Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances

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The aim of broth and agar dilution methods is to determine the lowest concentration of the assayed antimicrobial agent (minimal inhibitory concentration, MIC) that, under defined test conditions, inhibits the visible growth of the bacterium being investigated. MIC values are used to determine susceptibilities of bacteria to drugs and also to evaluate the activity of new antimicrobial agents. Agar dilution involves the incorporation of different concentrations of the antimicrobial substance into a nutrient agar medium followed by the application of a standardized number of cells to the surface of the agar plate. For broth dilution, often determined in 96-well microtiter plate format, bacteria are inoculated into a liquid growth medium in the presence of different concentrations of an antimicrobial agent. Growth is assessed after incubation for a defined period of time (16–20 h) and the MIC value is read. This protocol applies only to aerobic bacteria and can be completed in 3 d.

INTRODUCTION

Agar dilution and broth dilution are the most commonly used techniques to determine the minimal inhibitory concentration (MIC) of antimicrobial agents, including antibiotics and other substances that kill (bactericidal activity) or inhibit the growth (bacteriostatic activity) of bacteria. The methods described here are targeted for testing susceptibility to antibiotic agents as opposed to other antimicrobial biocides such as preservatives and disinfectants. However, there are no major reasons why they cannot be used for these other antimicrobials. For agar dilution, solutions with defined numbers of bacterial cells are spotted directly onto the nutrient agar plates that have incorporated different antibiotic concentrations. After incubation, the presence of bacterial colonies on the plates indicates growth of the organism. Broth dilution uses liquid growth medium containing geometrically increasing concentrations (typically a twofold dilution series) of the antimicrobial agent, which is inoculated with a defined number of bacterial cells. The final volume of the test defines whether the method is termed macrodilution, when using a total volume of 2 ml, or microdilution, if performed in microtiter plates using $\leq$500 µl per well. After incubation, the presence of turbidity or a sediment indicates growth of the organism. In both the agar and the broth dilution approaches, the MIC is defined as the lowest concentration (in mg l^{-1}) of the antimicrobial agent that prevents visible growth of a microorganism under defined conditions.

In clinical practice, this *in vitro* parameter is used to classify the tested microorganism as either clinically susceptible, intermediate or resistant to the tested drug. The interpretative standards for these classifications are published by different national organizations such as the Clinical and Laboratory Standards Institute (CLSI)[1] in the USA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[2]. Breakpoints (the particular MIC that differentiates susceptible, and assumingly treatable, from resistant and assumingly untreatable organisms) are derived from microbiological and clinical experience, and can vary according to the particular species being examined and the particular antimicrobial agent. Features that define these breakpoints are MIC distributions of relevant species, pharmacodynamics and pharmacokinetics of the antimicrobial agent, and clinical outcome data. Resistance (above the breakpoint) is associated with a high likelihood of therapeutic failure, whereas susceptibility is associated with a greater probability of therapeutic success. For isolates classified as intermediate, the therapeutic effect is uncertain[3].

MIC determinations can be used for monitoring the development of antibiotic drug resistance. MIC wild-type distribution databases are available for relevant species–drug combinations (http://www.eucast.org). The highest MIC of the wild-type population is defined as the 'epidemiological cut-off value' or wild-type (WT) cut-off value[3] (**Fig. 1**). Organisms with acquired resistance can be easily identified by showing higher MIC values than the epidemiological cut-off value. As even slight changes may become clinically relevant, the determination of MIC is a valuable means for resistance surveillance, as well as providing a valuable comparator

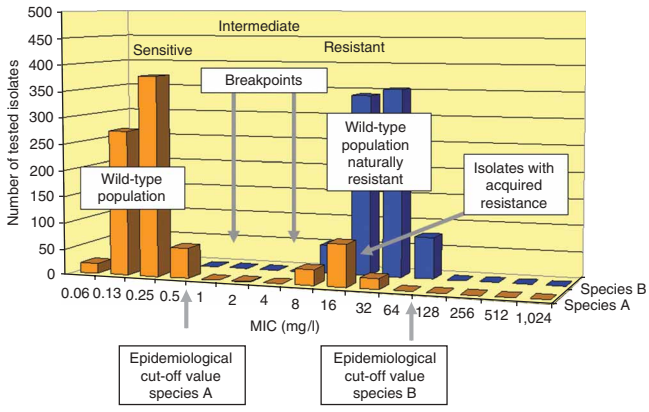

Figure 1 | Distribution of MIC values for different isolates for given species (modified from Wiegand and Wiedemann[27]).

for variants of a given antimicrobial agent and/or species with differential susceptibility. Indeed for new drug candidates, the MIC determination is one of the first steps to evaluate the antimicrobial potential.

Specialized protocols can also allow inferences to be drawn regarding resistance mechanisms. For example, results from broth dilution MIC determination with certain β-lactam antibiotics (cefotaxime, cefpodoxime and/or ceftazidime) in the presence or absence of an inhibitor (clavulanic acid) can indicate the production of extended-spectrum β-lactamases when MICs are at least three twofold concentration steps lower in the presence of the β-lactamase inhibitor[1]. Epidemiological resistance data furthermore provide the basis for appropriate first-line therapy recommendations for empirical treatment.

Dilution methods are considered as reference methods for *in vitro* susceptibility testing and are also used to evaluate the performance of other methods of susceptibility testing.

Crucial parameters

As the test results vary widely under different test conditions, the procedures have to be standardized for intra- and inter-laboratory reproducibility. The protocols described here are adjusted to a step-by-step format and follow the guidelines of the two established organizations and committees, the CLSI[1] and EUCAST[2]. Modifications are introduced for testing the susceptibility to cationic antimicrobial peptides and other cationic agents that tend to bind to surfaces. If implemented rigorously according to the procedures described herein, these modifications allow the generation of reliable data that will be comparable between different laboratories.

The use of all methods of this protocol is limited to aerobic bacteria that grow well within 24 h in the CLSI and EUCAST recommended test Mueller–Hinton growth medium. Mueller–Hinton broth (MHB) is a general purpose medium that can be used for cultivation of a wide variety of nonfastidious microorganisms. For growth of fastidious organisms, such as *Streptococcus* spp., *Haemophilus influenzae*, *Neisseria gonorrheae*, *Helicobacter pylori* and *Campylobacter* spp., the broth needs to be supplemented; furthermore, enrichment of the incubation atmosphere with CO_2 and an extension of the incubation time may be necessary for growth. For these species, specific recommendations for medium composition and for test conditions can be found in the CLSI guidelines[1].

Medium

To produce accurate and reproducible results, a number of additional requirements must be fulfilled by the test medium for certain antibiotics or antibiotic/species combinations:

- Correct susceptibility testing of tetracyclines[4,5], daptomycin for gram-positive bacteria[6] and aminoglycosides for *Pseudomonas aeruginosa*[5] in broth medium is dependent on the content of Ca^{2+} and Mg^{2+} ions. Non-cation-adjusted (unsupplemented) MHB contains in general inadequate amounts of Ca^{2+} and Mg^{2+} ions (information given by manufacturer). The broth, therefore, needs to be supplemented with divalent cations when testing the abovementioned antibiotics and/or antibiotic-species combinations. The final concentration should be 20–25 mg Ca^{2+} and 10–12.5 mg Mg^{2+} per liter[1], which reflects the divalent cation concentration in blood. Cation-adjusted MHB is commercially available and only needs to be further supplemented with Ca^{2+} in case of daptomycin susceptibility testing, as the recommended calcium concentration for testing this antibiotic is 50 mg l^{-1} (ref. 1). Please note however that cation-adjusted MHB should not be used when testing the activity of cationic antimicrobial peptides, as the presence of Ca^{2+} and Mg^{2+} ions causes a substantial inhibition of the cationic peptides' activity[7,8].
- Mueller–Hinton agar (MHA) needs to be supplemented with 2% (wt/vol) sodium chloride for testing susceptibility of *Staphylococcus aureus* to methicillin, oxacillin and nafcillin[1]. Methicillin-resistant *S. aureus* (MRSA) are often heteroresistant with resistant and susceptible cells in the same culture and supplementation with NaCl enhances the expression of heterogeneous resistance[9].
- To avoid the adsorption of dalbavancin to plastic surfaces, the addition of polysorbate 80 to broth at a final concentration of 0.002% (vol/vol) is recommended. Refer to the CLSI guidelines[1] when testing this antibiotic.
- High levels of thymidine and thymine interfere with susceptibility testing of sulfonamides and trimethoprim[10]. Contrary to the Difco MHB (not cation-adjusted), the BBL MHB (not cation-adjusted) is not explicitly formulated to have a low thymine and thymidine content. So, according to the manufacturer, only the former may be used for broth dilution antimicrobial susceptibility testing.
- Tigecycline is prone to oxidation, and it seems that its activity is affected by the amount of dissolved oxygen in the medium, which increases with the age of the broth. So, for broth dilution MIC tests with tigecycline, it is necessary to use fresh cation-adjusted MHB (<12 h after autoclaving)[11].

Bacteria

The bacteria subjected to antimicrobial susceptibility testing must be isolated in pure culture and should have been identified at the genus and species level. Most organisms are available from hospital laboratories, the American Type Culture Collection or other national collections (see **Table 1**).

Inoculum

The standardization of the bacterial cell number used for susceptibility testing is of critical importance for obtaining accurate and reproducible results. The recommended final inoculum size for broth dilution is 5×10^5 colony-forming units (cfu) ml^{-1}; the

TABLE 1 | Control organisms for antimicrobial susceptibility testing.

	Identical to ATCC strain
Escherichia coli ATCC 25922	NCTC 12241, CIP 76.24, DSM 1103
Pseudomonas aeruginosa ATCC 27853	NCTC 12934, CIP 76.110, DSM 1117
Staphylococcus aureus ATCC 29213	NCTC 12973, CIP 103429, DSM 2569
Enterococcus faecalis ATCC 29212	NCTC 12697, CIP 103214, DSM 2570

ATCC, American Type Culture Collection, P.O. Box 1549, Manassas, VA 20108, USA; NTCT, National Collection of Type Cultures, Health Protection Agency, 61 Colindale Avenue, London NW9 5EQ, UK; CIP, Collection de l'Institut Pasteur, 25–28 Rue de Docteur Roux, 75724 Paris Cedex 15, France; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7B, 38124 Braunschweig, Germany.

appropriate cell number in agar dilution experiments is set at 10^4 cfu per spot.

Higher inocula can lead to an increase in the MIC particularly if the tested bacterium produces an enzyme capable of destroying the antibiotic. An inoculum effect (e.g., an eightfold or greater MIC increase upon testing with a 100-fold higher inoculum than recommended) is frequently seen when testing β-lactam susceptibility for isolates that produce β-lactamases that are able to inactivate β-lactam antibiotics[12]. Lighter inocula than recommended may give artificially lower MICs. Use of inocula with $<5 \times 10^5$ cfu ml^{-1} in broth microdilution can lead to false-susceptible results as described for the detection of methicillin resistance in *S. aureus*[13] and for resistance to certain β-lactams in β-lactamase overproducing *Klebsiella oxytoca* isolates[14].

A fresh pure culture should be used for the preparation of the inoculum. To avoid the selection of an atypical variant clone, bacteria from four to five normal-appearing colonies are taken to prepare a bacterial suspension with a density equivalent to 10^8 cfu ml^{-1}, which is later used for inoculation.

Several options are available for the generation of the bacterial suspension (direct colony suspension into liquid and growth methods using either fresh or overnight cultures). The density of the cell suspension can be assessed spectrophotometrically for testing a small number of different bacterial isolates ($n < 5$). For a larger number of different bacterial isolates, to save time, a turbidity standard can be used as a visual yardstick. Comparison between the standards and the turbidity of the bacterial suspensions will in fact point the researcher toward the appropriate dilution for the suspension. The turbidity of a so-called McFarland 0.5 standard is equal to $1–2 \times 10^8$ cfu ml^{-1}. McFarland 0.5 turbidity standards are commercially available from several manufacturers (e.g., bioMerieux, cat. no. 70900 or Scientific Device Laboratory). Alternatively, a $BaSO_4$ turbidity standard equaling the McFarland 0.5 standard can be prepared as described below.

Once the bacterial suspension is adjusted, it must be used within 30 min to avoid changes in the cell number[2].

All protocols described here contain a paragraph on how to determine whether the correct inoculum density was used for the susceptibility testing. If the MIC tests are carried out in a laboratory on a routine basis, the cell counts of the inoculum need to be determined only periodically. For all other users, we recommend validating the accuracy of procedures for every test.

Quality control

To verify that the susceptibility results are accurate, it is necessary to include at least one control organism with every batch of MIC determinations. Control organisms are available from different strain collections (**Table 1**). The MICs for routinely used antibiotics for the quality control organisms are published[1,2] and the test values for the control strains should be within the published range to be considered acceptable.

Limitations

The MIC value does not give an indication of the mode of action (cidal or static) of the antimicrobial agent. Within the MIC well or tube or on the agar plate with no visible growth, there may still be viable cells if the drug had a bacteriostatic effect on the bacterial species tested. Growth may resume after the removal of the drug. Alternatively, there may be partial inhibition resulting in impaired and reduced growth rates and consequently no visible growth within the time given. Both phenomena are different from the action of a bactericidal drug, which causes irreversible damage leading to cell death.

Furthermore, even with the knowledge of the mode of action of an antimicrobial agent, the MIC value alone is a poor predictor of the efficacy of the drug *in vivo*. Factors that affect the response to therapy are far more complex and include host defense mechanisms, underlying diseases of the patient, the site of infection, and the pharmacokinetic and pharmacodynamic properties of the drugs[15].

Alternative method for determining MIC values

MICs for commonly used antibiotics can be obtained using an agar diffusion method with commercially available strips containing an exponential gradient of antibiotic (Etest; AB Biodisk). The antibiotic diffuses into the agar medium inoculated with a lawn culture of the test organism. After overnight incubation, the MIC is read at the point of intersection of an elliptical growth inhibition zone with the strip that has an MIC scale printed on it. This test has been evaluated for a variety of bacteria/antibiotic combinations[16–21] and is rapid and easy to use; however, it is limited to the antibiotic range supplied by the manufacturer and is an expensive test to use for screening.

Several automated systems for antimicrobial susceptibility testing and identification of clinically relevant bacteria are now commercially available, e.g., Phoenix Automated Microbiology System (BD Diagnostic Systems), the VITEK 2 System (bioMérieux) and the MicroScan WalkAway-96 System (Dade Behring). These systems are cost effective for clinical laboratories with a high throughput of clinical specimens. Fully automated systems reduce the time for setup and, depending on the system, also reduce the time to produce results compared to conventional tests. Moreover, they offer convenient interfaces with laboratory and hospital information systems. However, when testing certain organism-antimicrobial combinations limitations on the accuracy of the assessment of MIC values by these systems are known[22,23].

Experimental design

As there are alternative routes for generating the bacterial suspension with different time requirements and alternative methods to determine MIC values with potential pause point options that require advance planning of the workflow, the experiment should be carefully designed by the user before starting the protocol. The flowchart in **Figure 2** illustrates how the different stages are coordinated. Please examine this figure carefully to make an informed choice as to which experimental approach to embark on.

