Global Salm-Surv

A global *Salmonella* surveillance and laboratory support project
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Laboratory Protocols

Level 1 Training Course

MIC determination by broth dilution using Sensititre. 4th Ed. April 2003

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Contents

1. Susceptibility testing: Determination of phenotypic resistance

The methods to be demonstrated at this course are

1) Agar diffusion with disk

2) Agar diffusion with E-test

3) MIC-determination using commercially prepared microtitre trays with dehydrated antibiotics in wells and using agar plates with 2-fold dilutions (Demonstrations). If home made microtitre plates are prepared for MIC determination, NCCLS guidelines should be consulted.

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.

2. MIC determination by broth dilution (using Sensititre)

Modified from Sensititre protocol

Materials:

Equipment

- McFarland standard 0.5
- Nephelometer or white paper with black lines
- Microtitre trays with dehydrated antibiotics in two-fold concentrations (Sensititre plates from Trek Diagnostic System Ltd., England)
- Disposable loops (1 µl and 10 µl)
- Multichannal pipette
- Microtitration reader with mirror
- Disposable reservoir for reagents
- Graduated pipettes (20 µl - 1000 µl)

Media

- Sterile normal saline, 4 ml volumes in tubes for nephelometer
- 10 ml cation adjusted Mueller-Hinton II broth in sensititre tubes
- Nutrient agar plates for purity control of inoculum suspensions

Bacterial strains

- *Salmonella* strains on non-selective agar
- 4 strains for quality control: *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922

Safety

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

Procedure

Day 1

Standardisation of inoculum
From a pure o/n culture, pick material from at least 3-4 colonies. Suspend totally in 4 ml saline in tubes. Mix.

Adjust to McFarland 0.5 (nephelometer): Calibrate the nephelometer before use and gently turn all suspensions upside-down before measuring. Adjust turbidity of inoculum to match that of standard.

If a nephelometer is not available: Compare visually with the McFarland 0.5 standard using white paper with black lines as background.

The McFarland 0.5 suspension is diluted as follows for the species tested at this course:

Gr-neg.: same as for Gram-positive
Gr-pos.: 50 µl McFarl. 0.5 into 10 ml broth

Mix. The suspension should be used for inoculation within 15 minutes.

Inoculation and incubation
The microtitre trays are inoculated with 50 µl of the inoculum suspension using a multi-channel pipette or Sensititre autoinoculator.

Plates are sealed and incubated at 37°C for 18-22 hours. Do not stack plates more than 2 high.

Purity control: Spread 10 µl of the inoculation-suspension on a nutrient agar plate. Incubate at 35°C overnight.

Remember to run the quality control strains in parallel to the test strains.

Theory / comments

This is done to avoid picking bacteria, which have lost their resistance.

McFarland 0.5 ~ approximately 10^8 CFU/ml. Standardisation of inoculum is essential because the interpretation of the results is based on a certain inoculum.

NCCLS recommends that each well contain approximately 5 x 10^5 CFU/ml after inoculation.

To avoid further growth

Each well is inoculated with approximately $2.5 \cdot 10^4$ cells.

The incubation time is extremely important to obtain reliable end points.

Procedure

Day 2

Reading MIC / interpretation of results
Check purity of the inoculum suspension.
If not OK, results cannot be reported.

Read plates as follows:

- Use the record sheet for orientation of the plates.
- Check growth in the 3 positive control wells.
- The MIC is read as the lowest concentration without visible growth.
- Reading rules are demonstrated in Figure 2.

Be aware of special readings for trimethoprim and sulphonamides. In these cases the MIC is recorded as the lowest concentration where a growth reduction of 80-90 % can be seen.

Further interpretation of the MIC is done according to the NCCLS recommendations. The breakpoints are also shown on the record sheet.

The acceptable MIC-ranges for the quality control strains as recommended by the NCCLS are given in Appendix 5.

Theory / comments

Strange patterns of growth in the microtitre trays are often caused by contamination.

The MIC is determined from two-fold dilutions of the antimicrobial agent. Be aware that "the true" MIC can be anywhere between the observed MIC and the dilution step below.

The antibiotic trimethoprim and the sulphonamides allow growth of the bacteria for several generations before inhibition occurs.

The NCCLS standard does not include breakpoint-recommendations for all of the compounds tested. In these cases, breakpoints are assigned in accordance to the population-distribution after testing a large number of isolates.

Figure 2. Demonstration of various growth patterns and guidelines to read the MIC

