Global Salm-Surv

A global *Salmonella* surveillance and laboratory support project of the World Health Organization

Laboratory Protocols

Level 1 Training Course

Agar diffusion using E-test

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Introduction

The MIC (Minimal Inhibitory Concentration) of a bacterium to a certain antimicrobial agent gives a quantitative estimate of the susceptibility.

MIC is defined as the lowest concentration of antimicrobial agent required to inhibit growth of the organism. The principle is simple: Agar plates, tubes or microtitre trays with two-fold dilutions of antibiotics are inoculated with a standardised inoculum of the bacteria and incubated under standardised conditions following NCCLS guidelines. The next day, the MIC is recorded as the lowest concentration of antimicrobial agent with no visible growth.

The MIC informs you about the degree of resistance and might give you important information about the resistance mechanism and the resistance genes involved. MIC-determination performed as agar dilution is regarded as the gold standard for susceptibility testing.

Agar diffusion tests are often used as qualitative methods to determine whether a bacterium is resistant, intermediately resistant or susceptible. However, the agar diffusion method can be used for determination of MIC values provided the necessary reference curves for conversion of inhibition zones into MIC values are available. After an agar plate is inoculated with the bacteria, a tablet, disk or paper strip with the antimicrobial agent is placed on the surface. During incubation the antimicrobial agent diffuses into the agar and inhibits growth of the bacteria if susceptible. Diffusion tests are cheap compared to most MIC-determination methods. E-test is a diffusion test, but has been developed to give an approximate MIC-value.

Well standardised methods are essential for all kinds of susceptibility testing, since the methods are highly sensitive to variations in several factors, such as size of inoculum, contents and acidity of the growth medium, time and temperature of incubation. The agar diffusion methods are also strongly influenced by factors, such as agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria.

The MIC-determination and disk diffusion methods described in this protocol are in accordance with the international recommendations given by the National Committee for Clinical Laboratory Standards (NCCLS). The NCCLS describes how to perform the tests and sets international guidelines for interpretation of the results. It should be noted that the WHO does not prescribe any specific method for performance and interpretation of susceptibility tests.

Internal quality control should be regularly performed as recommended by NCCLS.

References

1. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests, 7th ed. Approved standard. M2-A7. NCCLS, Wayne, Pennesylvania, 2000.

2. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard. M31-A, NCCLS, Wayne, Pennesylvania, 1998.

3. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 5th ed. Approved standard. M7-A5, NCCLS, Wayne, Pennesylvania, 2000.

1.2 Agar diffusion using E-test (determination of an approximate MIC-value)

Materials

Equipment

- E-test strips
- McFarland standard 0.5
- Scissors
- Forceps
- Loops (1 µl and 10 µl)
- Bunsen burner
- Small sterile cotton swabs
- White paper with black lines on

Media

- Sterile normal saline, 4 ml volumes in tubes
- Mueller Hinton II agar plates (15 cm and a uniform agar depth of 4 mm)
- Nutrient agar plates (9 cm)

Bacterial strains

- *Salmonella* or other non-fastidious cultures plated out for single colonies
- Strain for quality control: *Escherichia coli* ATCC 25922

Safety

Carry out all procedures in accordance with the local codes of safe practice.

Procedure

Day 1

Prepare inoculum

Remove the E-test package from the freezer (-20^{o}C) at least 30 minutes before required.

With a loop, touch the top of 3 or 4 individual colonies and transfer to a tube of saline. Emulsify the inoculum on the inside of the tube to avoid lumps.

Compare turbidity to that in the 0.5 McFarland standard. Adjust turbidity of inoculum to match that standard.

Inoculate agar plate

Swab plate within 15 minutes of preparing the adjusted inoculum:

Dip a sterile cotton swab into the inoculum and pulling out slightly, rotate the swab several times against the inside of the tube above the fluid level to remove excess liquid.

Streak the swab over the entire surface of the agar plate. Rotate the plate approximately 60^{o} then repeat streaking motion. Rotate 60^{o} again and repeat streaking. Complete inoculation by running the swab around the rim of the agar.

Leave the lid of the plate ajar for 5 minutes (no more than 15 minutes) to allow any excess moisture to be absorbed before applying strips.

Apply E-test strips

Open E-test package by cutting along the broken line.

Apply strips to agar surface using forceps (or E-test applicator if available). Place the strip with the 'E end' at the edge of the plate and with the scale visible. An example of antimicrobial strips placed on a plate is shown in figure 1.

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Theory / comments

Ensure the agar surface is dry, but not overly dry (all the constraints of disk diffusion test apply to E-test).

If strips stick together, they may be pulled apart by handling the section marked E. Do not touch any other area of the strip.

Use templates to position 4 to 6 strips onto a 150 mm plate or one (seldom two) strips onto a 90 mm plate. Do not remove a strip once it has touched the agar.

Procedure

Check purity of the inoculum:
Transfer inoculum from the tube onto a nutrient agar plate using a 10 µl loop.

