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CHAPTER 3

Minimal Inhibitory Concentration (MIC) Test and Determination of Antimicrobial Resistant Bacterial

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PRINCIPLE

The agar dilution technique is used to measure qualitatively the *in vitro* activity of an antimicrobial agent against the test bacteria. In this method, graded amounts of antibiotics are incorporated in agar plates and inoculated in spots with the organisms under study. If the organism under study is susceptible to the incorporated antibiotic, no bacterial growth is expected in agar plates with higher amounts of the drugs. Bacterial growth is observed as the antibiotic concentration in the agar plate diminishes. Inhibition of growth at the minimum or lowest concentration of antibiotic is regarded as the end point.

MEDIA

Refer to Appendix 3.1 for the different culture media, solvents, antimicrobial agents, control strains and apparatus needed for the minimal inhibitory concentration (MIC) test.

The minimal inhibitory concentration (MIC) test is performed using Mueller-Hinton Agar (MHA), which is the best medium for routine susceptibility tests because it has good reproducibility, low in sulfonamide, trimethoprim, and tetracycline inhibitors, and gives satisfactory growth of most bacterial pathogens.

The inoculum is prepared using a suitable broth such as heart infusion broth (HIB). This medium is prepared according to the manufacturer's instructions, dispensed in tubes at 3 ml and sterilized. Sterile 0.9% salt solution may also be used.

Media are supplemented with 1-2% sodium chloride (NaCl) if intended for marine species.

Preparation of antimicrobial agent stock solution
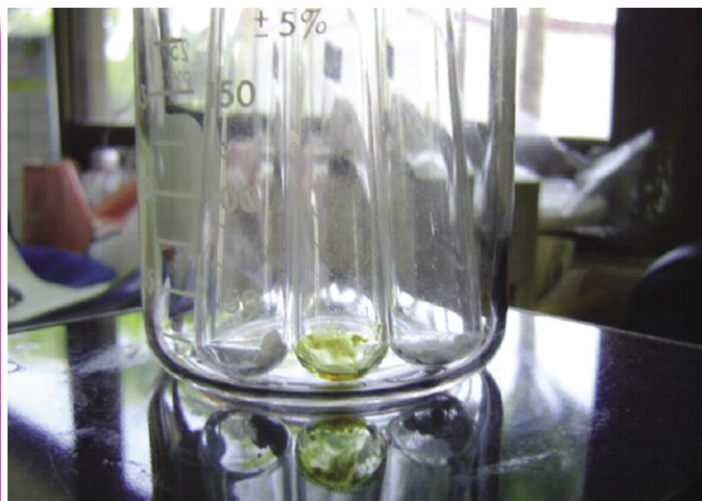
1 Remove the antimicrobial agent from the freezer, and warm to room temperature before opening to avoid condensation of water.

2   Weigh appropriate amount of the powdered antimicrobial agent.

NOTE: *The standard units of activity of the antimicrobial agents may differ widely by the actual weight of the powder or may differ within a drug production lot. Each laboratory must standardize the antimicrobial agent solutions whenever they are being used. See Appendix 3.2 for the formula used to determine the amount of powder needed for a standard solution.*

3   Dissolve the antimicrobial agent powder in solvent to make a concentration of 1,000 μg/ml.


NOTE: *Stock Solutions*

a.   *At least 1,000 μg/ml or 10 times the highest concentration to be tested is to be prepared as an antimicrobial agent stock solution. Some antimicrobial agents are of limited solubility. Therefore, lower concentration may be required. However, in all cases, drug manufacturers provide directions for determining solubility.*

b.   *Some drugs must be dissolved in solvents other than water. In such cases, it is necessary to:*

1)   *Use only enough solvent to dissolve the antimicrobial agent powder;*

2)   *Dilute to the final stock concentration with distilled water as indicated in Appendix 3.3;*

3)   *Prepare appropriate volumes of distilled water, the desired volume for serial dilution.*

