reflects the activity of the drug under the described test conditions, and can be interpreted for clinical management purposes by taking into account other factors, such as drug pharmacology or bacterial resistance mechanisms. This allows categorization of bacteria as “susceptible” (S), “intermediate” (I), or “resistant” (R). In addition, MIC distributions can be used to define wild type or non-wild type bacterial populations. Although clinical interpretation of the MIC value is beyond the scope of this part of ISO 20776, modifications of the basic method are required for certain antimicrobial agent - bacteria combinations to facilitate clinical interpretation. These modifications are included in a separate table. It is advisable to compare other susceptibility testing methods (e.g. routine methods or diagnostic test devices) with this reference method for validation, in order to ensure comparable and reliable results.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
antimicrobial agent
substance of biological, semi-synthetic or synthetic origin that inhibits the growth of or kills bacteria, and is thus of potential use in the treatment of infections

NOTE Disinfectants, antiseptics and preservatives are not included in this definition.

2.2 Antimicrobial agents — properties

2.2.1
potency
antimicrobially active fraction of a test substance, determined in a bioassay against a reference powder of the same substance

NOTE The potency is expressed as mass fraction in milligrams per gram (mg/g), or as activity content in International Units (IU) per gram, or as a volume fraction or mass fraction in percent, or as an amount-of-substance concentration (mass fraction) in mole per litre of ingredients in the test substance.

2.2.2
concentration
amount of an antimicrobial agent in a defined volume of liquid

NOTE 1 The concentration is expressed as mg/l.

NOTE 2 mg/l ≡ µg/ml but it is not recommended to use the unit µg/ml.

2.3
stock solution
initial solution used for further dilutions

2.4
minimum inhibitory concentration
MIC
lowest concentration that, under defined *in vitro* conditions, prevents visible growth of bacteria within a defined period of time

NOTE The MIC is expressed in mg/l.

2.5
breakpoint
BP
specific values of parameters, such as MICs, on the basis of which bacteria can be assigned to the clinical categories “susceptible”, “intermediate” and “resistant”

NOTE For current interpretive breakpoints, reference can be made to the latest publications of organizations employing this reference method (e.g. CLSI and EUCAST).

2.5.1
susceptible
S
bacterial strain inhibited *in vitro* by a concentration of an antimicrobial agent that is associated with a high likelihood of therapeutic success

NOTE 1 Bacterial strains are categorized as susceptible by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This breakpoint can be altered due to changes in circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.5.2
intermediate
I
bacterial strain inhibited *in vitro* by a concentration of an antimicrobial agent that is associated with uncertain therapeutic effect

NOTE 1 Bacterial strains are categorized as intermediate by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This class of susceptibility implies that an infection due to the isolate can be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of drug can be used.

NOTE 3 This class also indicates a “buffer zone”, to prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

NOTE 4 These breakpoints can be altered due to changes in circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.5.3
resistant
R
bacterial strain inhibited *in vitro* by a concentration of an antimicrobial agent that is associated with a high likelihood of therapeutic failure

NOTE 1 Bacterial strains are categorized as resistant by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This breakpoint can be altered due to changes in circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.6
wild type
absence of acquired resistance mechanisms to the antimicrobial agent for a given strain

2.7
reference strain
catalogued, characterized bacteria with stable, defined antimicrobial susceptibility phenotypes and/or genotypes

NOTE Reference strains are kept as stock cultures, from which working cultures are derived. They are obtainable from culture collections and used for quality control.

2.8 Susceptibility testing method

2.8.1
broth dilution
technique in which containers are filled with appropriate volumes of an antimicrobial solution, employing incrementally (usually two-fold) increasing concentrations of the antimicrobial agent and appropriate volumes of broth with a defined inoculum

NOTE The aim of this method is the determination of the MIC.

2.8.2
microdilution
performance of broth dilution in microdilution trays with a capacity of $\leqslant$ 200 µl per well

2.9
broth
fluid medium used for the *in vitro* growth of bacteria

2.10
inoculum
number of bacteria in a suspension, calculated with respect to the final volume

NOTE The inoculum is expressed as colony-forming units per millilitre (CFU/ml).

2.11
inoculum effect
change in MIC related to change in inoculum

3 Test procedures

3.1 General

The tests are performed in microdilution trays. The method is based on the preparation of antimicrobial agent working solutions, either in 50 µl volumes per well (with the addition of an inoculum also in a volume of 50 µl), or in a volume of 100 µl per well (with the addition of a maximum of 5 µl inoculum volume).

3.2 Medium

Mueller-Hinton broth shall be used (see Annex A for details).

3.3 Antimicrobial agents

3.3.1 General

Antimicrobial agents shall be obtained directly from the manufacturer or from reliable commercial sources; pharmaceutical preparations for clinical use are not acceptable. The antimicrobial agents shall be supplied with a lot number, potency, an expiry date and details of recommended storage conditions. Substances shall be stored in tightly closed containers in the dark, at 4 °C to 8 °C, with a desiccant unless otherwise recommended by the manufacturer. 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.

