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Laboratory Guide: Methodologies for Antimicrobial Susceptibility Testing

APEC Sub-Committee on Standards and Conformance

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Abbreviations

AMR	**Antimicrobial Resistance**
AST	**Antimicrobial Surveillance**
ATCC	**American Type Culture Collection**
CFU	**Colony-Forming Unit**
CLIA	**Clinical Laboratory Improvement Amendments**
CLSI	**Clinical and Laboratory Standards Institute (formerly NCCLS)**
EUCAST	**European Committee on Antimicrobial Susceptibility Testing**
FDA	**Food and Drug Administration**
MHA	**Mueller-Hinton Agar**
MHB	**Mueller-Hinton Broth**
MIC	**Minimal Inhibitory Concentration**
QA	**Quality Assurance**
QC	**Quality Control**
QS	**Quality System**

Introduction

Bacteria present in our environment may cause several diseases and mortality in farm animals, for this reason a wide range of antimicrobials are used to keep animals healthy. Antimicrobials in the last decades have been misused as some treatments have not been followed through, wrong dosage was used, or the same antimicrobial was used in several therapies without alternating active ingredients, which has led to longer recovery times from the diseases, and overall less effective and costlier treatments. More importantly, lack of veterinary guidance or ignorance regarding antimicrobials may increase the prevalence of transference of resistance genes to bacteria in the environment, rendering current antimicrobials ineffective. Resistant foodborne bacteria are capable of transmitting resistance genes to commensal bacteria in humans and to zoonotic bacteria, for this reason testing bacteria isolated from food samples is practical not only for its importance yielding useful information for animal welfare, but also because of its importance in public health.

There are several antimicrobials used in farm animals worldwide, therefore testing for susceptibility and resistance of bacteria to different drugs needs to be conducted to assess the effectivity of said antimicrobials against zoonotic and pathogenic bacteria. It is important to perform these tests to choose the right antimicrobial for each therapy and with that help reducing the likelihood of resistance.

Susceptibility and resistance can be assessed through different methods and each laboratory determines the method they use that better fits their practice, for this reason at this moment in time it is difficult to compare results between countries or regions of the world that use different antimicrobial susceptibility methods or breakpoints. Therefore, harmonized techniques and breakpoints along with comparability of results is needed to reach a higher level of antimicrobial resistance surveillance worldwide.

As for in vitro methods to determine susceptibility against antimicrobials, disk agar diffusion test is one of the two main techniques, this method consists of disks impregnated in antibiotic used with an agar medium. The second widely used in vitro method is the dilution technique in which the microorganism tested is exposed to increasing concentrations of an antibiotic in broth or agar.

Disk diffusion is a relatively inexpensive, easy to use and flexible agar-based method which provides qualitative results for rapidly growing aerobic bacteria (Schwalbe, et al., 2007). However, very few antimicrobials have veterinary-specific interpretive criteria for this susceptibility method, which forces practitioners to use human interpretive criteria for most antibiotics.

The agar and broth dilution susceptibility-testing methods are used for the determination of the minimal inhibitory concentration (MIC) of an antimicrobial agent required to inhibit the growth of a microorganism (Schwalbe, et al., 2007). This method is used to measure quantitatively the in vitro activity of antimicrobials, which allows for the determination of the minimum concentration of the drug needed to inhibit or eliminate the microorganism.

In this guide we will also review commercial systems, both manual and automated focusing on the automated MIC methods, which offer greater sensitivity with microbroth dilution and produces reproducible and accurate results with some to full automation. See Annex 4 for a summary of all methods reviewed in this manual.

Bacterial Isolation, Identification and Storage

The first approach to susceptibility testing is bacterial isolation, along with purification and identification. To get pure bacterial cultures the isolation process must be carried out and identification needs to take place to begin purification. With purified cultures different aspects of bacteria strains can be studied; such as morphology, physiology and antimicrobial susceptibility.