4   Dispense the stock solution into sterile diluent using two-fold dilution technique.

NOTE: *As an example, a range of 0.012-100 µg/ml two-fold dilutions may be used as test concentrations for different antibiotics. For Sulfadimethoxine and Sulfadiazine, 400 µg/ml may be used as the highest test concentration (see Appendix 3.4).*
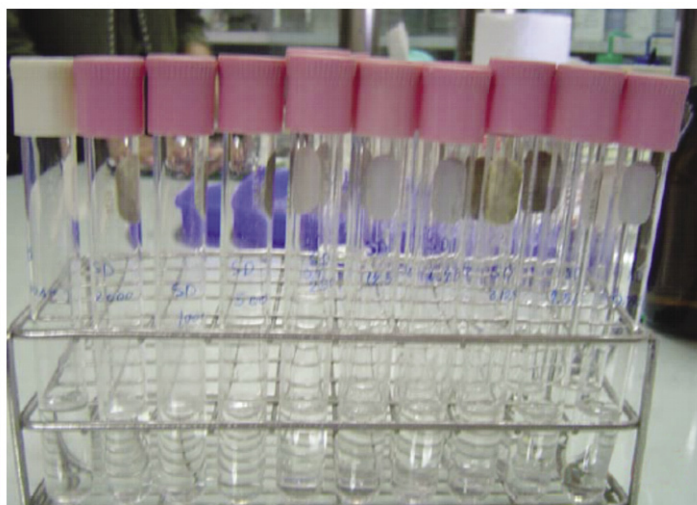
5   Set aside.

### ▬ Preparation of antimicrobial agar plates

1   Label each empty sterile plate in order to identify the antimicrobial agent and their concentrations.

Example: 0. 024 OTC means 0.024 µg/ml of oxytetracycline

2   Place the label on the upper portion of the bottom side of the petri dish to ensure that the plate is inserted at the correct point of the basal stand A of the multiple inoculating apparatus.
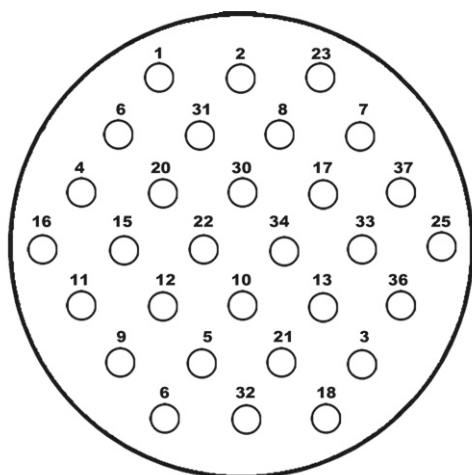
3 Draw a scheme to locate each bacterial strain in a well on a reference paper that will be used to read the results.

4 Prepare MHA following manufacturer's direction and supplement with 1-2% NaCl, if intended for marine organisms. Keep in a water bath at 48-50°C until use.

5 Pipette 1 ml of appropriate dilutions of the test antimicrobial agent (previously prepared, page 32) into the labeled plate (prepared in number 1). Two replicates must be made for each concentration.

6 Pipette 9 ml of MHA (keep warm at 48-50°C), add into the plate with appropriate dilution of the test antimicrobial agent and mix thoroughly.

7 Allow the agar to solidify at room temperature and use the plates immediately after the agar surface has dried completely. If necessary, dry the surface of agar in a laminar flow chamber under UV light, but avoid excessive drying.

Control agar plates/Drug-free agar plates

Prepare control agar plates by pipetting 10 ml of MHA into a sterile petri dish. Do not add any antimicrobial agent. There should at least be 2 control plates.

INOCULUM

Preparation

1 From a pure 18-24 hour bacterial culture get 4-5 isolated colonies (to minimize the risk of picking bacteria which have lost their resistance) and subculture to a tube with 3 ml HIB containing 0.3% yeast extract and 2% NaCl.

2 Shake vigorously in a water bath at 30°C until it achieves or exceeds the turbidity of 0.5 MacFarland standard (prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 0.36 NH_2SO_4; commercially available). The inoculum may also be standardized based on optical density [OD_{625} of 0.08-0.1 (1cm light path)] using a spectrophotometer. This is usually achieved after 18-24 hours.