3.3.2 Preparation of stock solutions

The use of a calibrated analytical balance is required to weigh antimicrobial agents. Allowance for the potency of the powder shall be made by use of the following formula to obtain the amount of antimicrobial agent substance or the volume of diluent needed for a standard solution:

$$m = \frac{V \times \rho}{P} \tag{1}$$

$$V = \frac{m \times P}{\rho} \tag{2}$$

where

ρ is the concentration of the stock solution, in mg/l;

m is mass of the antimicrobial agent (powder), in g;

P is the potency of the antimicrobial agent (powder), in mg/g;

V is the volume of diluent, in l.

Concentrations of stock solutions should be 1 000 mg/l or greater, although the solubility of some agents is a limiting factor. The actual concentrations of stock solutions depend on the method of preparing working solutions (serial dilutions). Agents should be dissolved and diluted in sterile distilled water unless the manufacturer states otherwise. Some agents require alternative solvents (see Table 1). Sterilisation of solutions is not usually necessary. If required, sterilisation should be done by membrane filtration, and samples before and after sterilisation should be compared by assay to ensure that adsorption has not occurred.

Unless information is available on stability of stock solutions under specified storage conditions, they should be prepared fresh for each test batch.

Table 1 — Examples of solvents and diluents for making stock solutions of selected antimicrobial agents

Antimicrobial agent	Solvent	Diluent
Amikacin	Water	
Amoxicillin	Phosphate buffer 0,1 mol/l, pH 6,0	Phosphate buffer 0,1 mol/l, pH 6,0
Ampicillin	Phosphate buffer 0,1 mol/l, pH 8,0	Phosphate buffer 0,1 mol/l, pH 6,0
Azithromycin	Ethanol volume fraction 95 % or glacial acetic acid[a]	Water
Azlocillin	Water	
Aztreonam	Saturated sodium bicarbonate solution	Water
Carbenicillin	Water	
Cefaclor	Water	
Cefamandole	Water	
Cefazolin	Phosphate buffer 0,1 mol/l, pH 6,0	Phosphate buffer 0,1 mol/l, pH 6,0
Cefdinir	Phosphate buffer 0,1 mol/l, pH 6,0	Water
Cefditoren	Phosphate buffer 0,1 mol/l, pH 6,0	Water
Cefepime	Phosphate buffer 0,1 mol/l, pH 6,0	Phosphate buffer 0,1 mol/l, pH 6,0
Cefetamet	Phosphate buffer 0,1 mol/l, pH 6,0	Water
Cefixime	Phosphate buffer 0,1 mol/l, pH 7,0	Phosphate buffer 0,1 mol/l, pH 7,0
Cefmetazole	Water	
Cefonicid	Water	
Cefoperazone	Water	
Cefotaxime	Water	
Cefotetan	Dimethyl sulfoxide	Water
Cefoxitin	Water	
Cefpodoxime	Mass concentration 0,1 % sodium bicarbonate solution	Water
Cefprozil	Water	
Ceftazidime	Saturated sodium bicarbonate solution	Water
Ceftibuten	1/10 volume of dimethyl sulfoxide	Water
Ceftizoxime	Water	
Ceftobiprole	Dimethyl sulfoxide plus glacial acetic acid[b]	Water, vortex vigorously
Ceftriaxone	Water	
Cefuroxime	Phosphate buffer 0,1 mol/l, pH 6,0	Phosphate buffer 0,1 mol/l, pH 6,0
Cephalothin	Phosphate buffer 0,1 mol/l, pH 6,0	Water
Chloramphenicol	Ethanol volume fraction 95 %	Water
Cinoxacin	Half volume of water, a minimum volume 1 mol/l NaOH to dissolve, then make up to total volume with water	Water
Ciprofloxacin	Water	
Clarithromycin	Methanol or glacial acetic acid[a]	0,1 mol/l phosphate buffer, pH 6,5
Clavulanic acid	Phosphate buffer 0,1 mol/l, pH 6,0	Phosphate buffer 0,1 mol/l, pH 6,0
Clinafloxacin	Water	
Clindamycin	Water	
Colistin[c]	Water	Water
Dalbavancin	Dimethyl sulfoxide	Water and dimethyl sulfoxide[d]

Table 1 (*continued*)