MATERIALS

REAGENTS

- MHB (Difco; BD Diagnostics, cat. no. 275730) sterilized by autoclaving
- Mueller–Hinton II broth (cation-adjusted (CAMHB); BBL, BD Diagnostics, cat. no. 212322) sterilized by autoclaving
- MHA (Difco; BD Diagnostics)
- Agar, Technical (Difco; BD Diagnostics, cat. no. 281230)
- Solution A: 0.02% acetic acid (Fisher) containing 0.4% BSA (Boehringer Mannheim)

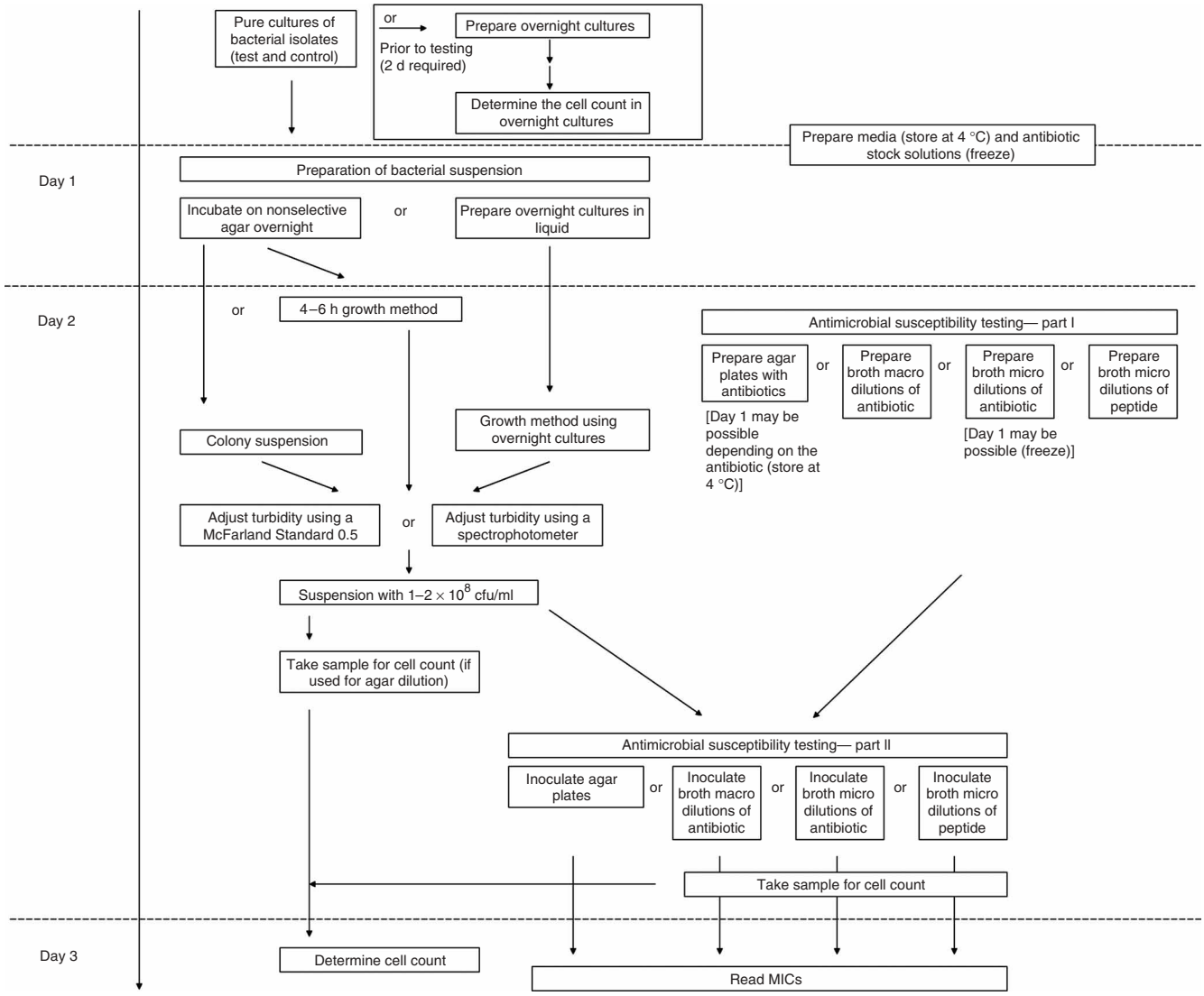

Figure 2 | Flowchart for antimicrobial susceptibility testing.

- Solution B: 0.01% acetic acid containing 0.2% BSA
- Preparation of McFarland Standard 0.5: $BaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, cat. no. B0750), H_2SO_4 (Fluka, cat. no. 84721) ! **CAUTION** H_2SO_4 is very corrosive/toxic; handling must be performed under the hood; wear acid-resistant gloves and protective clothing (see REAGENT SETUP).
- Cation adjustment: $MgCl_2 \cdot 6H_2O$ (Fluka, cat. no. 63072), $CaCl_2 \cdot 2H_2O$ (Fluka, cat. no. 21101)
- Physiological saline [0.9% (wt/vol) NaCl] sterilized by autoclaving

EQUIPMENT

- Spectrophotometer suitable for measuring at wave lengths of 600 and 625 nm
- For antibiotics: sterile 96-well microtiter plates. We recommend polystyrene plates (BD Falcon; Fisher Scientific, cat. no. 351177) for most antimicrobials as these are easier to read at the end of the experiment
- For cationic antimicrobial agents such as peptides: 96-well polypropylene microtiter plates (Costar, cat. no. 3790) ! **CAUTION** Avoid tissue culture treated or polystyrene plates as these are strongly negatively charged and will nonspecifically bind peptides.
- Eppendorf polypropylene microcentrifuge tubes, 1.5 ml (Fisher Scientific, cat. no. 05-402-24B), sterilized
- Screw-capped glass tubes, 13 × 100 mm (Fisher Scientific, cat. no. 14-930-10A)
- 48-Pin replicator (Boekel Scientific, cat. no. 140501) for inoculating agar dilution plates
- Shaker, suitable for test tubes 13 × 100 mm
- Parafilm (Pechiney Plastic Packaging; Fisher Scientific, cat. no. 13-374-10)
- Glass tubes, 13 × 100 mm (VWR International, cat. no. 47729-572) with cap (Utech Products, cat. no. 1017622), sterilized
- Erlenmeyer flasks
- Sterile petri dishes, 15 × 100 mm (Fisherbrand; Fisher Scientific, cat. no. 08-75-712)
- 0.2-µm pore size cellulose acetate filters (Nalgene; Fisher Scientific, cat. no. 190-2520)
- Cell spreader (Fisherbrand; Fisher Scientific, cat. no. 08-100-11)
- Inoculation loop or cotton swabs (sterilized by autoclaving)
- Vortex mixer

REAGENT SETUP

Preparation of McFarland 0.5 $BaSO_4$ turbidity standard Prepare a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution (0.048 mol l^{-1} $BaCl_2$) and a 1% (vol/vol) sulfuric acid (H_2SO_4) solution (0.18 mol l^{-1}, 0.36 N). Add 0.5 ml of the 1.175% $BaCl_2$ solution to 99.5 ml of the 1% H_2SO_4 solution with constant stirring to get a suspension. Measure the optical density of the turbidity standard using a spectrophotometer with a 1 cm light path length. The correct absorbance at 625 nm should be 0.08–0.13. Aliquot 4–6 ml into screw-capped glass tubes. The tubes should have the same size as those for preparing the bacterial suspension for inoculation. Seal tubes tightly with Parafilm and store in the dark at room temperature (20–23.5 °C). Standards are

stable for at least a month. ! CAUTION H_2SO_4 is corrosive/toxic; wear appropriate safety clothing when handling concentrated H_2SO_4.