NOTE: *Standardized inoculum has a concentration of $1\text{-}2 \times 10^8$ cfu/ml.*

3 Dilute the standardized inoculum 1:10 in sterile saline solution to obtain the desired concentration of 10^6 cfu/ml.

4 Pipette 0.1 ml of the 10^6 cfu/ml inoculum and transfer to a well, of a multi-dispenser containing 0.9 ml of HIB with 0.3% yeast extract.

In the absence of a multi-dispenser (photo at right), sterile test tubes of the same size, may be used to hold the diluted standardized inoculum.
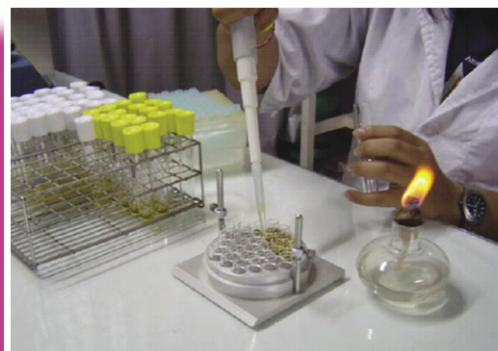
NOTE: *The final concentration of the bacterial suspension is approximately 10^5 cfu/ml well.*

5 Store at room temperature for not more than 2 hours before testing.

Inoculation volume

Inoculate plates with 1-3 μl of the inoculum if automatic multi-dispenser is used and 10 μl if done manually. Inoculated volume should be uniform for good results.

### Inoculation sequence

1 When using automatic multiple inoculating apparatus, drug free or control plate should be inoculated first, followed by the plate with the lowest antimicrobial concentration. Inoculate a second control agar plate last to ensure that there is no contamination or antimicrobial agent carry-over during the inoculation.

2 When inoculating manually, it is only important to include a drug free or control plate at the beginning of the inoculation series.

Inoculation

1 Place a completely dried agar plate on the basal stand A of the multiple inoculating apparatus. Insert the plate on the basal stand A of the multiple inoculating apparatus in such a way that the label on the plate is facing the front direction.
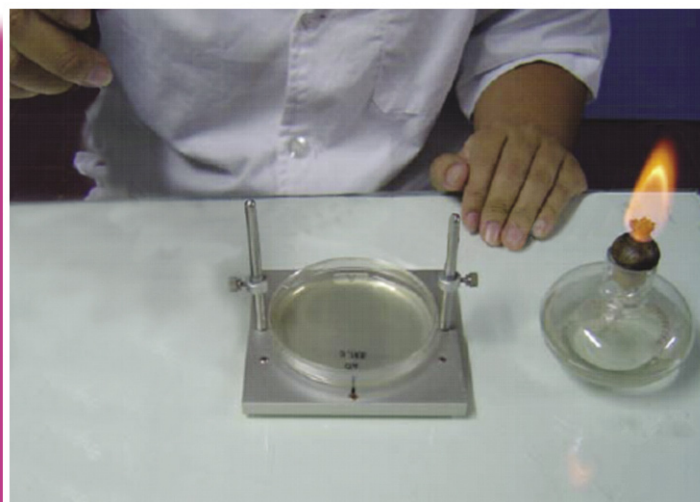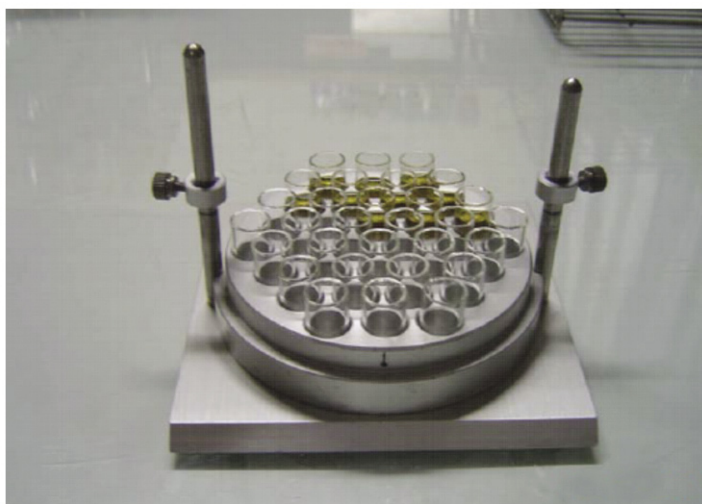
2 Place the multiple well tray of the multi-dispenser, which contain 1 ml of inocula, onto the basal stand B to ensure that the marking on the tray is arranged at the front direction.