Antimicrobial agent	Solvent	Diluent
Daptomycin	Water	Water
Dirithromycin	Glacial acetic acid[a]	Water
Doripenem	NaCl volume fraction 0,85 %	NaCl volume fraction 0,85 %
Doxycycline	Water	
Enoxacin	Half volume water, a minimum volume 0,1 mol/l NaOH to dissolve, then make up to total volume with water	Water
Ertapenem	Phosphate buffer 0,01 mol/l, pH 7,2	Phosphate buffer 0,01 mol/l, pH 7,2
Erythromycin	Ethanol volume fraction 95 % or glacial acetic acid[a]	Water
Faropenem	Water	Water
Fleroxacin	Half volume water, a minimum volume 0,1 mol/l NaOH to dissolve, then make up to total volume with water	Water
Fusidic acid	Ethanol volume fraction 95 %	Water
Garenoxacin	Water (with stirring)	
Gatifloxacin	Water (with stirring)	
Gemifloxacin	Water	
Gentamicin	Water	
Imipenem	Phosphate buffer 0,01 mol/l, pH 7,2	Phosphate buffer 0,01 mol/l, pH 7,2
Kanamycin	Water	
Levofloxacin	Half volume water, a minimum volume 1 mol/l NaOH to dissolve, then make up to total volume with water	Water
Linezolid	Water	
Loracarbef	Water	
Mecillinam	Water	
Meropenem	Phosphate buffer 0,01 mol/l, pH 7,2	Phosphate buffer 0,01 mol/l, pH 7,2
Methicillin	Water	
Mezlocillin	Water	
Minocycline	Water	
Moxalactam (diammonium salt)[e]	0,04 mol/l HCl (let sit for 1,5 h to 2 h)	Phosphate buffer 0,1 mol/l, pH 6,0
Moxifloxacin	Water	
Mupirocin	Water	
Nafcillin	Water	
Nalidixic acid	Half volume water, a minimum volume 1 mol/l NaOH to dissolve, then make up to total volume with water	Water
Netilmicin	Water	
Nitrofurantoin	Minimum volume dimethylformamide to dissolve, then make up to total volume with phosphate buffer 0,1 mol/l, pH 8,0	Phosphate buffer 0,1 mol/l, pH 8,0
Norfloxacin	Half volume of water, a minimum volume 1 mol/l NaOH to dissolve, then make up to total volume with water	Water
Ofloxacin	Half volume water, a minimum volume 1 mol/l NaOH to dissolve, then make up to total volume with water	Water
Oxacillin	Water	

Table 1 (*continued*)

Antimicrobial agent	Solvent	Diluent
Penicillin	Water	
Piperacillin	Water	
Polymyxin B	Water	Water
Quinupristin-dalfopristin	Water	
Rifampicin	Methanol	Water
Sparfloxacin	Water	
Sulbactam	Phosphate buffer 0,1 mol/l, pH 6,0	Phosphate buffer 0,1 mol/l, pH 6,0
Sulphonamides	Half volume water, a minimum volume 1 mol/l NaOH to dissolve, then make up to total volume with water	Water
Teicoplanin	Water	
Telavancin	Dimethyl sulfoxide	Water
Telithromycin	Glacial acetic acid[a]	Water
Tetracycline	Water	
Ticarcillin	Phosphate buffer 0,1 mol/l, pH 6,0	Phosphate buffer 0,1 mol/l, pH 6,0
Tigecycline	Water	Water
Tobramycin	Water	
Trimethoprim	Half volume water, a minimum volume 0,1 mol/l lactic acid or 0,1 mol/l HCl to dissolve, then make up to total volume with water	Water
Trimethoprim (if lactate)	Water	
Trospectomycin	Water	
Vancomycin	Water	

NOTE 1 The information regarding solvents and diluents in Table 1 was largely obtained from GLSI document M100-S16 (Performance Standards for Antimicrobial Susceptibility Testing; Sixteenth Informational Supplement)[7] with permission. This information is subject to periodic updates. Check the latest version of M100 available from CLSI (formerly NCCLS), 940 West Valley Road, Suite 1400, Wayne, PA 19087, USA.

NOTE 2 For further information on examples of solvents and diluents for making stock solutions of selected antimicrobial agents, consult the European Pharmacopoeia or the US Pharmacopoeia.

[a] For glacial acetic acid, use half volume of water, then add glacial acetic acid dropwise until dissolved, not to exceed 2,5 mg/l; add water to full volume. Glacial acetic acid is equivalent to acetic acid volume fraction > 99 %.

[b] For each 1,5 mg ceftobiprole, add 110 µl of a 10:1 mixture of dimethyl sulfoxide and glacial acetic acid. Vortex vigorously for 1 min, then intermittently for 15 min. Dilute to 1,0 ml with distilled water.

[c] The formulation of colistin used in antimicrobial susceptibility tests is colistin sulphate and not colistin methane sulfphonate (sulphomethate).

[d] Starting stock solutions of dalbavancin should be prepared at concentrations no higher than 1 600 mg/l. Intermediate 100 × concentrations should then be diluted in dimethyl sulfoxide. Final 1:100 dilutions should then be made directly into cation-adjusted Mueller-Hinton broth (CAMHB) supplemented with polysorbate-80 volume fraction 0,002 % so that the final concentration of dimethyl sulfoxide in the wells is no greater than 1 %.

[e] The diammonium salt of moxalactam is very stable, but it is almost pure R isomer. Moxalactam for clinical use is a 1:1 mixture of R and S isomers. Therefore, the salt is dissolved in 0,04 mol/l HCl and allowed to react for 1,5 h to 2 h to convert it to equal parts of both isomers.

3.3.3 Preparation of working solutions

The range of concentrations selected for testing depends on the organisms and antimicrobial agent. The chosen range shall allow full endpoint MIC determination for appropriate reference strains. A two-fold dilution series based on 1 mg/l is prepared in Mueller-Hinton broth. Dilutions should not be prepared by serial dilution steps, but according to the procedure outlined in Table 2. Working solutions shall be used the same day unless information is available on stability of the solutions under specified storage conditions.

Table 2 — Preparation of working dilutions of antimicrobial agents for use in broth dilution susceptibility tests[8]

Antimicrobial agent concentration in stock solution	Volume stock solution	Volume broth[a]	Antimicrobial agent concentration obtained
mg/l	ml	ml	mg/l
5 120	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

[a] Broth used for dilution is that used in the susceptibility test. Any supplementation shall take place before diluting the antimicrobial agent to maintain the required concentrations.