The obtention of reliable and reproducible results falls on the use of standardized methods throughout the whole process and having quality control within the laboratory and all the materials used. It's important to check the quality of the batches of test bacteria, its media and the overall performance of the assay protocol. Pure cultures can be obtained through different methods, some of the best are solid media, streak plate and the pour plate method. Streak plate tends to be the most practical one, in which the inoculum is placed close to the border of the plate with agar medium and then spread on the upper part of the plate with parallel strokes. The inoculum is also streaked on other parts of the plate to observe isolated colonies.

To have a clear diagnosis and achieve effective antimicrobial treatments, identification of bacterial agents is of importance. Different species of bacteria have distinctive morphological, physiological and biochemical characteristics, identification can be accomplished by testing for such characteristics.

Bacterial cultures must be stored in appropriate medium and there are different ways to do so, one of the methods is by sub-culturing or by transferring the purified culture into fresh solid medium with minimum nutrient content to prevent bacterial overgrowth. Another method is by freezing the bacterial culture, stocked in a broth medium with glycerol, which is added to prevent bacterial desiccation. (Ruangpang and Tendencia, 2004).

It is important to note that cultures should be properly labelled with permanent markers before storing, adding pertinent information such as sample source, date of isolation and identification.

Disk Diffusion Method

How it works

This method consists of inoculating the isolated bacteria onto a Mueller-Hinton agar plate, followed by placing antibiotic-impregnated paper disks on the surface of the agar. By incubating this plate, antibiotics will diffuse into the agar in a gradient, the antibiotic concentration will decrease as the distance from the disk increases. Antibiotic susceptibility is determined by measuring the diameter of the zones of bacterial inhibition around the antibiotic disks and comparing the diameter with disk diffusion interpretive criteria (Schwalbe, et al., 2007).

Materials See Annex 1.1

Media

Mueller-Hinton agar (MHA) is preferred for this method because of its reproducible results and its low sulfonamide, trimethoprim and tetracycline inhibitors which gives satisfactory growth of most bacteria, but other media such as MHA supplemented with blood may also be used as some bacteria have special requirements. Tryptic soy broth or 0.9% saline solution are suitable broths used for the inoculum of the disk diffusion method.

Preparation of Agar Medium

1. Prepare MHA medium according to manufacturer's instructions. Media must be prepared with distilled water or deionized water.

2. Bring to a boil agitating throughout the process until completely issolved. Sterilize in an autoclave at 121°C for 15 minutes.

3. After sterilization, check the pH of the preparation which should be 7.2-7.4 at room temperature.

4. Cool the agar medium to 40-50°C. Pour the agar into a sterile petri dish to a depth of 4mm.

5. Allow the agar to solidify.

6. Dry plates at 30-37°C in an incubator, with its lid slightly ajar until excess moisture has evaporated. Media must be free of water droplets so other bacteria don't contaminate the agar and get inaccurate results.

7. Test a couple of samples from each batch of plates for sterility by incubating at 35°C for 24 hours or longer. Discard these samples after testing.

Storage

1. Plates can be stored in a refrigerator if they are not used shortly after they are prepared. Plates are to be stored in airtight plastic bags or containers at 2-8°C for up to 4 weeks.

2. Media which has not been poured on a plate can be stored in a sealed bottle under the conditions specified by the manufacturer

Control

Before using the prepared plates, make sure the agar can support growth of control strains (such as Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus) by streaking the cultures on the medium.

Inoculum and inoculation

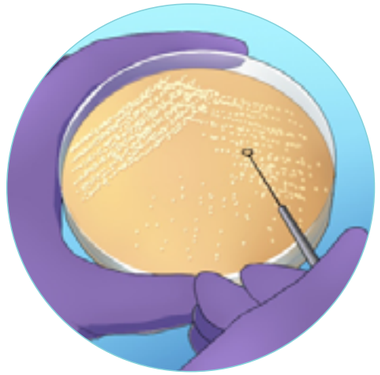
Preparation

1. From the prepared pure bacterial cultures, take 4-5 colonies with an inoculation loop.

2. Transfer colonies to 5 ml of Tryptic soy broth or 0.9% saline solution

3. Incubate broth or solution at 35°C (or optimum growth temperature for bacterial strain tested) until 0.5 McFarland standard turbidity is achieved or exceeded. The standardized inoculum has a concentration of 1-2 × 108 CFU/ml.