3 Set the tray to ensure that each inoculator stick will properly dip into the inoculum and carry a uniform volume of each test bacteria.
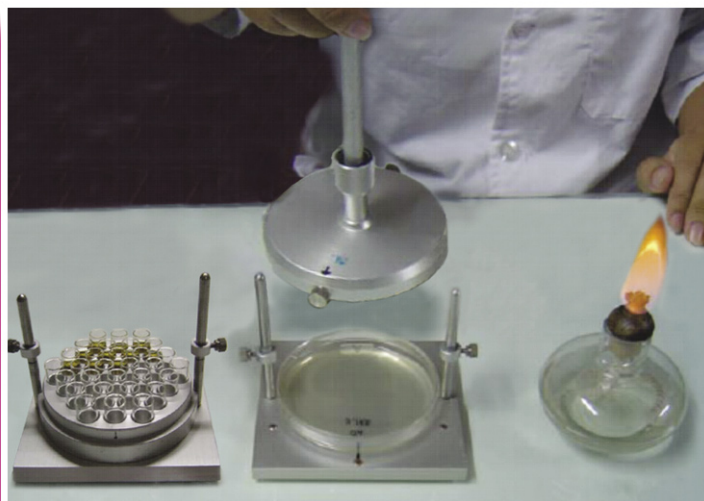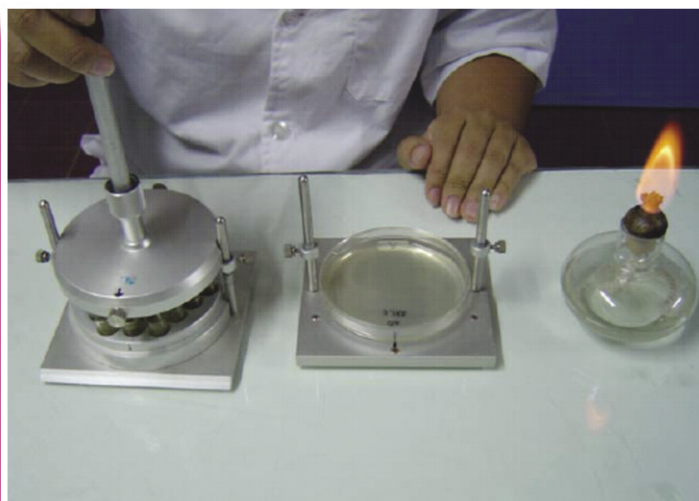
4 Set the apparatus to ensure that the surface of each multiple inoculator stick will properly touch the surface of the medium in agar plate.

5 Inoculate the bacterial suspensions onto the surface of the agar plate.

CONTROL PLATE

Include a control strain on each plate (see Appendix 3.1).

INCUBATION

1 Let the inoculated agar plates remain at room temperature until the moisture in the inoculum spot is absorbed by the agar or until all spots are dry.

2 Incubate the plates in an inverted position at 30°C for 18-24 hours

NOTE: *The incubation time is extremely important to obtain reliable end points when reading the results.*

READING OF MIC VALUES

1 Lay the agar plates on a non-reflecting dark surface and observe bacterial growth with the naked eye. Place the inoculated plate on or beside the reference paper to identify the spot or position of each tested bacteria.
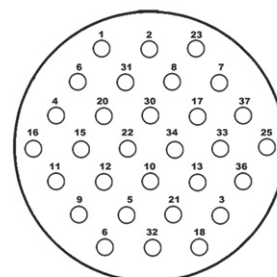
2 Check bacterial growth on the 2 control plates, disregard the results if no growth is observed in some control strains. The test should be repeated.