3.3.4 Preparation of microdilution trays

Working solutions are dispensed into microdilution trays at 50 µl per well with double the desired final concentrations of antimicrobial agent, or at 100 µl per well in the desired final concentrations.

At least one well, containing 50 µl or 100 µl of antimicrobial agent-free medium, should be included as a growth control for each strain tested. Likewise, a well containing 100 µl of antimicrobial agent-free medium should be included as an uninoculated negative control well for each strain tested.

3.3.5 Storage of microdilution trays

Filled trays may be used immediately or may be stored for up to three months. For storage the filled trays should be sealed in plastic bags and immediately placed in a freezer at $\leqslant -60$ °C unless the antimicrobial agents are known to be stable at higher temperatures.

Although the antimicrobial agents in frozen trays usually remain stable for several months, certain agents (e.g. clavulanic acid and imipenem) are more labile than others and should be stored at $\leqslant -60$ °C. Trays shall not be stored in a self-defrosting freezer, and thawed antimicrobial solutions shall not be refrozen, as repeated freeze-thaw cycles accelerate the degradation of some antimicrobial agents, particularly β-lactams.

3.4 Preparation of inoculum

3.4.1 General

Standardisation of the inoculum is essential for accurate and reproducible broth dilution susceptibility tests. Therefore purity checks and viable colony counts shall be performed on every isolate tested with this reference procedure.

The inoculum may be prepared by diluting a broth culture or by suspending colonies from an overnight culture on non-selective agar medium in broth or saline. For either method, four or five colonies of a pure non-selective nutritive agar medium are chosen to avoid selecting out an atypical variant.

The final inoculum shall be 5×10^5 CFU/ml (range 2×10^5 CFU/ml to 8×10^5 CFU/ml).

3.4.2 Broth culture method

Three to five colonies from a non-selective nutritive agar medium are touched with a loop and transferred to broth such as tryptic soy or brain heart infusion broth. The broth used shall not be antagonistic to the antimicrobial agent tested. The broth is incubated at 34 °C to 37 °C until the growth reaches a turbidity equal to or greater than that of a 0,5 McFarland standard. If needed, the culture is adjusted with saline or broth to give a turbidity equivalent to the 0,5 McFarland standard. This can be done by means of a photometric device (using 625 nm wavelength and a 1 cm path cuvette, the absorbance will be 0,08 – 0,13), or by employing a suitably calibrated nephelometer. Alternatively, this can be achieved visually by comparing the appearance of black lines through the inoculum and 0,5 McFarland standard suspensions (the inoculum and McFarland standard shall be in tubes of the same size) or any other method that gives reproducible CFU/ml.

NOTE A 0,5 McFarland standard can be produced by adding a 0,5 ml aliquot of 0,048 mol/l $BaCl_2$ (11,72 g/l $BaCl_2 \cdot 2H_2O$) to 99,5 ml of 0,18 mol/l H_2SO_4, with constant stirring to maintain a suspension.

3.4.3 Colony suspension method

Three to five colonies from a non-selective nutritive agar medium (incubated at 34 °C to 37 °C for 18 h to 24 h, unless longer incubation is required) 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 in 3.4.2 for the broth culture method.

For all organisms, the accurate concentration of viable cells in the final inoculum depends on the state of the culture. This effect is most pronounced for fastidious organisms such as *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 contains approximately 1×10^8 CFU/ml for the common relevant bacteria.

The adjusted inoculum prepared as above is diluted in broth to give a final cell number concentration of 5×10^5 CFU/ml (range 2×10^5 CFU/ml to 8×10^5 CFU/ml). The dilution required depends upon the organism being tested and the method used for inoculum delivery. Transfer of 0,1 ml of standardized organism suspension to a tube containing 9,9 ml (1:100 dilution) of broth results in a suspension of 1×10^6 CFU/ml which, when 50 µl is added to an equal volume (50 µl) of antimicrobial agent solution, results in a final inoculum of 5×10^5 CFU/ml with many Gram-negative bacteria (e.g. *Escherichia coli*). If the wells already contain 100 µl of antimicrobial agents in broth, an appropriate dilution to give the required final inoculum should be prepared prior to addition of up to 5 µl of the diluted suspension to each well. With Gram-positive organisms, a lesser dilution of the 0,5 McFarland suspension may be necessary, as determined by colony counts in preliminary tests.

3.5 Inoculation of microdilution trays

The trays shall be inoculated within 30 min of standardizing the inoculum suspension, in order to maintain viable cell number concentration. To each well containing 50 µl of diluted antimicrobial agent in broth (see 3.3), a volume of 50 µl of bacterial suspension (see 3.4) is added. For tray wells that contain 100 µl of diluted antimicrobial agent in broth, up to 5 µl of diluted inoculum suspension should be added.

Viable counts shall be performed on the test suspension to ensure that test wells contain approximately 5×10^5 CFU/ml. This shall be done by removing 10 µl from the growth control well 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. Twenty to eighty colonies would be expected from an acceptable test suspension. If this is not achieved, the results for this strain can not be used.