4. Compare turbidity of test bacterial suspension with 0.5 standard McFarland (shaken vigorously before use) against a white background with a contrasting black line.



Note: Alternatively, turbidity can be measured with a photometric device calibrated against 0.5 McFarland standard according to the manufacturer's instructions.

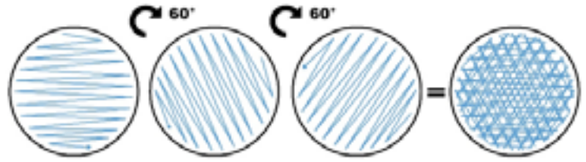
Inoculation of plates

1. Within 15 minutes of adjusting turbidity, dip a sterile cotton swab into the standardized bacterial suspension

2. Press the swab against the tube wall (above the fluid level) to remove excess inoculum.

3. Inoculate the agar by streaking the swab against the plate.

4. Rotate the plate by 60° and repeat the step twice, for an even distribution of inoculum.



5. Allow the surface to dry for 3-5 minutes but no longer than 15 so excess moisture is absorbed.

Antimicrobial disks

Selection

A limited number of antimicrobials should be tested, preferably choosing only one representative of each group of drugs. Antimicrobials of veterinary use and those used for epidemiological or research purposes should be the priority. Only use antibiotic disks purchased from reliable manufacturers, expired disks must not be used.

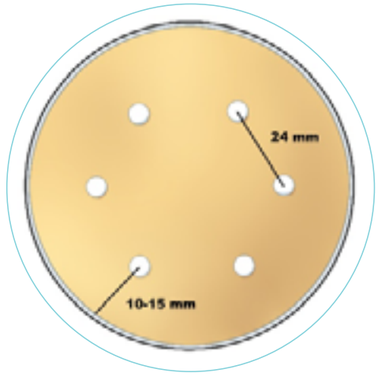
To properly store disks, use airtight containers with a desiccant at 2-8°C.

Application

1. With the help of a sterile forceps or disk dispenser, place antibiotic disk on the dried plate

2. Lightly press down the disk to make sure it is in contact with the surface of the plate. Do not move the disk once it has been placed, since some diffusion might happen.

3. Place disks in a way that between two centers of antibiotic disks there is at least 24 mm, and no less than 10–15 mm from the edge of the plate. A maximum of 6 antibiotic disks can be placed in a 9 cm petri dish. Number of disks must be reduced per plate if overlapping zones of inhibition are found.



Control Plate

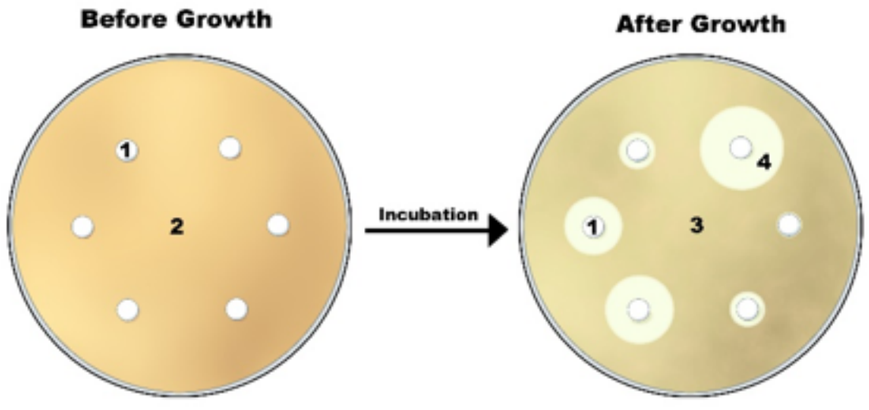
One plate inoculated with a control strain ATCC® is to be included in every batch of inoculated plates.