3 Read and record the MIC at the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the naked eye.

Example:

a) *No growth in plates with 250 μg/ml OTC, this means that OTC at 250 μg/ml completely inhibited all strains.*
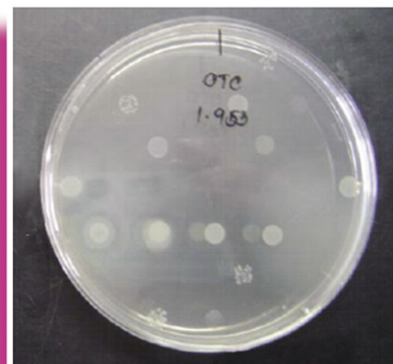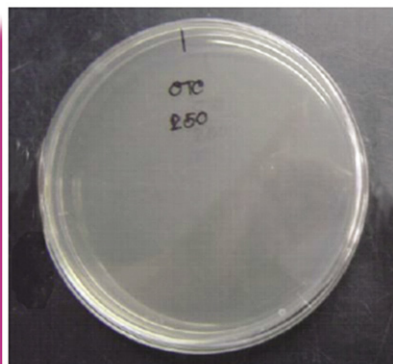
b) *Based on the reference paper, spots inoculated with bacterial strains # 1, 2, 31, 4, 30, 37, 15, 22, 34, 33, 36, 5, 31, 3 and 18 did not grow in plates with 1.953 μg/ml OTC, this means that OTC at 1.953 μg/ml inhibited the growth of these strains.*
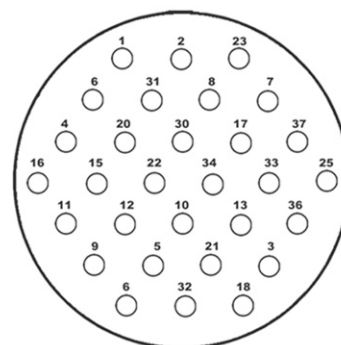
4   The MIC is the lowest concentration of antimicrobial agent that completely inhibits colony formation.

5   Disregard a single colony or a faint haze caused by the inoculum.

6   Repeat the test if two or more colonies persist in concentrations of the agent beyond an obvious end point; that is, if there is no growth at lower concentration but there is growth at higher concentrations.

7   When reporting the MIC values of antimicrobial agents that will prevent the growth of bacterial strains, include important information such as the locality and date the samples were isolated or collected.

8   Evaluate the MIC range of each antimicrobial agent.

9   Compare the MIC breakpoint of the test isolates with those in the chart (Appendix 3.5).

10   Report result as Resistant (R), Intermediate (I) or Susceptible (S).

Example:

Antibiotic:	oxytetracycline
MIC breakpoint:	0.2 μg/ml
Interpretation:	susceptible

NOTE: *The "true" MIC value is the lowest test concentration that completely inhibits the growth of organisms in both replicate plates. However, if the "observed" MIC values of the tested plates are different, for example, two-fold dilutions were used to test OTC and the "observed" MIC value of OTC in the first plate was 6.25 μg/ml, and the second plate was 3.125 μg/ml, the " true" MIC of OTC would be between 6.25 μg/ml and 3.125 μg/ml.*

Determination of Antimicrobial Resistant Bacteria

This activity will determine and provide baseline data on bacterial drug resistance that could be useful to policy makers and surveillance teams or systems.

INHIBITORY CONCENTRATION AT 50% AND 90%

After the MIC values are read, further interpretation of the inhibitory concentration at 50% (IC_{50}) and 90% (IC_{90}) of the bacterial strains are carried out.

Basically, the IC_{50} or IC_{90} is used to determine the dosage regimen of antimicrobial agent for *in vivo* treatment against the pathogen in the field to know the effectiveness of each drug. The formula used to determine IC_{50} and IC_{90} values are presented in Appendix 3.6.

When the IC_{50} and IC_{90} values could not be determined from any value of the MIC series results, these must be determined from the average value between the nearest percent that is lower and higher than the IC_{50} and IC_{90} values (see Appendix 3.6).

DETERMINATION OF ANTIMICROBIAL RESISTANT BACTERIA

The results for antimicrobial resistant bacteria can be evaluated according to the NCCLS standard for interpretative categories and correlative minimal inhibitory concentration (MICs)(NCCLS, 1998). Since the NCCLS standard does not include breakpoint recommendations for all of the agents tested, and the data are based on human drugs, which are recently used in shrimp production activity, therefore, data shown in Appendix 3.5 are standard categories used for determining drug resistance for bacteria which are close to food borne pathogenic bacteria, e.g., *Vibrio cholerae*.

