



## WHO Global Foodborne Infections Network (formerly WHO Global Salm-Surv)

"A WHO network building capacity to detect, control and prevent foodborne and other enteric infections from farm to table"

### **Laboratory Protocol: "Susceptibility testing of Enterobacteriaceae using disk diffusion"**

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WHO Global Foodborne Infections Network Laboratory Sub-Committee

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|---|---------------------------------|
| <b>Title:</b> Susceptibility testing of Enterobacteriaceae using disk diffusion |                                 |
| <b>SOP Number:</b> 2010GFNLAB002  | <b>Effective Date:</b> Feb 2010 |

### LABORATORY SOP

|   |                                 |
|---|---------------------------------|
| <b>Title:</b> Susceptibility testing of Enterobacteriaceae using disk diffusion |                                 |
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|   | <b>Revision Number:</b>         |

### REVISION HISTORY

| HISTORY OF CHANGES |                  |                                 |      |          |
|--------------------|------------------|---------------------------------|------|----------|
| Rev. Level         | Sections Changed | Description of Change (From—To) | Date | Approval |
| 0                  |                  |                                 |      |          |
| 1                  |                  |                                 |      |          |

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## I. PURPOSE

A standardised method for determining the antimicrobial susceptibility profile of Enterobacteriaceae, e.g. *Salmonella* and *Shigella*.

## II. TEST PRINCIPLES

Disk diffusion testing is one of several phenotypic assays which can be utilised to determine the antimicrobial resistance profile (antibiogramme) of an organism. Disk diffusion tests estimate *in vitro* susceptibility.

The principle of agar diffusion is simple: Agar plates are inoculated with a standardised inoculum of the bacteria and an antimicrobial disk is placed on the inoculated agar plate. The disks used for a disk diffusion assay contain a standardised known amount of an antimicrobial agent, which diffuses into the agar when in contact with the agar surface. The plate is incubated under standardised conditions following Clinical and Laboratory Standards Institute (CLSI) guidelines. During incubation, the antimicrobial agent diffuses into the agar and inhibits growth of the bacteria, producing a “zone of inhibition” around the disk. Following incubation, the diameter of this zone is measured and the results are interpreted as resistant, intermediate, or susceptible using standard guidelines (e.g. CLSI M100).

The size of the inhibition zone indicates the degree of resistance, and might also give important information about the resistance mechanism and the resistance genes involved. In addition, the disk 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.

Highly standardised methods are essential for all types of susceptibility testing. These assays are highly sensitive to variations in: inoculum density, media formulation, media pH, and incubation conditions. In addition, agar diffusion methods are strongly influenced by agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria. To insure the accuracy and reproducibility of antimicrobial susceptibility test results, internal quality control testing must be regularly performed as recommended by CLSI (CLSI M2).

Diffusion tests are low-cost compared to most MIC determination methods, but MIC determination performed as agar dilution is regarded as the gold standard for susceptibility testing. It should be noted that the WHO-GFN does not recommend any specific method for the susceptibility testing of microorganisms.

The disk diffusion method described in this protocol is in accordance with the international recommendations given by CLSI (M2). The CLSI sets international guidelines for interpretation of the results (e.g. M7, M31, and M100). The most recent update of the guidelines should be used as reference.

## III. RESPONSIBILITIES

### A. Staff Responsibilities

Refer to applicable manuals within the facility/location for complete set of responsibilities to properly conduct this procedure.

|   |                                 |
|---|---------------------------------|
| <b>Title:</b> Susceptibility testing of Enterobacteriaceae using disk diffusion |                                 |
| <b>SOP Number:</b> 2010GFNLAB002  | <b>Effective Date:</b> Feb 2010 |

## **B. Specific Safety Requirements and Responsibilities**

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

## **IV. SAMPLE PREPARATION**

The test material must be a pure 18- to 24-hour culture of the Enterobacteriaceae grown on non-selective agar. Single, isolated colonies should be present.

