NATIONAL CENTRE FOR ANIMAL HEALTH,
NATIONAL VETERINARY LABORATORY,
MICROBIOLOGY: BACTERIOLOGY

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SOP No.	Title	Version No.	Total Pages
NCAH/LAB/BACTO 37	SOP on Antimicrobial disk susceptibility tests	2	7

Issue Date	Effective Date	Review Period

Function	Name	Designation	Signature
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REVISION SUMMARY

Version No	Effective Date	Reason for Change	Details of the change
02		Original release	• Antimicrobial panels revised based on animal species • Revision in scope, objective, method and result Interpretation as per CLSI M02, 2018

1. Scope

This SOP describes the procedures for conducting CLSI agar disk diffusion test to obtain the in vitro antimicrobial susceptibility of bacteria that grow aerobically and includes;

- Agar plate preparation
- Testing conditions, including inoculum preparation and standardization, incubation time, incubation temperature,
- Results interpretation
- QC procedures
- Antibiotic panels for small animal, ruminants, poultry, pig, horses

2. Objective

1. To determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds in order to assist a veterinarian in selecting treatment options for his or her patients. The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism.
2. To standardize the antibiotic panels tested for bacteria against animal species among the veterinary laboratories for routine test

3. Principles

A standardized inoculum of the organism is swabbed onto the surface of a Mueller-Hinton agar plate. Antimicrobial impregnated filter paper disks of standardized concentration are placed on the agar. The presence or absence of growth around the disks is observed and measured using a scale in millimeters after 24 hours of incubation. The diameter of the zone of inhibition is interpreted using latest CLSI guideline to obtain report as susceptible, intermediate or resistant.

4. Equipment and Consumables

4.1 Antibiotic disc dispenser or Forceps
4.2 Ruler to measure inhibition zones
4.3 Vortex mixer
4.4 Incubator
4.5 Turbidity meter for McFarland's turbidity determination.
4.6 Sterile petri plates
4.7 Sterile cotton swab

5. Culture media and reagents

5.1 Mueller-Hinton Agar
5.2 Sheep blood
5.3 Normal saline
5.4 Antibiotic discs

6. Disk Diffusion Antibiotic Susceptibility Testing Process

6.1 Preparation of Inoculum

6.1.1 Using sterile loop or swab, transfer four or five isolated colonies of similar colony morphology grown overnight from nonselective medium to 5ml sterile normal saline aliquot.

6.1.2 Vortex for 15-20 seconds to mix well and adjust the turbidity to 0.5 McFarland turbidity standards, which is equal to 1-2 x 10^8 CFU/ml for E.coli ATCC 25922. Optimally use the inoculum within 15 minutes.

6.2 Agar preparation

Muller-Hinton Agar is considered the best medium for routine AST of non-fastidious bacteria, aerobic or facultative bacteria. Certain fastidious species, such as *H. influenzae, H. parainfluenzae, Neisseria, Campylobacter and Streptococci*, do not grow sufficiently on unsupplemented MHA. These organisms need supplements (5% sheep blood) or different media to grow.

6.2.1 The agar medium should have a pH between 7.2 to 7.4 at room temperature.

6.2.2 Store prepared MHA plates at 2-8 C in sealed packages until needed.

6.2.3 Remove the MHA plates from refrigerator at least 15 minutes before use and let them warm to room temperature. Remove/dry plates if surfaces contain excess moisture in an incubator.

6.2.4 Avoid prolonged exposure to elevated temperatures.

6.3 Inoculating Plates

6.3.1 Dip a sterile cotton swab into the inoculum and rotate it against the wall of the tube above the liquid to remove excess inoculum.

6.3.2 Swab entire surface of agar plate three times, rotating plate approximately 60 degrees between streaking to ensure even distribution. Avoid hitting sides of petri plate and creating aerosols.