In case a number of bacterial strains have been tested using MIC standardized method, the MIC values obtained from the study can be used to determine the breakpoint of each agent and interpretation of results for drug resistance.

The following steps should be applied:

1   A large number of bacterial strains (at least 300 strains) should be tested for each antimicrobial agent using MIC standardized method.

2   Arrange the MIC values of each bacterial strain against an agent and fill into the assigned table (see example in Appendix 3.7).

3 Analyze the results by using a program in WHONET to evaluate the breakpoint of each antimicrobial agent. To access the program: go to www.who.int, click drug resistance on the right side of the web page. On the left side of the web page is the table of contents, click # 2 How to obtain WHONET 5 through the internet. This will give you instructions on how to download the software.

4 Determine the resistant bacterial strains according to the breakpoint of each antimicrobial agent.

Some notes on laboratory practices are presented in Appendix 3.8.

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APPENDIX 3.1. List of culture apparatus, culture media, solvents, control strains, and antimicrobial agents needed for the minimal inhibitory concentration (MIC) test.

Apparatus:

1. MIC multiple inoculating apparatus set
2. Autoclave
3. Incubator
4. Analytical balance
5. Laminar flow hood
6. Water bath shaker
7. Refrigerator
8. Micropipette (20-100 and 200-1000 µl)
9. Vortex mixer
10. Glass pipette 2, 5, 20 ml
11. Test tubes
12. Dissecting set
13. Petri dishes (glass and disposable)

Culture media:

1. Thiosulphate-citrate-bilesalt-sucrose agar (TCBS, Difco)
2. Glutamate starch phenol-red agar (GSP, Merck)
3. Muller Hinton Agar (MHA, Merck)
4. Tryptic soy agar (TSA, Difco)
5. Sterile normal saline solution (NSS)
6. Heart infusion agar (HIA, Difco)
7. Heart infusion broth (HIB, Difco)
8. Sodium chloride (NaCl)

Solvents:

1. Distilled water
2. 0.1 N sodium hydroxide (NaOH)
3. Absolute ethanol
4. Dimethyl sulfoxide
5. N-N-Dimethyl formamide

APPENDIX 3.1. Continuation

Control bacterial strains:

1. *Enterococcus faecalis* ATCC 29212
2. *Escherichia coli* ATCC 25922

Antimicrobial agents:

1. Chloramphenicol (CP)
2. Erythromycin (E)
3. Furazolidone (FD)
4. Oxolinic acid (OA)
5. Oxytetracycline (OTC)
6. Norfloxacin (NFX)
7. Prefloxacin (PFX)
8. Trimethoprim   (TM)
9. Sulfadiazine (SD)
10. Sulfadimethoxine (S)

NOTE: *Each laboratory should consider which agents to routinely test or report, in accordance with the needs of each institute's study plan or of the farmers in particular aqua culture areas. The test reports should help to minimize the resistant strains due to misuse or overuse of the agents. At present, except for CP and FD, the listed antibiotics are FDA approved agents for use in food animal production (NCCLS, 1998).*

APPENDIX 3.2. Preparation of antimicrobial agent stock solution.

Formula to determine amount of antimicrobial agent powder needed for a standard solution:

$$\text{Volume (ml)} = \frac{\text{weight (mg)} \times \text{assay potency }(\mu g/mg)}{\text{concentration }(\mu g/ml)}$$

Example:

To prepare 10 ml (volume) of a stock solution containing 1,000 μg/ml (desired concentration) of antimicrobial agent with a potency of 94.5 μg/ml:

$$\text{Volume} = \frac{\text{actual weight} \times \text{potency}}{\text{desired concentration}}$$

$$10\ \text{ml} = \frac{\text{actual weight (mg)} \times 94.5\,\mu g/ml}{1,000\,\mu g/ml}$$

$$\text{Actual weight} = \frac{1,000}{94.5}$$
$$= 105.8201\ \text{mg}$$

Therefore, 105.8201 mg of powdered antimicrobial agent is needed for a 10 ml stock.