3.6 Incubation of microdilution trays

Microdilution trays should be sealed in polyethylene bags or fitted with a tight lid or adhesive seal before incubation, in order to prevent desiccation. In order to avoid uneven heating, microdilution trays should not be stacked more than five high.

Unless otherwise specified, microdilution trays are incubated at 34 ºC to 37 ºC in ambient air for (18 ± 2) h for most antimicrobial agent-bacteria combinations. A CO_2-enriched atmosphere should not be used.

3.7 Reading results

Results shall only be read when there is sufficient growth of the test organism (i.e. obvious button or definite turbidity in the positive growth control), when there is no growth in the uninoculated or negative growth control (where present) and when purity and the appropriate cell number concentration of the inoculum has been established. The amount of growth in each well is compared with that in the positive growth control, and the MIC recorded is the lowest concentration of the agent that completely inhibits visible growth.

3.8 Special test situations where the MIC result might give unreliable results

In some cases, the MIC value may not reflect relevant activity, therefore the interpretations of test results with some antimicrobial agents may need to be modified for clinical application. In those situations, the reference method has to be modified e.g. by changes in incubation conditions or adjustments to the media. In addition, certain resistance mechanisms may not always be expressed using the standard reference dilution method, e.g. the expression of some *β*-lactamases, efflux pumps or drug target site modifications. In those cases, the MIC should be interpreted with caution, or other information used instead, to guide clinical therapy. In Table 3, several antimicrobial agent-bacteria combinations are listed that require special attention.

Table 3 — Special test situations

Antimicrobial	Bacteria	Remarks
Aminoglycosides	*Enterococcus* spp.	The median MICs of gentamicin and tobramycin are 8 mg/l to 16 mg/l, and of streptomycin 8 mg/l to 32 mg/l for wild type *Enterococcus faecalis* and *Enterococcus faecium.* Aminoglycosides demonstrate a synergistic effect when combined with cell wall active agents (e.g. penicillins, carbapenems, glycopeptides). Some strains show high-level resistance to aminoglycosides (MIC > 500 mg/l). In such isolates, there is no synergistic effect. When testing *Enterococcus* spp., the range of dilutions should be sufficient to detect high-level resistance. The incubation time should be 24 h for gentamicin and 48 h for streptomycin.
β-lactams	All	The MIC may not form a valid basis for predicting the therapeutic value of a drug if the bacteria produce certain *β*-lactamases; therefore the MIC must be interpreted with caution.
Methicillin Oxacillin	*Staphylococcus* spp.	Broth microdilution may not reliably detect resistance conferred by the *mec*A gene. The following variations of the test method may enhance the detection of resistance: — incorporation of NaCl at a final concentration of 20 g/l in the broth; — incubation of tests for a full 24 h. — incubation temperature of 30 °C to not more than 35 °C. — use of the direct suspension method for preparing bacterial inocula rather than the growth method Detection of the *mec*A gene is the reference method for detection of methicillin/oxacillin resistance.
Daptomycin	All	Medium shall be supplemented to a final concentration of 50 mg/l Ca^{++}.
Fosfomycin	All	Broth microdilution may not give reliable results. Agar dilution should be used as the reference method[5][9]. The test agar should be supplemented with 25 mg/l glucose-6-phosphate.
Glycopeptides	All	The MIC should be read after 24 h incubation to give more consistent and reliable results.
	Staphylococcus aureus	Broth microdilution does not reliably detect heterogeneously resistant glycopeptide intermediate *S. aureus*.
Glycylcyclines Tigecycline	All	Freshly prepared (< 12 h) test medium shall be used.
Lincosamides	All	The MIC may not predict clinical utility if the strain is able to produce an inducible methylase (MLS_B resistance).
Sulphonamides and Trimethoprim	All	The MIC should be read at the lowest concentration that inhibits approximately 80 % of growth as compared with the growth control well.

4 Quality control

The quality of test results is monitored by the concomitant use of control strains (see Table 4). Stock control strains should be stored lyophilised or frozen (at −60 °C or below). Prepare working cultures by subculture of stock strains on a non-selective nutritive agar medium. Further subcultures may be made, from the first working culture only, for up to one week. When available, at least two relevant QC strains should be tested every day that testing is carried out. Test colonies of control cultures are processed in the same way as routine cultures. MICs of antimicrobial agents for control organisms should be within the ranges given in Table 4.