6.3.3 Allow inoculated plate to stand for at least 3 minutes but no longer than 15 minutes before applying disks.

6.4 Application of Antimicrobial disks to agar plate

6.4.1 After removing unopened containers of disks from freezer or refrigerator, allow them to equilibrate to room temperature (requires at least 1 hr.) prior to opening to minimize condensation. Do not use disks beyond expiry date

6.4.2 Apply disks to agar surface by using antibiotic disk dispenser or sterile forceps.

6.4.3 Apply gentle pressure with sterile forceps to ensure complete contact of disk with agar.

6.4.4 Do not place disks closer to each other than 24mm from center to center.

6.4.5 Place no more than 9 disks on a 150-mm plate or place no more than 5 disks on a 90-mm plate.

6.4.6 Do not relocate a disk once it has made contact with agar surface, because antimicrobial diffusion begins instantly.

6.5 Incubation

6.5.1 Incubate plates within 15 minutes of disk application.

6.5.2 Invert plates and stack them no more than 5 high.

6.5.3 Incubate for 16 to 18 hours at 35±2˚C in an ambient air incubator.

6.5.4 In case of *Campylobacter spp*. incubate in an anaerobic condition.

6.6 Reading plates

6.6.1 Each plate should be examined after incubating for 16 to 18 hours, for confluent lawn of growth and circular zones of inhibition.

6.6.2 The measuring device should be held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, nonreflecting background.

6.6.3 The diameters of the zones of complete inhibition, including the diameter of the disk, should be measured to the nearest whole millimeter with Vernier calipers or a ruler.

6.6.4 If blood was added to the agar base (as with streptococci), the zones must be measured from the upper surface of the agar illuminated with reflected light and with the cover removed.

6.6.5 For coagulase-negative Staphylococcus spp. with cefoxitin, 24 hours of incubation are needed before reporting as susceptible. Other agents should be read and reported at 16 to 18 hours. With cefoxitin, the zone of diameter need to be read with plate held up to the light.

6.6.6 When blood supplemented medium for testing streptococci is used, the zones of growth inhibition, not the zone of inhibition of hemolysis, should be measured

6.7 Reporting Results

6.7.1 Reference Range:

Use CLSI standard (Latest version) guidelines for interpretation, by referring to M100 tables 2A through 2I.

6.7.2 Reporting Format:

6.7.2.1 Report results as susceptible, intermediate, or resistant along with zone diameter according to laboratory practice and format.

6.7.2.2 Report any multidrug resistant (MDR) isolate to National veterinary reference laboratory (NVL) for further confirmation. (MDR- if the isolate are resistant to three or more antibiotic class)

6.7.2.3 Perform ESBL testing if the isolates are resistant to 3rd generation cephalosporin (Ceftriaxone)

7. Quality Control

7.1 QC Strains

7.1.1 Reference strains used are recommended in CLSI Standard (Latest version).

7.1.1.1 *Escherichia coli* ATCC 25922

7.1.1.2 *Staphylococcus aureus ATCC 25923*

7.1.2 Maintenance of QC strains

7.1.2.1 Maintain permanent stock cultures at –20˚C or –70˚C in tryptic soy broth with 15-20% glycerol.

7.1.2.2 Maintain working stock cultures for up to 1 week. Subculture each week for no more than 3 successive weeks.

7.2 Frequency of QC Testing

7.2.1 Perform QC daily until acceptable results from 20 (or 30) consecutive days of testing have been obtained.

7.2.2 Proficiency in performing QC tests is confirmed if for each drug, no more than 1 out of 20 or 3 of 30 results are outside the accuracy limits.

7.2.3 After validation of proficiency, frequency of QC testing can be reduced from daily to weekly.

7.2.4 Results are reviewed for acceptability and recorded on the Quality Control log sheets before reporting results.

8. Antibiotic Panels

Antibiotic panels are specific for animal species and selected based on antibiotic available for prescription.