Column 1 **Typical pattern**
MIC = Well D

Column 2 **Fading end point**
MIC = Well C

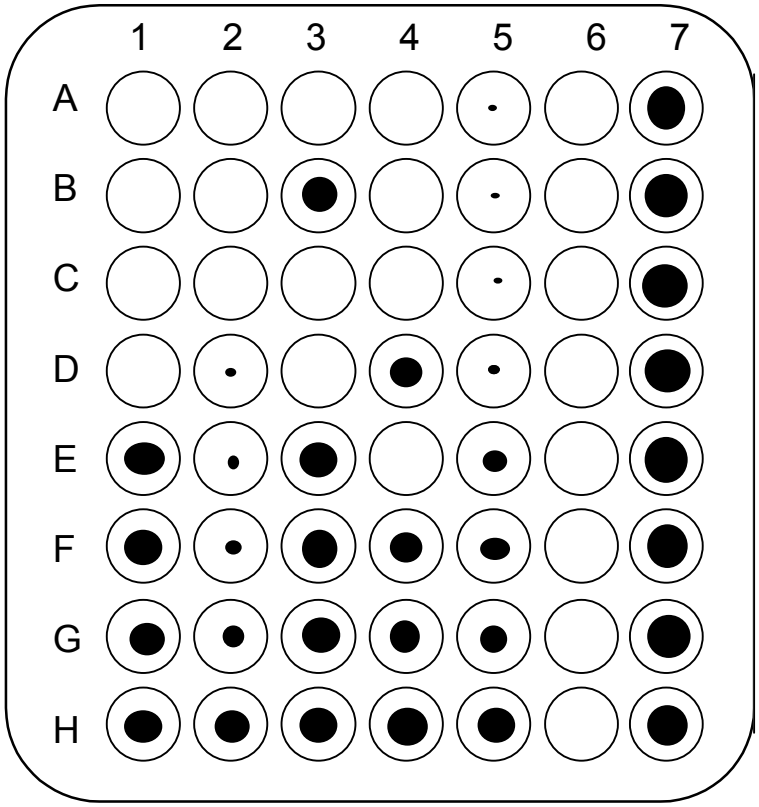
Column 3 **Single well contamination**
MIC = Well D

Column 4 **Skip**
MIC = Well C

Column 5 **Typical pattern for trimetho-prim and sulphonamides**
MIC = Well D

Column 6 **No growth at all in the test range**
MIC = smaller than or equal to the lowest concentration e.g. MIC≤1 μg/ml

Column 7 **Growth in all wells**
MIC = greater than the highest concentration e.g. MIC>512 μg/ml



● = Growth

3. 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.

APPENDIX 4

Quality control ranges for MIC determination (µg/ml)

ANTIMICROBIAL AGENT	*Enterococcus faecalis* ATCC 29212	*Staphylococcus aureus* ATCC 29213	*Pseudomonas aeruginosa* ATCC 27853	*Escherichia coli* ATCC 25922	*Escherichia coli* ATCC 35218
Amoxicillin clavulanate	-	-	-	-	4/2-16/8
Ampicillin	0.5-2	0.5-2	>32	2-8	-
Chloramphenicol	4-16	2-8	> 64	2-8	-
Ciprofloxacin	0.25-2	0.12-0.5	0.25-1	0.004-0.015	-
Colistin	>32	>16	≤2	≤2	-
Florfenicol	2-8	2-8	>16	2-8	-
Gentamicin	4-16	0.12-1	0.5-2	0.25-1	-
Kanamycin	16-64	1-4	>128	1-4	-
Nalidixic Acid	>128	16-64	≥ 128	1-4	-
Streptomycin	32-128	2-8	16-64	4-16***	-
Sulphamethoxazole	>512	>512	>512	8-32	-
Tetracycline	8-32	0.12-1	8-32	0.5-2	-
Trimethoprim	≤1	1-4	>64	0.5-2	-

Grey area: *NCCLS recommendations*
White area: *Quality control range assigned by the Danish Veterinary Laboratory*
****:* *Quality control range assigned to the Sensititre system by Trek Diagnostic Systems Ltd.*

Date: _____**Record sheet:**

Initials:_____**MIC determination by micro broth dilution using Sensititre**

Strain: _____

Put a cross on the lowest concentration of antibiotic without growth (the MIC) or above, e.g. GEN 32, if you find growth in all wells. Grey means resistant, light grey means intermediate and white means sensitive.
Write the MIC value underneath the abbreviation, e.g. AMP, and write RES if it is interpreted as resistant

Sensititre plate code: DKSVSR1 (for *E. coli* and *Salmonella* isolates)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SPE 64										SMX 256
C	CIP 1	SPE 32		AMP 8					AMC 8			SMX 128
D	CIP 0.5	SPE 16	NAL 16	AMP 4	CHL 8	FFN* 8	GEN 4	NEO 4	AMC 4	TET 4	STR 8	SMX 64
E	CIP 0.25	SPE 8	NAL 8	AMP 2	CHL 4	FFN* 4	GEN 2	NEO 2	AMC 2	TET 2	STR 4	SMX 32
F	CIP 0.125	SPE 4	NAL 4	AMP 1	CHL 2	FFN* 2	GEN 1	TMP 4	TMP 8			POS KON
G	CIP 0.06	SPE 2	COL 4	COL 8				T/S 1	T/S 2			POS KON
H	CIP 0.03	CEF 0.5	CEF 1	CEF 2			APR 4	APR 8				POS KON

** No international breakpoints for florfenicol (FFN) have been developed yet*

Code	Antimicrobial	Testrange (µg/ml)
AMC	AMOXICILLIN+CLAVULANAT	2-32
AMP	AMPICILLIN	1-32
APR	APRAMYCIN	4-64
CEF	CEFTIOFUR	0.5-8
CHL	CHLORAMPHENICOL	2-64
CIP	CIPROFLOXACIN	0.03-4
COL	COLISTIN	4-64
FFN	FLORFENICOL	2-64
GEN	GENTAMICIN	1-32
NAL	NALIDIXAN	4-128
NEO	NEOMYCIN	2-32
SPE	SPECTINOMYCIN	2-128
STR	STREPTOMYCIN	4-64
SMX	SULPHAMETHOXAZOLE	32-512
TET	TETRACYKLIN	2-32
TMP	TRIMETHOPRIM	4-32
T/S	TRIMETHOPRIM+SULPHA	1-8