Incubation

1. Invert the plates and incubate them at 35°C or at an optimum growth temperature for the tested microorganism.

2. Incubate for 16–18 hours. Each laboratory must check requirements for the tested bacterial strain as some have special requirements.

3. The zone of inhibition may be observed after incubation.



1. Antibiotic disc, 2. Agar medium, 3. Bacterial growth, 4. No bacterial growth (zone of inhibition)

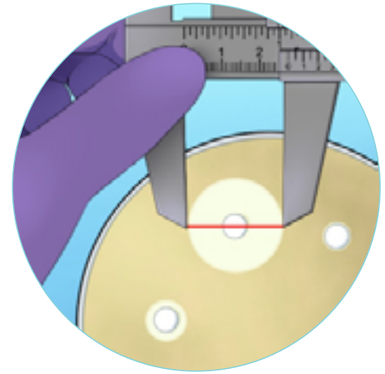
Reading and Measuring Zones of Inhibition

The "zone of inhibition" is a point at which no bacterial growth is visible to an unaided eye.

Reading

1. Read and register the diameter of zones of inhibition preferably using a Vernier scale (or a ruler graduated to 0.5 mm).

2. Round up the zone measurement to the nearest millimeter.



Interpretation of Results

1. Compare the diameter of the zone of inhibition of the test isolates with those of the interpretive criteria for veterinary pathogens from CLSI (see Annex 3).

2. Report results as Resistant (R), Intermediate (I) or Susceptible (S). Example of Results Reporting:

Disk used: Chloramphenicol, 30 µg (C-30)
Zone of inhibition: 16 mm
Result/Interpretation: Intermediate
(Note: Based on CLSI interpretive criteria)

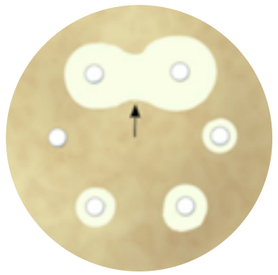
(Ruangpan and Tendencia, 2004)

3. Antimicrobial agents not yet listed with their own interpretive criteria are to be interpreted only qualitatively (presence or absence of a definite zone of inhibition) until its interpretative zones are established.

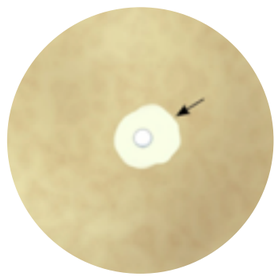
Rejection Criteria

1. Do not read plates which have isolated colonies or with less than uniform growth.

2. Do not read zones of inhibition in which two disks have overlapped.



3. Do not read zones of inhibition which are not circular or have distortion.



4. Reject all information from a batch if the zones of inhibition from a control strain plate are not within the appropriate limits.

Troubleshooting

See Annex 2.

Minimal Inhibitory Concentration Test

How it works

The minimal inhibitory concentration (MIC) is the lowest concentration of an antibiotic that inhibits the growth of a microorganism. This method can be performed on agar or liquid medium. The traditional method to determine the MIC is with a broth dilution technique, in which serial dilutions of antibiotics are incorporated into the broth. Each tube or well contains a different concentration of the antimicrobial agent and is inoculated with a fixed amount of the test bacteria. After incubation, the lowest concentration that shows no visible growth is considered the MIC. This is a quantitative test, in which the results are expressed in µg/ml (Schwalbe, et al., 2007).