Table 4 — MIC ranges (mg/l) for control strains

Antimicrobial Agent	*Staphylococcus aureus* ATCC 29213[a] NCTC 12973[b] CIP 103429[c] DSM 2569[d]	*Enterococcus faecalis* ATCC 29212 NCTC 12697 CIP 103214 DSM 2570	*Escherichia coli* ATCC 25922 NCTC 12241 CIP 7624 DSM 1103	*Pseudomonas aeruginosa* ATCC 27853 NCTC 12973 CIP 76110 DSM 1117	*Escherichia coli* ATCC 35218 DSM 5564	*Streptococcus pneumoniae* ATCC 49619 NCTC 12977
Amikacin	1-4	64-256	0,5-4	1-4	—	—
Amoxicillin-clavulanic acid (fixed 2:1 ratio)	0,12/ 0,06-0,5/0,25	0,25/0,12-1,0/0,5	2/1-8/4	—	4/2-16/8	0,03/ 0,015-0,12/ 0,06
Amoxicillin[e]	0,25-1	—	4-16	—	—	0,03-0,12
Ampicillin	0,5-2	0,5-2	2-8	—	—	0,06-0,25
Ampicillin-sulbactam (fixed 2:1 ratio)	—	—	2/1-8/4	—	8/4-32/16	—
Azithromycin	0,5-2	—	—	—	—	0,06-0,25
Azlocillin	2-8	1-4	8-32	2-8	—	—
Aztreonam	—	—	0,06-0,25	2-8	—	—
Carbenicillin	2-8	16-64	4-16	16-64	—	—
Cefaclor	1-4	—	1-4	—	—	1-4
Cefamandole	0,25-1	—	0,25-1	—	—	—
Cefazolin	0,25-1	—	1-4	—	—	—
Cefdinir	0,12-0,5	—	0,12-0,5	—	—	0,03-0,25
Cefditoren	0,25-2	—	0,12-1	—	—	0,015-0,12
Cefepime	1-4	—	0,015-0,12	1-8	—	0,03-0,25
Cefetamet	—	—	0,25-1	—	—	0,5-2
Cefixime	8-32	—	0,25-1	—	—	—
Cefmetazole	0,5-2	—	0,25-1	> 32	—	—
Cefonicid	1-4	—	0,25-1	—	—	—
Cefoperazone	1-4	—	0,12-0,5	2-8	—	—
Cefotaxime	1-4	—	0,03-0,12	8-32	—	0,03-0,12
Cefotetan	4-16	—	0,06-0,25	—	—	—
Cefoxitin	1-4	—	2-8	—	—	—
Cefpodoxime	1-8	—	0,25-1	—	—	0,03-0,12
Cefprozil	0,25-1	—	1-4	—	—	0,25-1
Ceftazidime	4-16	—	0,06-0,5	1-4	—	—
Ceftibuten	—	—	0,12-0,5	—	—	—
Ceftizoxime	2-8	—	0,03-0,12	16-64	—	0,12-0,5
Ceftobiprole	0,25-1	0,06-0,5	0,03-0,12	1-4	—	0,004-0,003
Ceftriaxone	1-8	—	0,03-0,12	8-64	—	0,03-0,12
Cefuroxime	0,5-2	—	2-8	—	—	0,25-1

Table 4 (*continued*)

Antimicrobial Agent	*Staphylococcus aureus* ATCC 29213[a] NCTC 12973[b] CIP 103429[c] DSM 2569[d]	*Enterococcus faecalis* ATCC 29212 NCTC 12697 CIP 103214 DSM 2570	*Escherichia coli* ATCC 25922 NCTC 12241 CIP 7624 DSM 1103	*Pseudomonas aeruginosa* ATCC 27853 NCTC 12973 CIP 76110 DSM 1117	*Escherichia coli* ATCC 35218 DSM 5564	*Streptococcus pneumoniae* ATCC 49619 NCTC 12977
Cephalexin[e]	—	—	4-16	—	—	—
Cephalothin	0,12-0,5	—	4-16	—	—	0,5-2
Chloramphenicol	2-16	4-16	2-8	—	—	2-8
Cinoxacin	—	—	2-8	—	—	—
Ciprofloxacin	0,12-0,5	0,25-2	0,004-0,015	0,25-1	—	—
Clarithromycin	0,12-0,5	—	—	—	—	0,03-0,12
Clinafloxacin	0,008-0,06	0,03-0,25	0,002-0,015	0,06-0,5	—	0,03-0,12
Clindamycin	0,06-0,25	4-16	—	—	—	0,03-0,12
Colistin	—	—	0,25-1	0,25-2	—	—
Dalbavancin	0,03-0,12	0,03-0,12	—	—	—	0,008-0,03
Daptomycin[f]	0,25-1	1-4	—	—	—	0,06-0,5
Dirithromycin	1-4	—	—	—	—	0,06-0,25
Doripenem	0,015-0,06	1-4	0,015-0,06	0,12-0,5	—	0,03-0,12
Doxycycline	0,12-0,5	2-8	0,5-2	—	—	0,015-0,12
Enoxacin	0,5-2	2-16	0,06-0,25	2-8	—	—
Ertapenem	0,06-0,25	4-16	0,004-0,015	2-8	—	0,03-0,25
Erythromycin	0,25-1	1-4	—	—	—	0,03-0,12
Faropenem	0,03-0,12	—	0,25-1	—	—	0,03-0,25
Fleroxacin	0,25-1	2-8	0,03-0,12	1-4	—	—
Fusidic acid[e]	0,06-0,25	1-4	—	—	—	—
Garenoxacin	0,004-0,03	0,03-0,25	0,004-0,03	0,5-2	—	0,015-0,06
Gatifloxacin	0,03-0,12	0,12-1,0	0,008-0,03	0,5-2	—	0,12-0,5
Gemifloxacin	0,008-0,03	0,015-0,12	0,004-0,015	0,25-1	—	0,008-0,03
Gentamicin	0,12-1	4-16	0,25-1	0,5-2	—	—
Grepafloxacin	0,03-0,12	0,12-0,5	0,004-0,03	0,25-2,0	—	0,06-0,5
Imipenem	0,015-0,06	0,5-2	0,06-0,25	1-4	—	0,03-0,12
Kanamycin	1-4	16-64	1-4	—	—	—
Levofloxacin	0,06-0,5	0,25-2	0,008-0,06	0,5-4	—	0,5-2
Linezolid	1-4	1-4	—	—	—	0,5-2
Lomefloxacin	0,25-2	2-8	0,03-0,12	1-4	—	—
Loracarbef	0,5-2	—	0,5-2	> 8	—	2-8
Mecillinam	—	—	0,03-0,25	—	—	—
Meropenem	0,03-0,12	2-8	0,008-0,06	0,25-1	—	0,06-0,25
Methicillin	0,5-2	> 16	—	—	—	—