Preparation of antibiotic-free nutrient-rich agar plates Prepare agar medium according to the manufacturer's instructions. Alternatively, use nutrient-rich broth according to the manufacturer's instructions and add 1.7% agar (17 g agar per liter) before autoclaving. Approximately 20–25 ml is necessary to pour one 15 × 100 mm petri dish. After autoclaving (e.g., 121 °C, 15 min, 1 bar), cool the medium to 50–60 °C. Pour agar into the petri dishes and allow to set. Dry the surface of the agar plates either in an incubator or in a laminar air flow hood for 30 min with the lid ajar. Store agar plates in plastic bags in inverted position (bottom facing up) at 4 °C.

Adjustment of cation content of MHB medium (20–25 mg Ca^{2+} and 10–12.5 mg Mg^{2+} per liter) Prepare a 10 mg ml^{-1} Mg^{2+} stock solution by dissolving 8.36 g of $MgCl_2 \cdot 6H_2O$ in 100 ml deionized water. Prepare a 10 mg ml^{-1} Ca^{2+} stock solution by dissolving 3.68 g of $CaCl_2 \cdot 2H_2O$ in 100 ml deionized water. Filter-sterilize both stock solutions using 0.2-µm pore size cellulose-acetate filters. Prepare MHB according to the manufacturer's instructions, autoclave and cool the medium to 2–8 °C before the addition of the cation solutions. Add 100 µl of stock solution per 1 mg l^{-1} needed for 1 l of medium. For example, add 2 ml of Ca^{2+} stock solution if 20 mg needs to be added to 1 l MHB.

Stock solution of the antimicrobial agent Antimicrobial agents should be stored in the dark at 4 °C in sealed containers containing a desiccant unless recommended otherwise by the manufacturer. We advise the storage of antimicrobial peptides at 20 °C. Before weighing the antimicrobial agent, let the container warm at room temperature for ~2 h to avoid condensation of water on the powder. Antibiotics are generally supplied by the manufacturer with the information about the potency (µg per mg powder) that needs to be taken into consideration when weighing the agent. For antibiotics, prepare a stock solution at 10 mg ml^{-1} when planning to use it for agar dilution (Step 4A). For broth dilution tests (Steps 4B and 4C) set up a stock solution with a concentration at least ten times higher than the highest concentration to be tested. For testing antimicrobial peptides (Steps 4D and 4E), prepare a 20-fold concentrated stock solution. Use the following formula for calculating the right amount of antibiotic to be weighed (this does not apply to antimicrobial peptides):

$$W = \frac{(C \times V)}{P}$$

where, W = weight of antimicrobial agent in milligram to be dissolved; V = desired volume (ml); C = final concentration of stock solution (µg ml^{-1}); P = potency given by the manufacturer (µg mg^{-1}).

Use sterile containers and spatula for weighing the antimicrobial agent and dissolve in sterile distilled water or in the recommended solvent. A list of solvents for frequently used antibiotics is found in ref. 24. Antibiotic solutions can be filter-sterilized using a 0.2-µm pore size cellulose-acetate filter. However, it has to be ascertained that the antibiotic does not bind to cellulose acetate (information that is sometimes given by the manufacturer). Do not filter-sterilize antimicrobial peptides, which tend to bind to anionic surfaces like cellulose acetate. Always use the fresh antibiotic stock solution for broth microdilution if it is planned to freeze the antibiotic containing microtiter plates at 70 °C for later usage. For other applications, aliquot the stock solution. The volume of the aliquots depends on the downstream applications and in general one aliquot should contain the volume needed for one test. Containers need to be sterile, cold resistant and should seal tightly (e.g., for smaller volumes sterile Eppendorf tubes can be used). Store the aliquots at 20 °C or below unless it is instructed otherwise by the manufacturer. Most antimicrobial agents are stable at −60 °C for at least 6 months. Stability and storage information for frequently used antibiotics can be found in ref. 24. Do not refreeze thawed stock solutions. Some antimicrobials, particularly β-lactams antibiotics, can degrade when thawed and refrozen repeatedly[1].

PROCEDURE

Preparation of the bacterial suspension ● TIMING ~5 min per isolate/overnight pause point

1| Streak the bacterial isolates to be tested (including a control organism) onto nutrient-rich (e.g., Mueller–Hinton) agar plates without inhibitor to obtain single colonies.

2| Incubate plates for 18–24 h at 37 °C.

3| Different methods for the preparation of the inoculum can be used. Direct suspension of overnight colonies into broth or sterile saline solution (option A) is a very convenient method that can be used for most bacterial species. It is particularly recommended for fastidious organisms such as *Streptococcus* spp., *Haemophilus* spp. and *Neisseria* spp. For some strains within a species, unpredictable clumping can occur with option A. Consequently, when colonies are difficult to suspend and an even suspension is difficult to achieve, freshly grown broth cultures can be diluted (option B). As an alternative to a freshly grown culture, an overnight broth culture can also be used (option C) according to the user's preference. This method is not part of CLSI or EUCAST recommendations; however, the option to use overnight cultures is given in the guide to antimicrobial susceptibility testing of the British Society of Antimicrobial Chemotherapy[24].

(A) Colony suspension method ● TIMING ~5 min per isolate

(i) Prepare the antibiotic or peptide dilutions.

(ii) For each isolate, select three to five morphologically similar colonies from the fresh agar plate from Step 2 and touch the top of each selected colony using a sterile loop or cotton swab. Transfer the growth into a sterile capped glass tube containing sterile broth or saline solution. Mix using a vortex mixer.

(iii) Turbidity can be assessed visually by comparing the test and the McFarland Standard. Mix the McFarland 0.5 $BaSO_4$ standard vigorously using a vortex mixer. Please note that commercially available standards containing latex particles should not be vortexed, but gently inverted several times. Comparison against a white background with contrasting black lines and good lighting are helpful. Alternatively, the turbidity can be verified measuring the absorbance of the suspension spectrophotometrically. The absorbance should be in the same range as that of the McFarland standard 0.5 (OD625 nm should be at 0.08–0.13).

(iv) Adjust the suspension's turbidity to that of a McFarland Standard 0.5 by adding sterile distilled water, saline or broth, if the turbidity is too high, or by adding more bacterial material if is too low.

▲ CRITICAL STEP After turbidity adjustment, the bacterial suspension should be used within 30 min, as the cell number might otherwise change.

(B) Growth method ● TIMING 2–6 h

(i) For each isolate, select three to five isolated colonies of the same morphological appearance from the fresh agar plate (from Step 2), touch them using a sterile loop or swab and transfer into a tube containing 3–4 ml of a suitable nutrient-rich medium (e.g., MHB). Mix using a vortex mixer.

(ii) Incubate the broth at 35–37 °C in a shaker at 225 r.p.m. until it reaches a visible turbidity that is equal to or greater than the turbidity of a McFarland Standard 0.5. Please note that the culture growth will require 2–6 h depending on growth rate of the bacteria to be tested. This pause period can be used to prepare the antibiotic or peptide dilutions.

(iii) Check the turbidity according to Step 3A(iii).

(iv) Adjust the suspension's turbidity to that of a McFarland Standard 0.5 by adding sterile distilled water, saline or broth, if the turbidity is too high, or by further incubating the bacterial culture if the turbidity is too low.
▲ CRITICAL STEP After turbidity adjustment, the bacterial suspension should be used within 30 min as the cell number might otherwise change.

(C) Growth method using overnight cultures ● TIMING ~5 min per isolate Steps (i)–(iii)/overnight pause point/~15 min per isolate Steps (iv)–(viii)/overnight pause point/~20 min per isolate Steps (ix)–(xiv)/overnight pause point/~5 min per isolate Steps (xv)–(xvi)

(i) Determine the correlation between OD600 of an overnight culture and the microbial number for each isolate performing the following steps (Steps (ii) through (xi)) in triplicate. This procedure is necessary because liquid overnight cultures may prove less viable than freshly grown broth cultures making a simple turbidity test unreliable.