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$$\text{(A) Weight (mg)} = \frac{\text{(Volume (ml)}\times\text{Concentration (µg/ml))/}}{\text{(Potency (µg/mg))}}$$

or

$$\text{(B) Volume (ml)} = \frac{\text{(Weight (mg)}\times\text{Potency (µg/mg))/}}{\text{(Concentration (µg/ml))}}$$

Materials

See Annex 1.2

Media

Different media can be used for the MIC test, but MHA is the preferred medium for routine susceptibility testing because it has good reproducibility and is low in sulfonamide, trimethoprim, and tetracycline inhibitors, which gives an effective growth of most bacterial pathogens. (CLSI, 2005)

Preparation of antimicrobial agent stock solution

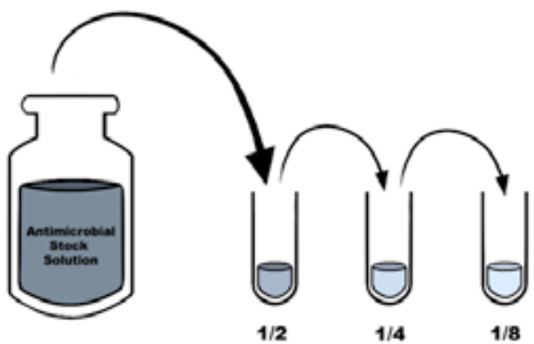
1. Weigh appropriate amount of the powdered antimicrobial agent.

2. Dissolve antimicrobial agent powder in solvent as indicated by manufacturer to make a concentration of at least 1,000 µg/ml or at least 10 times the highest concentration to be tested.

3. Dispense small volumes of the sterile stock solutions into sterile glass, polypropylene, polystyrene, or polyethylene vials. Carefully seal and store preferably at -60 °C or below.

   Note: Vials may be thawed and used the same day. Any unused stock solution should be discarded at the end of the day.

4. Prepare intermediate (10x) antimicrobial agent solutions by making successive (two-fold) 1:2, 1:4, and 1:8 dilutions into sterile diluent.



5. Set aside for now.

Inoculum Preparation

1. Grab 3-5 well-isolated colonies from a pure overnight bacterial culture and subculture it to a tube containing 4-5 ml of a suitable broth medium such as tryptic soy broth.

2. Incubate the broth culture at 35 ± 2°C until it achieves or exceeds a McFarland turbidity of 0.5

3. Adjust turbidity of the inoculum with sterile saline or broth to achieve a turbidity of a 0.5 McFarland standard. Use a photometric device or adequate light to compare the inoculum tube and the 0.5 McFarland standard against a paper with white background and contrasting black lines.

   Note: this results in a suspension containing approximately 1-2 × 108 CFU/ml.

Agar Dilution

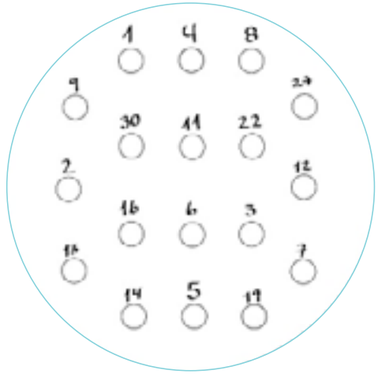
In this technique, antimicrobial agents are incorporated into the agar medium with each different plate containing different concentrations of the drug. The inoculum can be applied effortlessly with an inoculator or manually.

Preparation of Antimicrobial Agar Plates

1. Label each empty plate to be able to identify the antimicrobial agents and their concentrations.

2. Plan on a reference paper the arrangement of numbered bacterial strains, which will be used to read the results later.

3. Prepare MHA medium according to manufacturer's instructions, keep it in a water bath at 45–50°C until used.



4. Add appropriate dilutions of antimicrobial solution (previously prepared in "Preparation of antimicrobial agent stock solution") to molten test agars.

5. Mix the agar and antimicrobial solution thoroughly and pour into Petri dishes on a level surface to result in an agar depth of 3 to 4 mm.

6. Allow the agar to solidify at room temperature and use the plates immediately after the agar surface has dried completely, avoid excessive drying.