Table 4 (*continued*)

Antimicrobial Agent	*Staphylococcus aureus* ATCC 29213[a] NCTC 12973[b] CIP 103429[c] DSM 2569[d]	*Enterococcus faecalis* ATCC 29212 NCTC 12697 CIP 103214 DSM 2570	*Escherichia coli* ATCC 25922 NCTC 12241 CIP 7624 DSM 1103	*Pseudomonas aeruginosa* ATCC 27853 NCTC 12973 CIP 76110 DSM 1117	*Escherichia coli* ATCC 35218 DSM 5564	*Streptococcus pneumoniae* ATCC 49619 NCTC 12977
Mezlocillin	1-4	1-4	2-8	8-32	—	—
Minocycline	0,06-0,5	1-4	0,25-1	—	—	—
Moxalactam	4-16	—	0,12-0,5	8-32	—	—
Moxifloxacin	0,015-0,12	0,06-0,5	0,008-0,06	1-8	—	0,06-0,25
Mupirocin[e]	0,06-0,25	—	—	—	—	—
Nafcillin	0,12-0,5	2-8	—	—	—	—
Nalidixic acid	—	—	1-4	—	—	—
Netilmicin	$\leqslant$ 0,25	4-16	$\leqslant$ 0,5-1	0,5-8	—	—
Nitrofurantoin	8-32	4-16	4-16	—	—	4-16
Norfloxacin	0,5-2	2-8	0,03-0,12	1-4	—	2-8
Ofloxacin	0,12-1	1-4	0,015-0,12	1-8	—	1-4
Oritavancin	0,5-2	0,12-1	—	—	—	0,008-0,06
Oxacillin	0,12-0,5	8-32	—	—	—	—
Penicillin	0,25-2	1-4	—	—	—	0,25-1
Piperacillin	1-4	1-4	1-4	1-8	—	—
Piperacillin-tazobactam (fixed inhibitor concentration 4 mg/l)	0,25/4-2/4	1/4-4/4	1/4-4/4	1/4-8/4	0,5/4-2/4	—
Pipemidic acid[e]	—	—	0,5-2	—	—	—
Polymyxin B	—	—	0,25-2	0,25-2	—	—
Quinupristin-dalfopristin	0,25-1	2-8	—	—	—	0,25-1
Rifampin	0,004-0,015	0,5-4	4-16	16-64	—	0,015-0,06
Sparfloxacin	0,03-0,12	0,12-0,5	0,004-0,015	0,5-2	—	0,12-0,5
Streptomycin[e]	—	—	4-16	—	—	—
Sulfisoxazole	32-128	32-128	8-32	—	—	—
Teicoplanin	0,25-1	0,06-0,25	—	—	—	—
Telavancin	0,12-1	0,12-0,5	—	—	—	0,002-0,015
Telithromycin	0,06-0,25	0,015-0,12	—	—	—	0,004-0,03
Tetracycline	0,12-1	8-32	0,5-2	8-32	—	0,12-0,5
Ticarcillin	2-8	16-64	4-16	8-32	—	—
Ticarcillin-clavulanic acid (fixed inhibitor concentration 2 mg/l)	0,5/2-2/2	16/2-64/2	4/2-16/2	8/2-32/2	8/2-32/2	—

Table 4 (*continued*)

Antimicrobial Agent	*Staphylococcus aureus* ATCC 29213[a] NCTC 12973[b] CIP 103429[c] DSM 2569[d]	*Enterococcus faecalis* ATCC 29212 NCTC 12697 CIP 103214 DSM 2570	*Escherichia coli* ATCC 25922 NCTC 12241 CIP 7624 DSM 1103	*Pseudomonas aeruginosa* ATCC 27853 NCTC 12973 CIP 76110 DSM 1117	*Escherichia coli* ATCC 35218 DSM 5564	*Streptococcus pneumoniae* ATCC 49619 NCTC 12977
Tigecycline	0,03-0,25	0,03-0,12	0,03-0,25	—	—	0,015-0,12
Tobramycin	0,12-1	8-32	0,25-1	0,25-1	—	—
Trimethoprim	1-4	⩽ 1	0,5-2	> 64	—	—
Trimethoprim-sulfamethoxazole (fixed 1:19 ratio)	⩽ 0,5/9,5	⩽ 0,5/9,5	⩽ 0,5/9,5	8/152-32/608	—	0,12/2,4-1/19
Trospectomycin	2-16	2-8	8-32	—	—	1-4
Trovafloxacin	0,008-0,03	0,06-0,25	0,004-0,015	0,25-2	—	0,06-0,25
Vancomycin	0,5-2	1-4	—	—	—	0,12-0,5