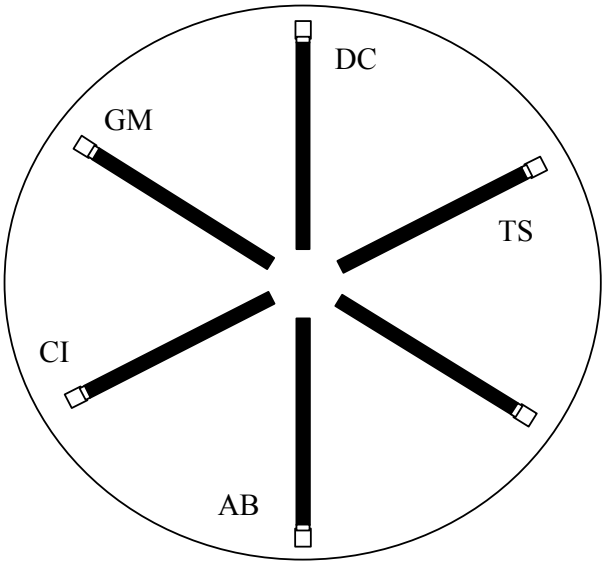
Incubate plates at 35°C for 16 to 20 hours in ambient air.

Place any open packages in separate airtight containers containing active desiccant and store at -20°C.

Theory / comments

Otherwise the expiry date will not be the same as for the original package.

Figure 1. Example of antimicrobials for *Enterobacteriaceae* susceptibility testing (ref. 2). Abbreviations are shown in table 4.



Procedure

Day 2: Results

Read MIC at the point where ellipse intersects the scale. If a MIC value between two two-fold dilutions is seen, always round up to the highest value.

Remember to read the MIC value at **complete inhibition** of all growth including isolated colonies.
If the intersect differs on either side of the strip, read the MIC as the greater value.

Ignore any growth at the edge of the strip. Refer to E-test reading guide if necessary.

Consult the appropriate application sheet to find breakpoint values telling at which MIC values the bacteria should be interpreted as S (Susceptible), I (Intermediate) or R (Resistant). An example for *Enterobacteriaceae* according to the NCCLS guideline is shown in table 4.

Theory / comments

Read results only if a good inhibition ellipse is visible, e.g. inoculum is sufficient.

However, remember that sulfonamide and trimethoprim should be read at **80% of growth** and that swarming of *Proteus* should be ignored.

This is caused by organisms growing in a ‘tunnel’ of water.

Table 4. An example of criteria for interpretation of susceptible, intermediate or resistant bacteria and the accepted range of MIC for the *E. coli* ATCC 25922 reference strain.

Antimicrobial		NCCLS MIC interpretive criteria (μg/ml)			NCCLS QC ranges
		S	I	R	*E. coli* ATCC 25922. MIC (μg/ml)
AM	Ampicillin	≤ 8	16	≥ 32	2-8
CL*	Chloramphe-nicol	≤ 8	16	≥ 32	2-8
CI*	Ciprofloxacin	≤ 1	2	≥ 4	0.004-0.016
NA	Nalidixic acid	≤ 16	-	≥ 32	1-4
SU	Sulphadiazine	≤ 256	-	≥ 512	8 - 32
TC	Tetracycline	≤ 4	8	≥ 16	0.5 - 2

* Notice the difference between these two abbreviations.

References

1. Etest technical guide 3B. Etest for MIC determination. AB Biodisk.
2. Etest Gram negative application sheet. AB Biodisk.

2. Composition and preparation of culture media and reagents

If no reference is given, it is the procedure used at DVL.
The media and reagents are available from several companies including Oxoid, Merck and Difco. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also, the media should be prepared according to the manufacturers description if it differs from the description given here. Refer to Appendix 2 for a colour presentation of growth of *Salmonella* on selective agar media and positive and negative reactions of biochemical tests.

Mueller Hinton II agar (e.g. from BBL)

Beef extract	2.0 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1000 ml

Preparation:
Dissolve the dehydrated medium in water by heating if necessary. Adjust pH to 7.2 - 7.4, transfer into bottles and autoclave at 110^{o}C for 20 min.

Saline solution

Sodium chloride	8.5 g
Water	1000 ml

Preparation:
Dissolve the sodium chloride in the water by heating if necessary. Adjust pH to ~ 7.0 after sterilisation. Dispense the solution into tubes so 4 ml is obtained after autoclaving at 121^{o}C for 20 min.

References

1. Post D. E. (1997) Food-borne pathogens monograph number I *Salmonella*. Oxoid limited, Hampshire, England.
2. ISO 6579 :1993(E) 3rd ed. Microbiology - General guidance on methods for the detection of *Salmonella.*
3. NMKL method no. 71, 2nd ed., 1999: *Salmonella*. Detection in food. Nordic committee on food analysis.

Date:_____ **Record sheet:**

Initials:_____ **Disk diffusion susceptibility testing**

Agar diffusion with E-test

Antimicrobial	Strain: DVL #13	
	MIC (μg/ml)	Interpretation (R-I-S)
Ampicillin		
Chloramphenicol		
Ciprofloxacin		
Nalidixic acid		
Sulphonamides		
Tetracycline		

Antimicrobial	Strain: ATCC 25922	
	MIC (μg/ml)	Within the QC range
Ampicillin		
Chloramphenicol		
Ciprofloxacin		
Nalidixic acid		
Sulphonamides		
Tetracycline		