1. Antibacterial Power Evaluation Test Methods for Antibacterial Agents

(1) Minimal Growth Inhibitory Concentration Determination Method I (2012 Version)

MIC determination method based on liquid medium dilution

1. Scope

This test method shall be applied to slightly soluble antibacterial agents.

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) L-shaped test tubes (made of glass, with stoppers, length 130 to 140 mm, height 110 to 120 mm, outside diameter 18 mm)
(2) Shaking incubator (a model that can be operated at an accuracy within ±1°C)
(3) Constant-temperature chamber (a model that can be operated at an accuracy within ±1°C)

3.2 Culture media

(1) Nutrient agar medium (NA medium)

Meat extract	5.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1,000 ml
pH	7.0 to 7.2

(2) Mueller-Hinton broth medium (MHB medium)[2]

Meat extract	300.0 g
Casamino acid	17.5 g
Soluble starch	1.5 g
Purified water	1,000 ml
pH	7.3±0.1

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

[2] MHB agar medium is used for the MIC determination method of the Japan Society of Chemotherapy. MHB medium is commercially available from Eiken Chemical Co., Ltd. (MHB agar medium only), DIFCO, BBL, Merck and others.

4. Test Procedures

4.1 Incubation of the test microorganisms

Transplant each test microorganism to nutrient agar medium and incubate at 35 to 37°C for 24 hours. Inoculate a platinum loopful of this culture to MHB medium and incubate at 35 to 37°C for 16 to 20 hours.

4.2 Preparation of liquid inoculum

Dilute the culture broth using MHB medium[1] to obtain a cell count of 1.0 to 5.0 × 10^4 cells/ml.

4.3 Preparation of test culture media

Dispense 10 ml of sterile MHB medium to a sterile L-shaped test tube.[2] Add the sample[3] to this medium[4] to prepare a series of test culture media (concentrations obtained by multiplying or dividing the basic concentration of 100 μg/ml by a common factor of 2)[5]. Measure the pH of each medium and make any necessary adjustment if the pH is not in the range of ±0.5 compared to the level before sample addition. Inoculate 0.1 ml of the inoculum liquid[6] to each test culture medium.

4.4 Cultivation

Perform shaking culture to achieve sample homogenization at a shaking rate of 100 to 200 rpm (horizontal or vertical shaking) and an amplitude of 40 to 60 mm at 35 to 37°C for 24 hours.

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[1] The MHB medium used for dilution has been chosen in accordance with the standard method of the Japan Society of Chemotherapy. The cell count of the liquid inoculum was set at nearly the same level as the standard method.

[2] Before using in the test, the L-shaped test tubes shall be sterilized with dry heat (at 160°C to 180°C for 120 minutes or more), the MHB medium shall be sterilized with high-pressure steam (at 121°C for 15 minutes), and the stoppers shall be sterilized by an appropriate method chosen in consideration of their heat resistance etc.

[3] 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*.

[4] It is desirable that control experiments be performed without adding the sample.

[5] Since the specification value is 800 μg/ml, the test may be performed at practically selected concentrations of 3,200, 1,600, 800, 400, 200, 100, 50, and 25 μg/ml.

[6] To obtain nearly the same cell count as the standard method of the Japan Society of Chemotherapy, 0.1 ml of the inoculum liquid is prepared.

4.5 Judgement

After cultivation, macroscopically examine each test culture medium for growth of each test microorganism[1], determine the minimum concentration of the sample showing no growth, and use this concentration as the minimal growth inhibitory concentration.

[1] The minimum cell count allowing the macroscopic confirmation of growth of test microorganism is approximately 10^6 cells/ml.