(3) Minimal Bactericidal Concentration Determination Method I (2012 Version)

Minimal bactericidal concentration (MBC) determination method Abolish

1. Scope

This test method shall be applied to antibacterial agents etc. that do not allow a determination of minimal growth inhibitory concentration (MIC).

2. Test Strains[1]

(1) *Staphylococcus aureus* NBRC 12732 (ATCC 6538P)
(2) *Escherichia coli* NBRC 3972 (ATCC 8739)

3. Procurement of Supplies

The reagents, instruments and other supplies used in this test method shall be in conformity with the Japan Industrial Standards or the Japanese Pharmacopoeia unless otherwise specified.

3.1 Instrumentation and equipment

(1) Glass conical flasks (300-ml capacity)
(2) Sterile synthetic resin centrifugal tubes with stoppers[2] (50-ml polypropylene centrifugal tubes)
(3) Sterile synthetic resin tubes with stoppers (13-ml polypropylene tubes)
(4) Glass test tubes (length 170 to 200 mm, outside diameter 18 mm)
(5) Shaking incubator (a model that can be operated at an accuracy within ±1°C)
(6) Constant-temperature chamber (a model that can be operated at an accuracy within ±1°C)
(7) Synthetic resin pipettes or synthetic resin pipetters (allowing dispensing of 0.1 to 1.0 ml)
(8) Conradi rods

3.2 Culture media

(1) Nutrient broth medium (NB medium)

Meat extract	5.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Purified water	1,000 ml
pH	7.0 to 7.2

(2) Nutrient agar medium (NA medium)
NB medium (1) supplemented with 1.5% of agar

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[1] These test microorganisms were chosen to represent Gram-positive and -negative bacteria, respectively.

[2] In view of silver adsorption, supplies made of synthetic resin were used as the containers and instruments that come in contact with the sample or sample liquid. In the experiments by this test method, centrifugal tubes (# 25331CTF50) and tubes (# 25226ST17PO) manufactured by CORNING were used.

4. Test Procedures

4.1 Incubation of the test microorganisms

Place 50 ml of NB medium in a 300-ml conical flask and sterilize. Subsequently, transfer a platinum loopful of each test microorganism to the medium, and perform shaking culture at 35 to 37°C for 18 hours (shaking rate 80 to 100 rpm). Inoculate 0.1 ml of this culture broth to 50 ml of a fresh supply of NB medium, and perform shaking culture at 35 to 37°C for 2 to 4 hours until the cell count[1] becomes 10^8 cells/ml.

If the absorbance at 660 nm does not reach about 0.2 even after 2 to 4 hours of incubation, discontinue the incubation and proceed to re-incubation using a fresh supply of NB medium (50 ml).

4.2 Preparation of liquid inoculum

Centrifuge the culture broth under cooling[2] (below 10°C) at 2,000 rpm or more for 10 minutes, discard the supernatant, and harvest the cells. To eliminate the medium components from the cells harvested, suspend the cells in sterile purified water[3] being cooled below 10°C, centrifuge in the same manner as described above, and again harvest the cells. Repeat these steps in three cycles.

Finally, suspend the cells thus deprived of the medium components in sterile purified water being cooled below 10°C. Dilute this suspension as appropriate to obtain a cell count of 2.0 to 4.0 × 10^6 cells/ml, and use this dilution as the liquid inoculum.

The prepared liquid inoculum must be immediately stored under refrigeration and used within 1 hour. The viable cell count of this liquid inoculum shall be measured by the pour plate culture method at the time of use.

4.3 Preparation of test liquids

Weigh out 64 mg of the sample[4] in a synthetic resin centrifugal tube (50-ml capacity), add 10 ml of sterile purified water, stir using a touch mixer or the like, and then sonicate the mixture over 5 minutes to obtain a thoroughly dispersed liquid.

Retain this dispersion (containing the sample at 6,400 μg/ml) at 30±1°C for 2 hours[5], then stir using a touch mixer or the like, and sonicate the mixture over 5 minutes to obtain a sample dispersion.

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1 When the absorbance at 660 nm is about 0.2, the cell count is about 10^8 cells/ml.

2 If a centrifuge not equipped with cooling apparatus is used, the culture broth shall be cooled with ice for 5 to 10 minutes in advance, and then centrifuged immediately.

3 The purified water used shall be a supply conforming to the Japanese Pharmacopoeia.

4 Because the sample is placed directly in the medium, microorganisms possibly derived from the sample can influence the test results. It is desirable, therefore, that the sample be germfree. To this end, it is recommended that the sample be sterilized in advance if possible (in addition to dry heat sterilization, high-pressure steam sterilization, gas sterilization and other methods of sterilization are available).

① Samples allowing high-temperature heating

Heat the sample at 160 to 180°C for 120 minutes or more to achieve sterilization and drying. After drying, allow the sample to cool in a silica gel-containing desiccator.