Note: use the plates either immediately or store them in sealed plastic bags at 2–8°C for up to five days for reference work, or longer for routine tests.

Inoculation

Dilution of Inoculum Suspension
Cultures adjusted to the 0.5 McFarland standard contain approximately 1–2 x 108 CFU/ml with most species, and the final inoculum required for a 5–8 mm spot is 104 CFU/spot.

»When using replicators with 3 mm pins that deliver 2 µl, dilute the 0.5 McFarland suspension 1:10 in sterile broth or saline to obtain a concentration of 107 CFU/ml which will give a final inoculum on the agar of approximately 104 CFU per spot.

»When using replicators with 1 mm pins that deliver 0.1–0.2 µl, do not dilute the initial suspension.
»When doing manual inoculation dilute the 0.5 McFarland suspension 1:10 in sterile broth or saline and deliver 10 µl of the suspension.

Sequence

The first plate to inoculate must be the control plates, to avoid contamination of the plates with antimicrobial agents. It is important to include the drug-free plate at the beginning of the inoculation process.

1. Arrange the tubes containing the adjusted and diluted bacterial suspensions (107 CFU/ml) in order in a rack.

2. On a fully dried agar plate inoculate the specified amount described on "Dilution of Inoculum suspension", with an inoculum replication device or standardized loops or pipettes.

Note: final concentration of spots should be 104 CFU/spot.

3. Allow the inoculum to be absorbed into the agar before incubation.

Control Agar Plates

Antibiotic free plate: pipette MHA into a sterile petri dish, without any antimicrobial agent.
Growth-control plate: Inoculate a growth-control plate (no antimicrobial agent).
Mixed cultures plate: streak a sample of each inoculum on a suitable non-selective agar plate and incubate overnight to detect mixed cultures.

Incubation

1. Allow the inoculated plates to rest at room temperature until no moisture from the inoculum is visible or until all spots are dry.

2. Incubate the plates in an inverted position at 35 ± 2°C for 16-20 hours (or for longer, depending on the tested microorganism).

Note: do not incubate the plates in an atmosphere with increased CO2 when testing non-fastidious organisms as the surface pH may be altered.

Control Strains

When testing samples, the corresponding QC organism should be tested concurrently. To consider a result valid, the MIC of the QC organism must fall within the acceptable ranges for quality control strains stated in the CLSI guidelines.

Reading MIC Values

1. Place the agar plates on a non-reflective black surface and observe bacterial growth without visual aids. Use the reference paper previously made on Step 2 of "Preparation of Antimicrobial Agar Plates" to locate the position of the test bacteria.

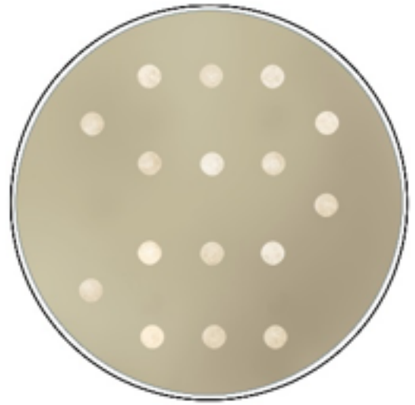
2. Check bacterial growth on the control plates, reject results if no growth is detected in some control plates as the test must be repeated.

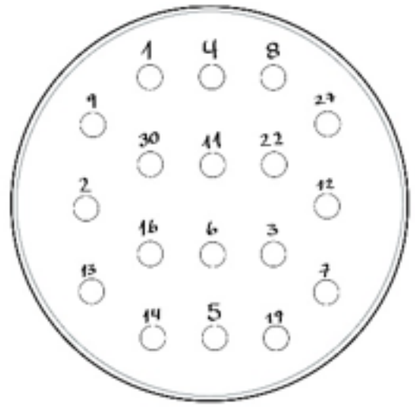
3. Register the MIC (lowest concentration of antibiotic that completely inhibits bacterial growth) detected without visual aid.