(ii) Select one colony from the fresh agar plate, touch it using a sterile loop or swab and inoculate a sterile glass tube with cap (13 × 100 mm) containing 3 ml nutrient-rich medium (e.g., MHB).

(iii) Incubate the tubes overnight in a shaker at 225 r.p.m. at 37 °C.

(iv) Measure the OD600 of the overnight cultures. Because of the loss of linearity at OD600 values >1.0, it is necessary to dilute the sample until the OD600 value is below 1.0. For most bacterial species, a dilution of 1:30 might be appropriate (33 µl in 967 µl broth).

(v) Dilute the overnight culture 1:100 using sterile tubes and nutrition-rich broth or sterile saline solution (dilution: 10–2)

(vi) Dilute stepwise the solution from Step (v) five times 1:10, using sterile tubes and nutrition rich broth or sterile saline solution until you reach a dilution of 10–7.

(vii) Plate 100 µl of the last four of the 1:10 dilutions (10–4 to 10–7) evenly onto nutrient-rich agar plates using a sterile cell spreader starting with the dilution 10–7.

(viii) Incubate plates overnight at 37 °C.

(ix) Count colonies on plates (taking into account only plates with up to 500 colonies).

(x) It is best to determine the relationship between OD600 and the microbial number basing the calculation on plates displaying a number of colonies between 100 and 400. Higher numbers do not accurately represent the original suspension as errors created by coincidence of colonies and nutrient limitation lead to numbers that are lower than expected. Relying on fewer than 100 colonies might, on the other hand, lead to incorrect conclusions due to the addition of statistical and methodological errors while performing the dilutions.

(xi) Calculate the cfu per ml that were in the overnight culture according to the following formula:

$$N = \frac{C \times 10}{10^{-D}}$$

where, N = cfu ml^{-1}; C = number of colonies per plate; D = number of the 1:10 dilution.

(xii) Average the results from three tests performed with the microbial isolate and correlate this number with the OD600 value obtained from the overnight culture. This relationship holds true for subsequent cultures of the same bacterium grown in the same way.

(xiii) For the generation of the overnight cultures to be used for preparation of the bacterial suspension for MIC tests, select three to five morphologically similar colonies for each isolate from a fresh agar plate. Touch the top of each selected colony using a sterile loop or cotton swab. Inoculate a sterile glass tube with cap (13 × 100 mm) containing 3 ml nutrient-rich medium (e.g., MHB).

(xiv) Incubate overnight at 37 °C. Before starting with Step 3C(xv), prepare the antibiotic or peptide dilutions.

(xv) Measure the OD600 of the overnight culture (dilute your sample until OD600 value is <1.0).

(xvi) Calculate a dilution factor of the overnight culture using the correlation from Step 3C(xii) to obtain a solution that will contain 1 × 108 cfu ml^{-1} and adjust accordingly.
▲ CRITICAL STEP After turbidity adjustment, the bacterial suspension should be used within 30 min as the cell number might otherwise change.

Antimicrobial testing

4| Broth and agar dilution are routinely used methods for antimicrobial susceptibility testing. As larger volumes of antimicrobial agents are needed for agar dilution, this method is applicable to antibiotics available in ample amounts. The main advantage of the agar dilution (option A below) is the option to test a large number of bacterial isolates simultaneously under exactly identical conditions (e.g., 48 spots can be placed onto one agar plate using petri dishes of 100 × 15 mm). Agar dilution is not the method of choice if the susceptibility to a wide range of different antibiotics is to be tested on a smaller number of bacterial isolates. Broth dilution (options B and C below) is equally applicable for either testing different antibiotics or a large number of bacterial isolates. Using a microtiter format has the advantage that plates can be prepared in advance, stored at 20 °C and can be used at the time point needed. Broth macrodilution (option B) requires large volumes of antibiotic. We recommend using broth microdilution (option C) for most routine procedures. Please note that neither option B nor option C, but modified versions of the microdilution procedure (options D and E) should be used for antimicrobial peptides and highly cationic antibiotics (e.g., polymyxin B, gramicidin S, cecropin). The modifications in the microdilution procedure for antimicrobial peptides and other cationic antimicrobials (options D and E) are intended to avoid the possibility of the antimicrobials binding to the plastic dishes and furthermore to reduce the amount of substance needed for testing, while still producing acceptable results. **Table 2** illustrates the substantial variations in MIC that can occur with different peptide solvents and when the incorrect type of test vessel is utilized. Some peptides can precipitate when diluted into Mueller–Hinton medium which can be avoided by adding acetic acid/BSA[25]. Whether antimicrobial peptides require (option D) or not (option E) the addition of acetic acid/BSA has to be determined by trial and error. If the MIC of an antimicrobial peptide determined by options D and E for the same test isolate is two or more dilution steps higher for option E (no BSA/acetic acid) compared to option D (with the addition of acetic acid/BSA) it can be assumed that the antimicrobial peptide requires acetic acid/BSA. Irrespective of the method used, twofold dilution series with around 10–12 dilutions up and/or down from 1 mg l^{-1} (1 μg ml^{-1}) are conventionally used for most antibacterial agents. However, the concentration range will depend on the bacterial isolates to be tested and the antibacterial agent (see ref. 24 for suggestions).

(A) Agar dilution ● TIMING 4–6 h

(i) Prepare MHA medium according to the manufacturer's instructions. Alternatively, use the MHB according to the manufacturer's instructions and add 1.7% agar (17 g agar per liter) before autoclaving. Around 25 ml is necessary to pour one 15 × 100 mm petri dish to produce the required depth of 3–4 mm.

(ii) After autoclaving (e.g., 121 °C, 15 min, 1 bar), cool the medium to 50 °C.
▲ **CRITICAL STEP** Higher temperatures might inactivate the antibiotic, whereas at lower temperatures the agar will begin forming solid clumps making it difficult to pour homogeneous plates.

(iii) As the medium cools down, calculate the amount of antibiotic solutions (10, 1 and 0.1 mg l^{-1}) needed. **Table 3** gives an example of the volumes needed for a concentration range between 0.125 and 128 mg l^{-1} for one 25 ml agar plate per concentration. Adjust if a different concentration range is needed. The volumes of antibiotic and agar can also be varied depending on the number of plates to be poured. As each agar plate can be used for up to 48 tests, more than one plate might be necessary if a larger amount of isolates is going to be tested.

(iv) Label 12 (adjusting the number if more or less than 12 different concentrations are going to be tested) sterile containers appropriately (e.g., glass Erlenmeyer flasks closed with metal caps, tinfoil caps or cotton buds) with the final antibiotic concentration. The size of the flask depends on the total volume needed; e.g., use a 100-ml flask for 50 ml of agar.

(v) Dilute the 10 mg ml^{-1} antibiotic stock solution 1:10 in sterile broth or water to achieve a 1 mg ml^{-1} solution.

(vi) Dilute the 1 mg ml^{-1} solution 1:10 in sterile broth or water to achieve a 0.1 mg ml^{-1} solution.

(vii) Dispense appropriate amounts of antibiotic solution (**Table 3**) into the respective containers.

(viii) For each agar plate, add 25 ml agar (now at a temperature of ~50 °C) into the container, mix well (avoid bubbles) and pour 25 ml into a petri dish labeled with the respective antibiotic concentration. Use a 25-ml pipette if the container contains >25 ml agar in situations where more than one plate with the same antibiotic concentration is going to be poured (see Step 3A(iii))

(ix) Pour a control agar plate without any antibiotic. Adjust the number if necessary (see Step 3A(iii)).

TABLE 2 | Effect of MIC determination conditions on the MIC value recorded for cationic antimicrobial peptides.

Peptide solvent	Microtiter plate	MIC of CP-26 (μg ml^{-1}) (α-helical peptide)	MIC of CP-11CN (μg ml^{-1}) (extended peptide)
Acetic acid/BSA	Polypropylene	0.5	2.0
dH_2O	Polypropylene	1.0	4.0
Acetic acid	Polypropylene	2.0	4.0
Acetic acid/BSA	Polystyrene*	1.0	4.0
dH_2O	Polystyrene*	8.0	16.0

*Described as 'tissue-culture treated'.