## **V. MATERIALS/SUPPLIES**

### **Media**

- Sterile saline solution (0.85%) 3-4 mL each tube
- Mueller-Hinton agar plates (with a uniform agar depth of approximately 4 mm)
- Antimicrobial Disks
- Nutrient agar plates or other non-selective agar

### **Bacterial strains**

- Enterobacteriaceae strains on non-selective agar (colonies from selective isolation plates should not be used because they could be mixed cultures and could influence on the susceptibility test result)
- Strain for quality control: *Escherichia coli* ATCC 25922

## **VI. EQUIPMENT**

- McFarland standard 0.5 (the tube containing the McFarland standard **must** be the same type/material and diameter as the tubes used for the test suspension)
- Nephelometer or white paper with black lines
- Vortex
- Scissors
- Disk dispenser (alternatively, forceps can be used)
- Forceps
- Loops (1 µl and 10 µl)
- Bunsen burner (or other to secure sterilisation of forceps and loops)
- Small sterile cotton swabs or drigalski (hockeystick)
- Ruler or calliper
- CLSI M100: Performance Standards for Antimicrobial Susceptibility Testing
- CLSI M2: Performance Standards for Antimicrobial Disk Susceptibility

|   |                                 |
|---|---------------------------------|
| <b>Title:</b> Susceptibility testing of Enterobacteriaceae using disk diffusion |                                 |
| <b>SOP Number:</b> 2010GFNLAB002  | <b>Effective Date:</b> Feb 2010 |

## VII. QUALITY ASSURANCE

Each lot of media utilized in this SOP will be quality control tested prior to use. The results of QC testing (performance characteristics and sterility) will be recorded. Only media which has passed QC will be used for testing.

Quality assurance of the susceptibility testing of Enterobacteriaceae includes testing of *E. coli* ATCC 25922. The test results from this microorganism must be within the QC ranges set by the CLSI. The test thereby serves as quality assurance that the method was carried out with all variable factors standardized according to the CLSI guidelines. If the test results of the *E. coli* ATCC 25922 QC strain are out of range, do not report results from test strains and proceed with the troubleshooting guidelines according to CLSI (M100, Table 3C).

Reference strains should be stored in a suitable stabilizer such as 50% fetal calf serum in broth, 10-15% glycerol in tryptic soy broth, defibrinated sheep blood or skim milk. Store at -20°C to -80°C (preferably -70°C to -80°C).

Before using rejuvenated strains for QC, subculture to check for purity and viability. Set up on agar slants with appropriate medium, store at 4-8°C and subculture weekly. Replace the working strain with a stock culture at least monthly. If a change in the organisms inherent susceptibility occurs, obtain a fresh stock culture or a new strain from a reference culture collection e.g. ATCC.

Antimicrobial discs should be stored in a freezer (-10°C to -20°C) until needed. A small working supply of discs can be kept in a refrigerator, in containers with a desiccant. To prevent condensation, the jars and disc dispensers should be allowed to warm to room temperature before being opened. The unused portion of the discs should be put back into the refrigerator as soon as possible to minimize the exposure to room temperature and humidity. Only those discs that have not reached the manufacturer's expiration date stated on the label may be used. Discs should be discarded on the expiration date.

## VIII. PROCEDURE

### Day 1

#### Standardisation of inoculum:

Prior to preparing the inoculum, visually examine the agar plates containing the test organism and control strain. If culture appears mixed, a fresh sub-culture will be prepared.

With a loop or sterile swab, touch the top of at least 4 to 5 well isolated colonies. Transfer the growth to the tube of saline. Emulsify the inoculum on the inside of the tube to avoid clumping of the cells.

*Picking cells from more than one colony ensures the selection of sufficient bacterial numbers and minimizes the risk of selecting bacteria that have lost their resistance.*

Adjust the inoculum standard to a 0.5 McFarland:

Compare turbidity to that in the 0.5 McFarland standard using a nephelometer or paper with black lines. Adjust turbidity of inoculum to match that standard.

*McFarland 0.5 equals approximately 10<sup>8</sup> CFU/mL. Use of a standardised inoculum is essential for the accurate performance of the assay. If the suspension is too light,*

|   |                                 |
|---|---------------------------------|
| <b>Title:</b> Susceptibility testing of Enterobacteriaceae using disk diffusion |                                 |
| <b>SOP Number:</b> 2010GFNLAB002  | <b>Effective Date:</b> Feb 2010 |

*confluent growth will not be obtained; if the suspension is too heavy, zone sizes will be artificially small (CLSI/Kirby-Bauer).*

#### Inoculation of Mueller-Hinton plate:

Visually examine the Mueller Hinton agar plates prior to use, insure plates are:

- free of visible contamination
- poured to a uniform depth of approximately 4mm
- not excessively wet
- not cracked or dry

Within 15 minutes of preparing the adjusted inoculum, dip a sterile cotton swab into the inoculum. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.