② Samples not allowing high-temperature heating

After sterilization by an appropriate method, dry the sample within a range of temperature and time that do not deteriorate the sample. Allow the sample to cool in a silica gel-containing desiccator. In this case, the drying and sterilization conditions shall be indicated. Be sure to avoid inadequate heating temperature and time because incorrectly sterilized samples allow the survival of spores of bacteria of the genus *Bacillus*.

5 To obtain a constant amount of silver dissolved, retain the sample at 30±1°C for 2 hours prior to dilution.

Add 1 ml of this dispersion to a previously provided synthetic resin tube (13-ml capacity) containing 1 ml of 30°C sterile purified water, and mix the contents to obtain a 2-fold dilution[1]. Serially dilute the sample 2 fold in the same manner to obtain a series of dispersions having sample concentrations of 3,200, 1,600, 800, 400, 200, 100, 50, 25, 12.5, and 6.25 μg/ml, respectively. Transfer 1 ml of each dispersion to a synthetic resin tube (13-ml capacity), add 1 ml of the liquid inoculum to each tube, and use these liquids as the test liquids[2].
Separately, provide a synthetic resin tube containing a blank test liquid having a sample concentration of 3,200 μg/ml (not supplemented with the liquid inoculum), and a synthetic resin tube containing a test liquid consisting of the liquid inoculum alone. For reference controls, prepare reference control test liquids by serially diluting the Standard Silver Solution for Atomic Absorption Analysis with sterile purified water[3].

4.4 Cultivation

Set the test liquid to a constant-temperature mechanical shaker[4] at 30±1°C immediately after preparation, and perform shaking culture at a shaking rate of 100 rpm (horizontal shaking) for 1 hour. Indicate the shaking conditions (shaking rate, amplitude) in the Test Results section.
After the shaking culture, uniformly apply 0.1 ml of the test liquid to a plate of NA medium using a Conradi rod, and culture at 35 to 37°C for 48 hours.
For the blank test liquid, uniformly apply 0.1 ml to each of a pair of plates of NA medium using a Conradi rod. Culture one plate as is at 35 to 37°C for 48 hours ("blank 1"[5]). For the other plate, uniformly apply 0.1 ml of a 1000-fold dilution of the test liquid consisting of the liquid inoculum alone using a Conradi rod, and culture at 35 to 37°C for 48 hours ("blank 2"[6]).
For the test liquid consisting of the liquid inoculum alone, determine the viable cell count by the pour plate culture method using NA medium[7] ("blank 3"[8]).

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1 To handle the dilution, a synthetic resin pipette or pipetter shall be used (avoid using glass pipettes).

2 The sample concentrations of the test liquid are 1,600, 800, 400, 200, 100, 50, 25, 12.5, 6.25, and 3.13 μg/ml.

3 Provide reference control test liquids having silver concentrations of 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, 0.1, 0.05, 0.025, and 0.013 μg/ml.

4 Because it is necessary to quickly adjust the test liquid to a temperature of 30±1°C, a constant-temperature water chamber is desirable but caution should be exercised to avoid contamination.

5 "Blank 1" determination is performed to confirm the sterility of the sample.

6 "Blank 2" determination is performed to confirm the growth of the test microorganism without being influenced by the sample.

7 For viable cell counting, standard agar medium, SCD agar medium and the like may be used.

8 "Blank 3" determination is performed to confirm the absence of a non-negligible change from the initial cell count even after shaking culture of the test microorganism at 30±1°C for 1 hour.

4.5 Requirements for test validity

The test shall not be regarded as valid unless all the following requirements for test validity[1] are fulfilled.

(1) In “blank 1 determination”, no microbial growth is observed on the NA medium plate applied with the blank test liquid.

(2) In “blank 2 determination”, not less than 30 colonies of the test microorganism are observed on the plate of NA medium applied with the test liquid.

(3) In “blank 3 determination”, the difference in antibacterial activity value between the initial viable cell count in the test liquid consisting of the liquid inoculum alone and the viable cell count after the cultivation is not more than ±30%[2].

(4) The MBC of silver tested using reference control test liquids shall lie in the range of 0.025 to 0.2 μg/ml[3].

4.6 Judgement

The minimum sample concentration of the test liquid with which no microbial growth is observed after the cultivation shall be established as the minimal bactericidal concentration (MBC). If the number of colonies of the test microorganism that have grown at the time of cell counting by the pour plate culture method is not more than 5, the rating shall be “no microbial growth observed”.

[1] Provided that the requirements for test validity (1) and (2) are fulfilled, it is judged that the sample is germfree, and that the test microorganism has grown without being influenced by the sample.

[2] Provided that these requirements are fulfilled, the test microorganism is considered to retain a cell count at nearly the same as the initial level. Therefore, if no microbial growth is observed in the test, it can be concluded that the test microorganism was killed by the bactericidal action of the sample.

[3] The MBC of silver was determined using reference control test liquids at four laboratories to be between 0.05 and 0.1 μg/ml. These values were divided or multiplied by a common factor of 2 to establish the range of 0.025 to 0.2 μg/ml with a safety margin for both the upper and lower limits.