(x) Allow agar to set.

(xi) Dry the surface of the agar plates either in an incubator or in a laminar air flow hood for 30 min. Leave the lid ajar.

(xii) Mark the bottom of the agar plates to define an orientation.

■ **PAUSE POINT** Plates should ideally be used on the same day. However, depending on the antibiotic used (see information occasionally provided by the manufacturer or ref. 26), the plates can be stored at 4–8 °C for up to 5 d.

(xiii) Mix the bacterial suspension, adjusted to 1 × 10^8 cfu ml^{-1} from Step 3A(iv), Step 3B(iv) or Step 3C(xvi), by vortexing and dilute it 1:10 into a cavity of a sterile 96-well microtiter plate by pipetting 10 µl into a well containing 90 µl of sterile broth or saline.

TABLE 3 | Antibiotic dilution chart for agar dilution method.

Antimicrobial concentration (mg l^{-1})	Volume of antibiotic stock solution[a] (µl)	Final concentration when adding 25 ml agar
10	320	128
10	160	64
10	80	32
10	40	16
1	200	8
1	100	4
1	50	2
0.1	250	1
0.1	125	0.5
0.1	62.5	0.25
0.1	31.25	0.125

(xiv) Repeat for each bacterial isolate to be tested. Be sure to make a note of the content of each well and to inoculate the microtiter plate in a way that the inocula can be transferred to the agar plates using a 48-pin replicator. For up to 48 tests, inoculate only within rows A–H, columns 1–6 of the microtiter plate. As an alternative to the replicator, a multichannel micropipette set at 1 µl can be used to deliver the spots.

▲ **CRITICAL STEP** Make sure that the required number of bacterial cells is going to be transferred. A 48-pin replicator with 1.5 mm pins delivers 1 µl. The final inoculum for a spot with a size of 5–8 mm should deliver the desired cell density of around 10^4 CFU per spot. Adjust the dilution of the bacterial suspension in Step 4A(xiii) if a replicator with different pin diameter is used.

(xv) Sterilize the 48-pin replicator by soaking the pins in 95% ethanol and passing them through a Bunsen burner flame. Hold the pins in upright position until the flame extinguishes. Let the pins cool in an inverted position to maintain sterility.

! **CAUTION** Care should be taken to hold the flaming replicator away from hair and clothing. In case the alcohol bath accidentally ignites, have a glass plate available that can be used to cover the alcohol bath to extinguish the fire.

(xvi) Place the sterilized replicator into the microtiter plate to soak the pins and transfer it onto the agar plate. Start by inoculating a growth control plate without antibiotic. Make sure that each agar plate has the same orientation while inoculating and to make a note of the orientation so that spots on the agar plate can be assigned to the respective isolate tested.

▲ **CRITICAL STEP** Make sure the microtiter plate containing the bacterial suspension is not cross-contaminated by liquid dripping from the pins.

(xvii) Inoculate the antibiotic-containing agar plates starting with the lowest concentration.

(xviii) Let the inoculum spots dry at room temperature before inverting the plates.

(xix) To confirm that the size of the bacterial inoculum was appropriate, determine the viable count of the bacterial suspension used for preparing the initial inoculum. Dilute this suspension 1:100 by pipetting 10 µl into sterile capped Eppendorf tubes containing 990 µl nutrient-rich broth or sterile saline solution (dilution:10^{-2}). Dilute this 10^{-2} solution sequentially 1:10 three times until you reach a dilution of 10^{-5}.

(xx) Plate 100 µl of the last two 1:10 dilutions (10^{-4} to 10^{-5}) evenly onto antibiotic-free nutrient-rich agar plates using a sterile cell spreader.

(xxi) Incubate agar plates at 37 °C for 16–20 h.

(xxii) Count colonies the next day with the same considerations as discussed in Step 3C(x).

(B) Broth macrodilution ● TIMING ~20–45 min per antibiotic

(i) Prepare antibiotic dilutions in sterile MHB in sterile test tubes according to **Table 4**. As the antibiotic solution is later inoculated with an equal amount of bacteria in broth, the dilutions are prepared at a concentration twice the desired final concentration. Start by dispensing sterile broth into twelve sterile 13 × 100 mm tubes closed with metal caps. A single 10 ml pipette can be used to pipette the 9 ml and 3 ml volumes of broth into the respective tubes (see **Table 4**). Use a single 1 ml pipette for pipetting the 1 ml of broth in stages 2, 5, 8 and 11.

(ii) It is possible to use the same pipette for pipetting 1 ml of the antibiotic stock solution into the first test tube. Mix thoroughly using a vortex mixer.

(iii) Use separate pipettes/pipette tips when preparing each of the other antibiotic solutions according to **Table 4**. Mix thoroughly using a vortex mixer.

TABLE 4 | Scheme for preparing antibiotic dilutions to be used in broth dilution susceptibility tests*.

Stage	Antimicrobial concentration (mg l^{-1})	Source	Volume of antibiotic stock solution† (ml) (+)	Volume sterile broth† (ml) (=)	Antimicrobial concentration obtained	Final concentration in test
1	1280	Stock	1	9	128	64
2	128	Stage 1	1	1	64	32
3	128	Stage 1	1	3	32	16
4	128	Stage 1	1	7	16	8
5	16	Stage 4	1	1	8	4
6	16	Stage 4	1	3	4	2
7	16	Stage 4	1	7	2	1
8	2	Stage 7	1	1	1	0.5
9	2	Stage 7	1	3	0.5	0.25
10	2	Stage 7	1	7	0.25	0.125
11	0.25	Stage 10	1	1	0.125	0.06
12	0.25	Stage 10	1	3	0.06	0.03

*Modified from Ericsson and Sherris[28]. †The volumes used can be any multiple of these figures depending on the number of the tests to be performed. For macrodilution, 1 ml of each antibiotic dilution is needed for one bacterial isolate to be tested.

(iv) For every bacterial isolate, label twelve sterile 13 × 100 mm test tubes closed with cotton buds or metal caps with the respective antibiotic concentration to be tested. Also label control tubes for bacterial growth (one for each isolate tested) and a single tube for sterility control for the entire measurement.

(v) Add 1 ml of each antibiotic dilution into one test tube for each isolate to be tested. Fill the control tubes with 1 ml sterile broth without antimicrobial agent. Mix the bacterial suspension adjusted to 1×10^8 cfu ml^{-1} from Step 3A(iv), Step 3B(iv) or Step 3C(xvi) by vortexing, and dilute it by a factor of 1:100 by adding 200 µl bacterial suspension to 19.8 ml sterile MHB in a sterile 50 ml Erlenmeyer flask to prepare a 20 ml inoculum. Mix well. Adjust volumes if necessary.

(vi) Inoculate each test tube containing the antibiotic solution and one control test tube (growth control) with 1 ml of the bacterial suspension from Step 4B(v). This results in the final desired inoculum of 5×10^5 cfu ml^{-1}.
▲ **CRITICAL STEP** Make sure that the bacterial suspension is well mixed before inoculating each tube.

(vii) Remove a 10 µl sample from the growth control tube immediately after inoculating and pipette it into a sterile Eppendorf tube holding 990 µl of sterile saline or broth. Mix well by vortexing. Make a further dilution of this suspension (1:10) by pipetting 100 µl into 900 µl of sterile saline or broth and mix well.

(viii) Plate 100 µl of each of the two dilutions from Step 4B(vii) onto two different nutrient-rich agar plates.

(ix) Incubate agar plates and tubes at 37 °C for 16–20 h, shaking the latter with at 225 r.p.m.