Streak the swab over the entire surface of the Mueller Hinton agar plate. Rotate the plate approximately 60° then repeat streaking motion. Rotate 60° again and repeat streaking for the third time. Complete inoculation by running the swab around the rim of the agar.

*Homogeneous plating is important to yield reliable results. An alternative method for obtaining a confluent lawn is to inoculate each 10 cm plate with 50 µL and use a drigalski (hockeystick) to create the uniform lawn. This method is a practical, and by most laboratories' experience, an acceptable deviation from the recommended standard.*

Allow any excess moisture on the agar surface to be absorbed prior to applying the antimicrobial disks. The lid of the plate may be left ajar for 3-5 minutes (no more than 15 minutes) to allow any excess moisture to be absorbed before applying disks.

#### Dispensing Antimicrobial Disks:

Dispense disks to the agar surface with a disk dispenser or sterile forceps (forceps can be sterilized by flaming with alcohol. Avoid using over-heated forceps). Do not relocate any disks after contact with the agar. After application, insure that the disk has made complete contact with the agar surface by touching the top of the disk with forceps.

*The disks cannot be moved after being placed onto the agar surface. Diffusion of the drug begins immediately when the disk contacts the agar. Moving the disks after contact with the agar will produce distorted zones and result in unreliable results.*

*Note: Selection of disks should be guided by plate size and the intended use of the results (clinical or epidemiological). Ordinarily, no more than 5 disks should be placed on a 10 cm agar plate and no more than 12 disks should be placed on a 15 cm agar plate. In a clinical setting, only ampicillin, a quinolone and/or fluoroquinolone, and trimethoprim-sulphamethoxazole should be reported for faecal isolates of Salmonella and Shigella. Chloramphenicol and a third-generation cephalosporin should also be tested and reported for extra-intestinal isolates of Salmonella. Other agents and drug classes (e.g. aminoglycosides) may provide valuable epidemiologic data; however in-vitro susceptibility of Salmonella to these agents may not correlate with in-vivo efficacy. The reader is advised to consult the current CLSI interpretative standards (M100) for further guidance.*

**Title:** Susceptibility testing of Enterobacteriaceae using disk diffusion

**SOP Number:** 2010GFNLAB002

**Effective Date:** Feb 2010

### Verifying Purity of Inoculum:

To verify the purity of the inoculum, the inoculum is plated to a nutrient agar plate (or other non-selective media).

Use a sterile 10  $\mu\text{L}$  loop, to collect inoculum from the tube and plate to a nutrient agar plate (or other non-selective media).

Incubate plates inverted at  $36\pm 1^\circ\text{C}$  for 16 to 18 hours in ambient air.

## **Day 2**

### Reading Results:

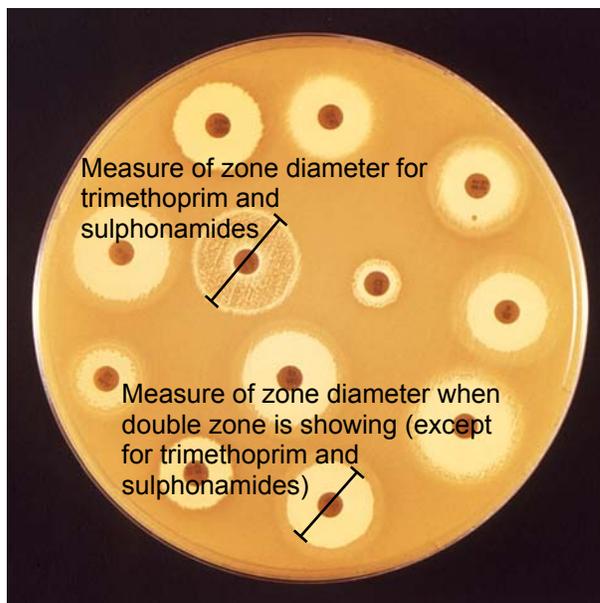
Check the purity of the purity control plate. If the growth appears mixed, attempt to obtain a pure culture.

Check that the growth is a confluent lawn. Individual colonies of resistant organisms may be observed around some antimicrobial disks; however, if individual colonies are dispersed across the plate, the inoculum was too light and the sample must be retested.