APPENDIX 3.3. List of solvents and diluents needed for the preparation of stock solutions of antimicrobial agents.

Antimicrobial agent	Solvent	Diluent
Oxolinic acid	0.1 N NaOH	Distilled water
Norfloxacin	0.1 N NaOH	Distilled water
Prefloxacin	0.1 N NaOH	Distilled water
Sulfadiazine	0.1 N NaOH	Distilled water
Sulfadimethoxine	0.1 N NaOH	Distilled water
Chloramphenicol	95 % ethanol	Distilled water
Erythromycin	95 % ethanol	Distilled water
Furazolidone	N-N-Dimethyl formamide	Distilled water
Oxytetracycline	Distilled water	Distilled water
Trimethoprim	Dimethyl sulfoxide or 0.05 N (0.05 mol/L) lactic or hydrochloric acid 10% of final volume	Distilled water

NOTE: *For antimicrobial agents which must be dissolved in solvents other than water, it is necessary to follow this guide:*

a. *Use only enough solvent to dissolve the powdered antimicrobial agent (in our case, 1 ml of solvent was used).*

b. *Dilute to the final stock concentration with distilled water.*

c. *If sterile solutions are desired, they are to be filtered through a membrane filter.*

d. *Use antimicrobial solution immediately after preparing and discard unused portion at the end of the day.*

e. *Stock solution of antimicrobial agents can be stored at – 70°C or colder for 6 months without significant loss of activity.*

Appendix 3.4. Preparation of dilutions of antimicrobial agents for use in the agar dilution method of minimal inhibitory concentration (MIC) test.

Step	Concentration (µg/ml)	Source	Volume (ml)	Distilledwater	Intermediate Conc. (µg/ml)	Final Conc. At 1: 10 dilution in Agar (µg/ml)
1	1000	Stock	10	10	1000	100
2	1000	Step 1	5	5	500	50
3	500	Step 2	5	5	250	25
4	250	Step 3	5	5	125	12.5
5	125	Step 4	5	5	62.50	6.25
6	62.5	Step 5	5	5	31.25	3.125
7	31.25	Step 6	5	5	15.625	1.56
8	15.625	Step 7	5	5	7.812	0.781
9	7.82	Step 8	5	5	3.906	0.390
10	3.906	Step 9	5	5	1.953	0.195
11	1.953	Step 10	5	5	0.977	0.098
12	0.977	Step 11	5	5	0.488	0.0481
13	0.488	Step 12	5	5	0.244	0.024
14	0.244	Step13	5	5	0.122	0.012

NOTE:

a. *Modified from Ericsson and Sherris (1971).*

b. *For S and SD, stock solutions were prepared at 4,000 µg/ml and the concentrations were made by the same method as shown in the scheme.*

APPENDIX 3.5. Interpretative categories and correlative minimal inhibitory concentration (MICs) for food-borne pathogens (source: NCCLS, 1998).

Antimicrobial agent	MIC Breakpoint µg/ml		
	Susceptible	Intermediate	Resistant
Chloramphenicol*	≤ 8	-	≥ 32
Oxytetracycline*	≤ 4	-	≥ 16
Erythromycin*	≤ 0.5	-	≥ 8
Furazolidone	≤ 0.5	-	≥ 128
Norfloxacin	≤ 0.5	-	≥ 16
Prefloxacin	≤ 0.5	-	≥ 16
Sulfadiazine	≤ 0.5	-	≥ 152
Sulfadimethoxine	≤ 256	-	≥ 350
Trimethoprim	≤ 2	-	≥ 16
Oxolinic acid	≤ 8	-	≥ 32

* based on NCCLS publication M31-A2- Performance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals: Approved Standard- Second Edition (ISBN 1-56238-461-9).

NOTE: *The data are based on human drugs.*

APPENDIX 3.6. Determination of inhibitory concentrations at 50% and 90% (IC_{50} and IC_{90} values).