NOTE Except where noted, MIC ranges were obtained with perission from CLSI document M100-S16 (*Performance Standards for Antimicrobial Susceptibility Testing*; Sixteenth Informational Supplement)[7]. Control ranges are for cation-adjusted Mueller-Hinton broth for all control organisms except *S. pneumoniae* ATCC 49619, which is tested using cation-adjusted Mueller-Hinton broth supplemented with 2,5-5 % lysed horse blood. Ranges are subject to periodic updates. Check the latest version of M100 available from CLSI (formerly NCCLS) for updated ranges. CLSI, 940 West Valley Road, Suite 1400, Wayne, PA 19087, USA.

[a] ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA.

[b] NCTC, National Collection of Type Cultures, Health Protection Agency Centre for Infections, 61 Colindale Avenue, London NW9 5HT, UK.

[c] CIP, Collection de Institut Pasteur, 25–28 Rue du Docteur Roux, 75724 Paris Cedex 15 France.

[d] DSMZ, Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, Mascheroder Weg 16, D-38124 Braunschweig, Germany.

[e] MIC range derived from the target MIC provided by EUCAST (Clin. Microbiol. Infec. 9:1-7, 2003). Check EUCAST website at http://www.eucast.org for latest published EUCAST control target values.

[f] Daptomycin QC ranges reflect MICs obtained when Mueller-Hinton broth is supplemented with calcium to a final concentration of 50 mg/l.

Annex A
(normative)

Requirements for Mueller-Hinton broth

A.1 General

Supplements other than divalent cations or other additional components should not be used unless necessary for growth of the test organism.

A.2 Testing of non-fastidious organisms in Mueller-Hinton broth

A.2.1 General

The standard medium for testing of non-fastidious organisms is Mueller-Hinton broth. The original formulation was prepared as follows[10]:

Dehydrated infusion from 300 g beef

Acid digest of casein 17,5 g

Corn starch 1,5 g

QSP distilled water 1 000 ml

pH 7,2-7,4

A.2.2 Cation supplementation and content

The broth should contain sufficient concentrations of cations to provide adequate growth, and to permit the user to determine MIC values for quality control strains within ranges identified in Table 4.

New lots of Mueller-Hinton broth may require testing for acceptable cation content. This may be accomplished by either Inductively Coupled Plasma (ICP) spectroscopy or Flame Atomic Absorption Spectrometry (FAAS)[11].

For calcium and magnesium ion supplementation, prepare 10 mg/l solutions of calcium chloride (3,68 g $CaCl_2 \cdot 2H_2O$ in 100 ml deionised water) and magnesium chloride (8,36 g $MgCl_2 \cdot 6H_2O$ in 100 ml deionised water), sterilise by membrane filter and store at 2 °C to 8 °C. Each 0,1 ml volume of 10 mg/l cation solution added to 1 l of broth increases the cation content by 1 mg/l. Add whilst stirring at 2 °C to 8 °C.

For most antimicrobial agent - bacteria combinations, addition of calcium and magnesium to a final concentration of 20 mg/l to 25 mg/l, and of 10 mg/l to 12,5 mg/l, respectively, has been shown to provide accurate quality control results[12][13].

For daptomycin, 50 mg/l final concentration of calcium ions in the medium is required[14].

For the carbapenem agents, imipenem and meropenem, it has been shown that the final zinc concentration should be less than 3 mg/l[15]. Mass concentrations of zinc required for optimal activity of other carbapenems have not yet been documented, but should be in the same range.

A.2.3 Testing of *Streptococcus* species

Lysed horse blood should be added to cation supplemented Mueller-Hinton broth medium to a final volume concentration of 2.5 % to 5 % for testing of *Streptococcus* species. The blood should be obtained from a reputable supplier. Haematocrit information should be available (not less than 30 %). To prepare the lysed blood, aseptically mix equal volumes of defibrinated blood and sterile distilled water. Freeze at –20 °C and thaw until cells are thoroughly lysed (may require five to seven cycles). Clarify by centrifugation. The resulting preparation of lysed blood is a 50 % volume concentration stock solution.

A.2.4 Supplementary medium issues

A.2.4.1 General

It is not possible at this time to identify all possibilities that may occur for quality control and reference antimicrobial susceptibility testing of aerobic and facultative anaerobic bacterial species. Some data on medium effects is contained in unpublished data. New agents will challenge the “standard” preparations of the medium. Users should ensure that standard quality control parameters are met and that new issues are communicated internationally, so that this part of ISO 20776 can be updated regularly.

A.2.4.2 Sulphonamides and trimethoprim

The medium shall have a thymidine mass concentration of less than 0,03 mg/l. Such a medium does not preclude use for testing of other antimicrobial agents.

A.2.4.3 Tigecycline

Tigecycline has to be added to the Mueller-Hinton broth within 12 h of preparation of the broth. Once prepared, the microdilution trays may be frozen.

A.2.4.4 Dalbavancin

For testing dalbavancin, cation-adjusted Mueller-Hinton broth should be supplemented with polysorbate-80 volume fraction 0,002 %.

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