*An overly dense inoculum will yield artificially small zones (this becomes apparent when comparing the results of the control strain, E. coli ATCC 25922, to the QC-ranges given in the CLSI guidelines M100, Table 3).*

Check that zones are round; not oval, deformed or have jagged edges.

*Sometimes when disks are placed closely together, interaction between antimicrobials may produce distortion of inhibition zones (i.e. antagonism, synergism, inhibition and/or induction). Such valuable additional information should not be considered in the reading of the inhibition zones but provides important information about the putative mechanism of resistance, bacterial ID, etc.*



Measure the diameter of inhibition zones. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye (a haze should be disregarded). The antibiotic trimethoprim and the sulphonamides allow growth of the bacteria for some generations before inhibition occurs, therefore for these antimicrobials, disregard slight growth (20% or less of the lawn of growth), and measure the more obvious margin to determine the zone diameter. If no inhibition is present (confluent growth is present up to the border of the disk), the diameter of the disk should be recorded (6mm).

**Photo:** Disk diffusion on a 150mm Mueller Hinton agar plate.  
From CDC Public Health Image Library (PHIL) [www.phil.cdc.gov](http://www.phil.cdc.gov)

## IX. INTERPRETATION OF RESULTS

CLSI Guideline M100, Table 2A ('Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae') is the interpretation guideline for the categorization of the test strain as sensitive, intermediate or resistant.

Interpretation of the inhibition zones is based on a confluent lawn of growth and on regression lines found by testing and comparing a large population of isolates.

## X. LIMITATIONS OF PROCEDURES

Disk diffusion testing, like other antimicrobial susceptibility testing assays, is an *in vitro* determination of antimicrobial susceptibility. These *in vitro* results may not always correlate with *in vivo* efficacy. Additionally, antimicrobial agents with limited clinical efficacy (aminoglycosides against *Salmonella* and *Shigella*) or antimicrobials which are subject to legal restrictions (chloramphenicol in food animals) often provide valuable markers for specific resistance phenotypes are still included in many susceptibility panels.

Susceptibility testing microorganisms by disk diffusion should be performed on a pure culture. An indication (non-reportable zone size) of the results can, however, be obtained even though the culture is not pure. To obtain a correct result, subculture the test strain and re-test.

To insure accurate and reproducible results, the agar used for the disk diffusion should follow international guidelines (CLSI M2):

- The pH of the Mueller-Hinton agar should be between 7.2 and 7.4 (at room temperature after gelling), otherwise some antimicrobials will appear to lose potency while other agents may appear to have excessive activity
- Excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides and trimethoprim, thus yielding smaller and less distinct zones, or even no zone at all, which may result in false-resistance reports
- Variations in the content in the Mueller-Hinton agar of Ca<sup>++</sup> and Mg<sup>++</sup> affect the zone sizes for tetracycline. The zones will be too small when the content of Ca<sup>++</sup> and Mg<sup>++</sup> is too high and too large when the content of Ca<sup>++</sup> and Mg<sup>++</sup> is too low

Testing the QC strain *E. coli* ATCC 25922 is the QC reference, and if problems with obtaining values within the CLSI acceptable zone diameters, proceed with the troubleshooting guidelines according to CLSI (M100, Table 3C).

## XI. REPORTING

Results are reported as S, I or R following interpretation according to the CLSI guidelines

## XII. REFERENCES

1. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests, 9<sup>th</sup> ed. Approved standard. M2-A9. CLSI, Wayne, Pennsylvania, 2006

|   |                                 |
|---|---------------------------------|
| <b>Title:</b> Susceptibility testing of Enterobacteriaceae using disk diffusion |                                 |
| <b>SOP Number:</b> 2010GFNLAB002  | <b>Effective Date:</b> Feb 2010 |

2. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 7<sup>th</sup> ed. Approved standard. M7-A7. CLSI, Wayne, Pennsylvania, 2006

3. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, 3<sup>rd</sup> ed. Approved standard. M31-A3. CLSI, Wayne, Pennsylvania, 2001

3. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 19<sup>th</sup> Informational Supplement. M100-S19. CLSI, Wayne, Pennsylvania, 2009

### **XIII. ATTACHMENTS**

Attachment 1: Composition and preparation of culture media and reagents

Attachment 2: Example of Record sheet

|   |                                 |
|---|---------------------------------|
| <b>Title:</b> Susceptibility testing of Enterobacteriaceae using disk diffusion |                                 |
| <b>SOP Number:</b> 2010GFNLAB002  | <b>Effective Date:</b> Feb 2010 |

### **Attachment 1: Composition and preparation of culture media and reagents**

If no reference is given, it is the procedure used at DTU Food, Copenhagen, Denmark.