(x) Count colonies the next day with the same considerations as discussed in Step 3C(x).

(C) Broth microdilution ● **TIMING** ~**20–45 min per antibiotic**

(i) Follow Steps 4B(i)–(iii) in order to prepare the antibiotic solutions, and adjust volumes if necessary. Note however that the 96-well microtiter plate format allows only testing ten different concentrations if following the outline of **Figure 3**. For this procedure, 50 µl of each dilution is needed for each bacterial isolate to be tested. In situations where one plate contains only dilutions of one antibiotic permitting the testing of eight different bacterial isolates, a multichannel

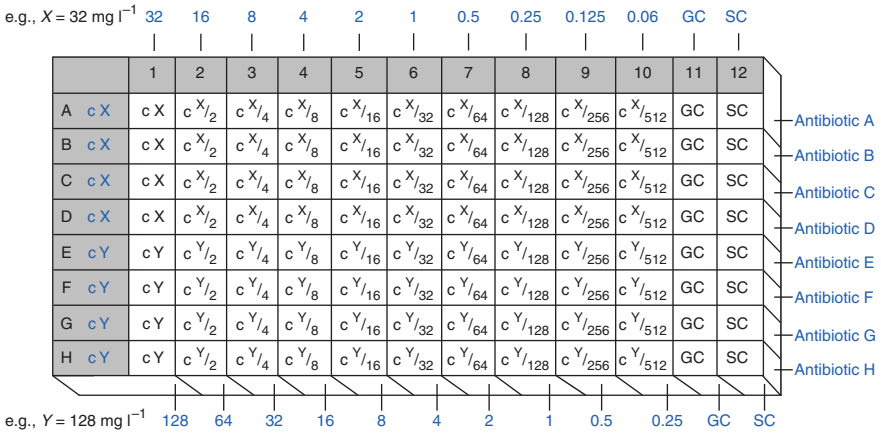

Figure 3 | Outline of the setup of a microtiter plate for antimicrobial susceptibility testing with doubling dilutions of eight different antimicrobial agents in two different concentration ranges with labeling suggestions in blue. c, concentration; X, Y, the highest concentrations tested (mg l^{-1}); GC, growth control (broth with bacterial inoculum, no antibiotic); SC, sterility control (broth only); blue, actual labeling. If more than two concentration ranges (e.g., with further different maximal concentrations c Z or c W, etc.) are going to be tested, write the value for c Z, c W, etc. next to column 1 that contains the highest concentration.

pipette or a multistep pipettor can be used to distribute the solutions. At least 400 µl of each antibiotic dilution is required per plate in this scenario. If a multichannel pipette is used, the liquids can be transferred into sterile petri dishes for dispensing and total volumes of 10 ml or more are recommended.

(ii) Remove a 96-well microtiter plate from its sterile packing. If the plate is not supplied with a lid, cover it with another sterile microtiter plate or with a microtiter plate that has been thoroughly wiped with 70% ethanol at the bottom.
▲ CRITICAL STEP Keep the lid closed when not handling the plate to avoid contamination by airborne pollutants.

(iii) Label the 96-well sterile microtiter plate with the respective antibiotic concentration according to **Figure 3**. Use one row for each test with up to 10 different dilutions. Pipette 100 µl of broth in the sterility control well (column 12) and 50 µl in the growth control well (column 11).

(iv) For each bacterial isolate to be tested, add 50 µl of each antibiotic dilution into the respective well.

(v) Mix the bacterial suspension adjusted to 1×10^8 cfu ml^{-1} from Step 3A(iv), Step 3B(iv) or Step 3C(xvi) by vortexing, and dilute it 1:100 according to Step 4B(v).

(vi) Inoculate each well containing the antibiotic solution and the growth control well with 50 µl of the bacterial suspension. This results in the final desired inoculum of 5×10^5 cfu ml^{-1}.

(vii) Remove a 10 µl sample from the growth control well immediately after inoculating the plate and pipette the sample into a sterile Eppendorf tube holding 990 µl of sterile saline or broth. Mix well by vortexing. Make a further dilution of this suspension (1:10) by pipetting 100 µl into 900 µl of sterile saline or broth and mix well.

(viii) Plate 100 µl of each of the two dilutions from Step 4C(vii) onto two different nutrient-rich agar plates.

(ix) Incubate microtiter plate and agar plates at 37 °C for 16–20 h.
▲ CRITICAL STEP Make sure that the liquid in the microtiter plate does not evaporate during incubation. If the plates are incubated in an incubator with low humidity, it is advisable to put the plates into a container that includes a moist paper towel. Do not stack more than four microtiter plates on top of each other.

(x) Count colonies the next day with the same considerations as discussed in Step 3C(x).

(D) Broth microdilution for antimicrobial peptides that require the presence of acetic acid/BSA ● TIMING **~20–45 min per antibiotic**

(i) Remove two 96-well sterile polypropylene microtiter plates from their packing. If the plate is not supplied with a lid, cover it with another sterile microtiter plate or with a microtiter plate that has been thoroughly wiped with 70% ethanol at the bottom.
▲ CRITICAL STEP Keep the lid closed when not handling the plate to avoid contamination by airborne pollutants.

(ii) Label microtiter plates with the respective peptide dilutions according to **Figure 3**. Use one row for each test with up to 10 different dilutions and a single test isolate.

(iii) Add 20 µl of the 20-fold concentrated peptide stock solutions into the wells of column 1 of plate 1. If the MIC test involves just one peptide instead of different peptides, then this volume (*V*) can be adjusted up to 100 µl that can be used for eight MIC tests (10 µl is needed for each single test) and only one row of the microtiter plate is used for preparing the peptide dilution.

(iv) Add the same volume (*V*) of solution A into the wells in column 1. Mix carefully by pipetting up and down 6–8 times.

(v) Add half the volume (*V*/2) of solution B into columns 2–10.

(vi) Withdraw *V*/2 from each well in column 1 and add this to the corresponding wells in column 2. This makes column 2 a twofold dilution of column 1. Mix carefully by pipetting up and down 6–8 times.

(vii) Repeat the procedure down to column 10. Discard the withdrawn solution from column 10.

(viii) Mix the bacterial suspension adjusted to 1×10^8 cfu ml^{-1} from Step 3A(iv), Step 3B(iv) or Step 3C(xvi) by vortexing and dilute it 1:200 in MHB. This results in the final desired inoculum of 5×10^5 cfu ml^{-1}. Each test requires 1,000 µl of this bacterial solution. Use a sterile petri dish or another sterile reservoir to hold the solution.

(ix) Pipette 90 µl of the microbial solution into each well of column 1–10 of microtiter plate 2. Add 100 µl into column 11 (growth control).

(x) Pipette 100 µl of MHB in the sterility control well (column 12) of microtiter plate 2. Use a sterile petri dish or another sterile reservoir to hold the liquid.

(xi) Transfer 10 µl of each of the 10 wells containing the peptide dilutions in plate 1 into the respective wells of the microtiter plate containing the microbial solution (plate 2). Do not add peptides in column 11 or 12 of plate 2.

(xii) Remove a 10 µl aliquot from the growth control well immediately after inoculating the plate and pipette it into a sterile Eppendorf tube holding 990 µl of sterile saline or broth. Mix well by vortexing. Make a further dilution of this suspension (1:10) by pipetting 100 µl into 900 µl of sterile saline or broth and mix well.

(xiii) Plate 100 µl of each of the two dilutions onto two different antibiotic-free nutrient-rich agar plates. Use a sterile cell spreader to spread the liquid.

(xiv) Incubate microtiter plate and agar plates at 37 °C for 16–20 h or until satisfactory growth is obtained.
▲ CRITICAL STEP Make sure that the volume in the microtiter plates does not evaporate during incubation. If the

plates are incubated in an incubator with low humidity, it is advisable to put the plates into a container that includes a moist paper towel. Do not stack more than four microtiter plates on top of each other.