Table 1: Antibiotic panel for small animal

SL.No	Gram positive panel	Gram negative panel
1	Cotrimoxazole(trimethoprim/sulfamethoxazole)	Ampicillin
2	Enrofloxacin(Ciprofloxacin)	Gentamicin
3	Tetracycline	Ciprofloxacin
4	Ceftriaxone	Cotrimoxazole
5	Penicillin-G	Tetracycline
6	Cloxacillin(Oxacillin)	Amoxicillin/Clavulanate
7	Amoxicillin/Clavulanate	Ceftriaxone

Table 2: Antibiotic panel for ruminants

Sl.no	Gram positive panel	Gram negative panel	Pasteurellaceae
1	Tetracycline	Ampicillin	Ampicillin
2	Ceftriaxone	Tetracycline	Tetracycline
3	Penicillin-g	Ceftriaxone	Ceftriaxone
4	Cloxacillin(oxacillin)	Sulfadimidine	Sulfadimidine
5	Sulfadimidine	Trimethoprim	Trimethoprim
6	Trimethoprim		Penicillin G

Table 3: Antibiotic panel for poultry

Sl.no	Gram positive panel	Gram negative panel
1	Tetracycline	Ampicillin
2	Ceftriaxone	Tetracycline
3	Ampicillin(Amoxycillin)	Ceftriaxone
4	Cotrimoxazole	Cotrimoxazole
5	Tylosin	Neomycin

Table 4: Antibiotic panel for pigs

Sl.no	Gram positive panel	Gram negative panel	Pasteurellaceae
1	Tetracycline	Ampicillin	Ampicillin
2	Ceftriaxone	Tetracycline	Tetracycline
3	Penicillin-G	Ceftriaxone	Ceftriaxone
4	Cloxacillin(Oxacillin)	Cotrimoxazole	Cotrimoxazole
5	Cotrimoxazole	Neomycin	Penicillin_G
6	Tylosin		

Table 5: Antibiotic panel for horses

Sl.no	Gram positive panel	Gram negative panel
1	Tetracycline	Tetracycline
2	Ceftriaxone	Ceftriaxone
3	Penicillin-G	Cotrimoxazole
4	Enrofloxacin(ciprofloxacin)	Gentamin
5	Cotrimoxazole	Enrofloxacin(ciprofloxacin)
6	Cephalexin	

9. Waste Disposal

All the waste generated are disposed in waste container and autoclaved.

10. References

10.1 Harmonized Test Protocol for Isolation, Identification and ABST profiling of Salmonella in Human, Animal and Food products in Bhutan through One Health approach-AGISAR, WHO, Bhutan.

10.2 CLSI Guidelines-M100S-Performance Standards for Antimicrobial Susceptibility Testing, 26th Edition.

10.3 CLSI, M02 Performace standards for Antimicrobial Disk Susceptibility tests 13th edition, January 2018

10.4 Standard Operating Procedures, Bacteriology, Antimicrobial Resistance Surveillance and Research Network, Indian Council of Medical Research, 2nd Edition, 2019.

Foreword

The current version of SOP on Antimicrobial disk susceptibility tests, BACTO 37 (version 2) replaces the older version of SOP on Antimicrobial sensitivity test by CLSI BACTO 37 (version 2018.1). This version is developed as per the CLSI document for performance standards for antimicrobial disk susceptibility tests M02, 13 editions, 2018. The antibiotic panels' specific for animal species was developed with the guidance from Fleming Fund fellowship mentors and as an activity under AMR Laboratory fellowship.

This SOP has several changes made;

General:

- Added scope to the document
- Deleted Introduction from the document
- Added objective to the document
- Revised principal of the document
- Deleted application from the document
- Revised equipment and consumables list
- Revised culture media and reagents list
- Revised the SOP format to new SOP format

Disk Diffusion Antibiotic Susceptibility testing process

- Renamed procedure to Disk Diffusion Antibiotic Susceptibility testing process
- Revised number format
- Revised 6.1 preparation of Inoculum method
- Added 6.2 Agar preparation method
- Revised 6.6 reading plates
- Revised reporting results
- Added quality control
- Revised references
- Deleted interpretive tables
- Deleted trouble shooting and risk assessment

Antibiotic panels for animal species

Classified antibiotic panel as per animal species based on available treatment to guide and assist the prescribing veterinarian on susceptible drugs

- Added Table 1; antibiotic panel for small animal
- Added Table 2; antibiotic panel for ruminants
- Added table 3. Antibiotic panel for poultry
- Added table 4; Antibiotic panel for pigs
- Added table 5; Antibiotic panel for horses