The media and reagents are available from several companies including Oxoid, Merck and Difco.

#### **Mueller Hinton Agar**

Mueller-Hinton Agar should be prepared from a commercially available base.

Only Mueller-Hinton agar formulations that have been tested according to, and meet the quality standards prescribed by CLSI document M6 *Protocols for Evaluating Dehydrated Mueller-Hinton Agar*, should be used.

The media should be prepared in accordance with the manufacturer's instructions.

Specifications: Final pH 7.2-7.4. Agar depth 4 mm

**Nutrient agar** (according to ISO 6579:2002 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.)

Meat extract 3.0g

Peptone 5.0g

Agar 12g to 18g<sup>1)</sup>

Water 1000 mL

<sup>1)</sup> Depending on the gel strength of the agar

Preparation: Dissolve the dehydrated medium in the water by heating. If necessary, adjust pH to ~7.0 after sterilisation, transfer into bottles and autoclave at 121°C for 20 min. Pour 15 mL of melted medium into each sterile 10 cm petri dish.

#### **Saline solution**

Sodium chloride 8.5g

Water 1000 mL

Preparation: Dissolve the sodium chloride in the water by heating if necessary. Adjust pH to 7.0. Dispense the solution into tubes so 4 mL is obtained after autoclaving at 121°C for 20 min

|   |                                 |
|---|---------------------------------|
| <b>Title:</b> Susceptibility testing of Enterobacteriaceae using disk diffusion |                                 |
| <b>SOP Number:</b> 2010GFNLAB002  | <b>Effective Date:</b> Feb 2010 |

**Attachment 2: Example of Record sheet**

|                       |                            |
|-----------------------|----------------------------|
| <h1>Record sheet</h1> | Date: _____<br>Name: _____ |
|-----------------------|----------------------------|

| Antimicrobial                                 | Strain 1:# _____   |                        | Strain2:# _____    |                        |
|---|--------------------|------------------------|--------------------|------------------------|
|   | Zone diameter (mm) | Interpretation (R-I-S) | Zone diameter (mm) | Interpretation (R-I-S) |
| Ampicillin (10µg)                             |                    |                        |                    |                        |
| Ceftriaxone (30µg)                            |                    |                        |                    |                        |
| Chloramphenicol (30µg)                        |                    |                        |                    |                        |
| Ciprofloxacin (5µg)                           |                    |                        |                    |                        |
| Nalidixic acid (30µg)                         |                    |                        |                    |                        |
| Trimethoprim-Sulphamethoxazole (1.25/23.75µg) |                    |                        |                    |                        |

| Method used*  | Antimicrobial                                 | Strain: <i>E. coli</i> ATCC 25922 |                     |
|---|---|-----------------------------------|---------------------|
|   |   | Zone diameter (mm)                | Within the QC range |
| <input type="checkbox"/> Cotton swab<br><input type="checkbox"/> 50µL<br><input type="checkbox"/> 100µL<br><input type="checkbox"/> 150µL<br><input type="checkbox"/> 200µL | Ampicillin (10µg)                             |                                   |                     |
|   | Ceftriaxone (30µg)                            |                                   |                     |
|   | Chloramphenicol (30µg)                        |                                   |                     |
|   | Ciprofloxacin (5µg)                           |                                   |                     |
|   | Nalidixic acid (30µg)                         |                                   |                     |
|   | Trimethoprim-Sulphamethoxazole (1.25/23.75µg) |                                   |                     |

\*Homogeneous plating is important to yield reliable results. An alternative method to the standard is to inoculate the plate with a certain volume of suspension (e.g. 50 µL pr. 10 cm agar plate) and use a drigalski (hockeystick) to create the uniform lawn. The method chosen as the routine method should ensure a confluent growth which yields zones for the reference strain *E. coli* ATCC 25922 within the QC-ranges given by the CLSI guidelines (in M100, Table 3). The obtained zone diameters for the *E. coli* reference strain should preferably be in the middle of the QC-ranges for as many antimicrobials as possible.