(xv) Count colonies the next day with the same considerations as discussed in 3C(x).

(E) Broth microdilution for antimicrobial peptides that do not require the presence of acetic acid/BSA

● TIMING ~20–45 min per antibiotic

(i) Remove a 96-well sterile polypropylene microtiter plates from its packing. If the plate is not supplied with a lid, cover it with another sterile microtiter plate or with a microtiter plate that has been thoroughly wiped with 70% ethanol at the bottom.

▲ CRITICAL STEP Keep the lid closed when not handling the plate to avoid contamination by airborne pollutants.

(ii) Label microtiter plates with the respective peptide dilutions according to **Figure 3**. Use one row for each test with up to 10 different dilutions and a single test isolate.

(iii) Pipette 50 µl of MHB into column 2–11 of microtiter plate 2. Add 100 µl into column 12 (sterility control).

(iv) Dilute the 20-fold concentrated peptide stock solution 1:10 into MHB in a polypropylene Eppendorf tube. For each test, 100 µl of this solution is needed.

(v) Add 100 µl of the thus twofold concentrated peptide solution into the wells in column 1.

(vi) Withdraw 50 µl from each well in column 1 and add this to the corresponding wells in column 2. This makes column 2 a twofold dilution of column 1. Mix by carefully pipetting up and down 6–8 times.

(vii) Repeat the procedure down to column 10. Discard the withdrawn solution from column 10.

(viii) Mix the bacterial suspension adjusted to 1×10^8 cfu ml^{-1} from Step 3A(iv), Step 3B(iv) or Step 3C(xvi) by vortexing and dilute it 1:100 according to Step 4B(v).

(ix) Inoculate each well containing the peptide solution and the growth control well with 50 µl of the bacterial suspension (columns 1–10). This results in the final desired inoculum of 5×10^5 cfu ml^{-1}.

(x) Remove a 10 µl aliquot from the growth control well immediately after inoculating the plate and pipette it into a sterile Eppendorf tube holding 990 µl of sterile saline or broth. Mix well by vortexing. Make a further dilution of this suspension (1:10) by pipetting 100 µl into 900 µl of sterile saline or broth and mix well.

(xi) Plate 100 µl of each of the two dilutions onto two different nutrient-rich agar plates. Use a sterile cell spreader to spread the liquid.

(xii) Incubate microtiter plate and agar plates at 37 °C for 16–20 h or until satisfactory growth is obtained.

▲ CRITICAL STEP Make sure that the volume in the microtiter plates does not evaporate during incubation. If the plates are incubated in an incubator with low humidity, it is advisable to put the plates into a container that includes a moist paper towel. Do not stack more than four microtiter plates on top of each other.

(xiii) Count colonies the next day with the same considerations as discussed in Step 3C(x).

? TROUBLESHOOTING

ANTICIPATED RESULTS

Agar dilution (Step 4A)

In order for the test to be valid, the agar plates for the cell count (Step 4A(xxii)) have to be checked to verify that the right number of cfu were used. The presence of around 100–200 colonies on the lower of the two dilutions (10^{-5}) of the initial bacterial suspension is expected when using the correct bacterial suspension density of $1–2 \times 10^8$ cfu ml^{-1}. If the cell numbers are within the desired range, the test can be analyzed to determine the MIC. Also check the antibiotic-free growth control plate. Visible growth needs to occur for the test to be valid.

The MIC is defined as the lowest concentration of the antimicrobial substance that inhibits visible growth of the tested isolate. The growth of a single colony or faint film caused by the inoculum should be disregarded. When growth of the tested organism occurs on all agar plates with antimicrobial agent, the MIC is recorded as greater than the highest concentration tested. The MIC is recorded as less than or equal to the lowest concentration when no growth occurs on any of the agar plates but the growth control.

Broth dilution (Steps 4B–4E)

In order for the test to be valid, the agar plates for the cell count (Steps 4B(x), 4C(x), 4D(xv) or 4E(xiii)) have to be checked to verify that the right number of cfu were used. The presence of around 50 colonies on the lower of the two dilutions (1:1000 of the initial inoculum) is expected when using the correct inoculum density of 5×10^5 cfu ml^{-1}.

Wipe off the bottom of the microtiter plate using a lint-free tissue to remove any condensation that may be present. Determine if there is sufficient growth in the growth control tube or in the growth control well. In order for the test to be valid, a definite turbidity or sediments (button size in microtiter plates ≥ 2 mm), need to occur. Do not read the MIC value if the sterility control (no bacterial inoculum) is turbid.

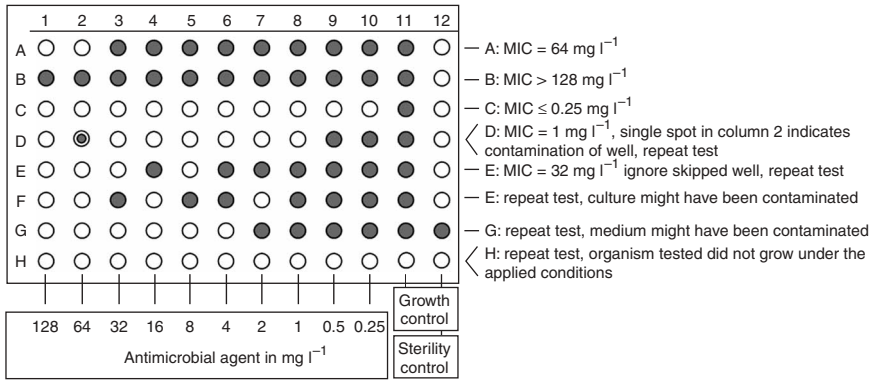

Figure 4 | Interpretation of possible growth patterns in MIC microtiter plates. When growth occurs in all dilutions containing the antimicrobial agent, the MIC is recorded as greater than the highest concentration. The MIC is recorded as less than or equal to the lowest concentration, when no growth occurs in any of the concentrations tested. A clear well in a series of wells with visible growth, e.g., growth at 1, 2 and 8 μg ml^{-1}, but not at 4 μg ml^{-1}, is called a skipped well and should be ignored. Spot growth in isolated wells indicates contamination. The test should be repeated.

The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye (**Fig. 4**). The MIC for the quality control organism should be within one twofold dilution of published values for routinely used antibiotics[1,2].

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

TABLE 5 | Troubleshooting table.

Problem	Possible reason	Solution
Microbial growth in sterility control	Broth was contaminated	Make sure to use an unopened batch of sterilized broth
	Pipettes/pipette tips used were not sterile	Make sure to use sterile pipettes/pipette tips
	Containers to hold solutions were not sterile	Make sure to use sterile containers
	For microdilution: Contamination with microbes in column 11	Avoid spills and aerosol formation when pipetting the bacterial solution
No growth in growth control	Inoculum was too low	Determine the cell count of the bacterial suspension for every test isolate used
	Growth conditions were not appropriate for microbial growth	Check if used broth and incubation temperature is sufficient for microbial growth

● TIMING

Steps 1 and 2: ~5 min per isolate/overnight pause point
Step 3A, colony suspension method: ~5 min per isolate
Step 3B, growth method: 2–6 h
Step 3C, growth method using overnight cultures: ~5 min per isolate (Steps 3C(i)–(iii))/overnight pause point/~15 min per isolate (Steps 3C(iv)–(viii))/overnight pause point/~20 min per isolate (Steps 3C(ix)–(xiv))/overnight pause point/~5 min per isolate (Steps 3C(xv)–(xvi))
Step 4A, agar dilution: 4–6 h
Step 4B, macrodilution: ~20–45 min per antibiotic
Step 4C, microdilution for antibiotics: ~20–45 min per antibiotic
Steps 4D and 4E, microdilution for antimicrobial peptides: ~20–45 min per antibiotic

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