Formula:

$$IC_{50} = \frac{A+B}{2} : \text{ when } A = \frac{50 \times \text{MIC value of the next \% lower than 50\%}}{\text{the next \% lower than 50\%}}$$

$$B = \frac{50 \times \text{MIC value of the next \% higher than 50\%}}{\text{the next \% higher than 50\%}}$$

$$IC_{90} = \frac{C+D}{2} : \text{ when } C = \frac{90 \times \text{MIC value of the next \% lower than 90\%}}{\text{the next \% lower than 90\%}}$$

$$D = \frac{90 \times \text{MIC value of the next \% higher than 90\%}}{\text{the next \% higher than 90\%}}$$

Example: How to find IC_{50} and IC_{90} of OTC from the following MIC values.

MIC (µg/ml)	% of Inhibiting Strains
0.0125	5
0.025	20
0.05	30
*0.1	46 *
**0.2	58 **
0.4	63
0.8	70
*1.6	84 *
**3.2	95 **
6.4	100
100	100

The next lower * and higher ** values of IC_{50}
The next % lower * and higher ** than 50%

The next lower * and higher ** values of IC_{90}
The next % lower * and higher ** than 90%

Calculation for IC_{50}

$$A = \frac{50 \times 0.1}{46} = 0.1087$$

$$B = \frac{50 \times 0.2}{58} = 0.1724$$

$$IC_{50} \text{ of OTC } = \frac{0.1087 + 0.1724}{2}$$

$$= 0.1406 \, \mu g/ml$$

Calculation for IC_{90}

$$C = \frac{90 \times 1.6}{84} = 1.7143$$

$$D = \frac{90 \times 3.2}{95} = 3.0316$$

$$IC_{90} \text{ of OTC } = \frac{1.7143 + 3.0316}{2}$$

$$= 2.3729 \, \mu g/ml$$

APPENDIX 3.7. Table of assigned values to evaluate the breakpoint, which correlates to the minimal inhibitory concentration (MIC).

Example: The MIC values of the bacterial strains against OTC, OA, S, SD, CP and TM in various provinces of Thailand in 2002.

No. of Strain	Source of Sample	MIC Values (µg/ml)					
		OTC	OA	S	SD	CP	TM
01	Chanthaburi	0.2	0.2	>400	>400	0.1	25
02	Chanthaburi	0.4	0.1	200	>400	0.4	50
03	Chanthaburi	0.1	0.4	>400	200	0.4	12.5
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
81	Samutsakhon	0.8	0.2	200	200	0.4	6.28
82	Samutsakhon	0.2	0.4	>400	>400	0.2	3.50
83	Samutsakhon	0.2	0.1	>400	200	1.25	25
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
150	Phuket	0.1	0.2	>400	>400	0.2	50
151	Phuket	0.1	0.4	>400	>200	0.4	25
152	Phuket	0.4	0.2	200	>400	0.4	25

APPENDIX 3.8. Notes on laboratory practice (adapted from NCCLS, 2002)

1) Guidelines for Quality Control

The goal of a quality control program is to monitor the following:

a. The precision and accuracy of the MIC test procedure;

b. The performance of reagents and the viability of the microorganisms used in the test; and

c. The performance of personnel who carry out the tests and interpret the results.

2) Responsibilities of the Laboratory

The following are the responsibilities of the laboratory:

a. Storage to prevent drug deterioration;

b. Personnel proficiency;

c. Adherence to procedure [e.g., inoculum standards, incubation conditions such as time, temperature, and atmosphere (aerobic or anaerobic)]; and

d. Media preparation and storage.

3) Common Sources of Error

The following are common sources of error and should be checked whenever the results of MIC are outside the accuracy control limits:

a. Reader error in observation of the apparent bacterial growth on each concentration;

b. Contamination or other changes in the control plates;

c. Inoculum adjusted too heavy or too light;

d. Failure to thoroughly mix the antimicrobial dilution tube;

e. Variability in the performance of antibiotic media. Each new lot should be checked before use;

f. Loss of drug potency during handling or storage in the laboratory; and

g. Mistake in labeling antimicrobial concentration and/or bacterial code.

4) Safety in Laboratory Practice

Carry out all procedures in accordance with the local code of practice of a microbiological laboratory such as ISO/IEC standard.