4. Compare the MIC breakpoint of the test isolates with those of the interpretive criteria for veterinary pathogens from CLSI (see Annex 3).

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

Example:
Antibiotic: Oxytetracycline
MIC breakpoint: 0.2 µg/mL
Interpretation: Susceptible
(Ruangpan and Tendencia, 2004)





Broth Microdilution

This method involves the use of small volumes, hence the name "microdilution". The broth is dispensed in sterile plastic microdilution trays for which each well should contain 0.1 ml of broth.

Preparing and Storing Diluted Antimicrobial Agents

1. Make intermediate two-fold dilutions of antimicrobial agent volumetrically in broth or sterile water.

Note: use one pipette for measuring all diluents and then for adding the stock antimicrobial solution to the first tube. For each subsequent dilution step, use a new pipette.

2. Dispense the antimicrobial-broth solutions into the plastic microdilution trays.

3. Use a multichannel pipette for preparing microdilution trays as it is the most convenient method. Antimicrobial dilutions made in at least 10 ml of broth should be used.

Note: the dispensing device then delivers 0.1 (± 0.02) ml into each of the 96 wells of a standard tray

4. Compare the MIC breakpoint of the test isolates with those of the interpretive criteria for veterinary pathogens from CLSI (see Annex 3).

Control

Each tray should include a growth control well and a sterility (uninoculated) well.

Inoculum and Inoculation

1. Prepare a standardized inoculum as directed in "Inoculum Preparation"

2. Within 15 min of preparation, dilute colonies in broth, water or saline so after inoculation, each well contains approximately 5×105 CFU/ml (2–8×105 CFU/ml)

Note: the dilution procedure to obtain this final inoculum varies according to the method of delivery of the inoculum to the individual wells and it must be calculated for each situation.

Example: If the volume of broth in the well is 0.1 ml and the inoculum volume is 0.01 ml, then the 0.5 McFarland suspension (1×108 CFU/ml) should be diluted 1:20 to yield 5×106 CFU/ml. When 0.01 ml of this suspension is inoculated into the broth, the final test concentration of bacteria will be 5×105 CFU/ml approximately (or 5×104 CFU/well)

3. Within 15 minutes of inoculum standardization, inoculate each well of a microdilution tray.

»Inoculator device: use it so it delivers a volume that does not exceed 10% of the volume in the well (e.g., ≤10 µl of inoculum in 0.1 ml antimicrobial agent solution).
»Micropipette: if a 0.05 ml pipette is used, it results in a 1:2 dilution of the contents of each well (containing 0.05 ml).

4. Perform a purity check of the inoculum suspension by subculturing an aliquot onto a nonselective agar plate for incubation.

Incubation

Incubate the inoculated microdilution trays within 15 minutes of adding the inoculum at 35 ± 2°C for 16 to 20 hours in an ambient air incubator (or more depending on needs of the microorganism).

Note: to maintain the same incubation temperature for all cultures, do not stack more than four microdilution trays.

Control Strains:

When testing samples, the corresponding QC organism should be tested concurrently. To consider a result valid, the MIC of the QC organism must fall within the acceptable ranges for quality control strains stated in the CLSI guidelines.

Reading MIC Values

1. Wells containing QC strains must be checked to ensure their MIC values are within acceptable ranges

2. Antibiotic-free wells must be checked to ensure bacterial growth is present.

3. Broth control wells must remain clear (no growth).

Note: growth in this well is an indicative of contamination, if growth is found the test must be repeated as its results are invalid.

4. Compare wells with the negative control included in the test, the MIC is detected when there is lack of visual turbidity, matching the negative control. A spectrophotometer can also be used.

5. Compare the MIC breakpoint of the test isolates with those of the interpretive criteria for veterinary pathogens from CLSI (see Annex 3).

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

Example:
Antibiotic: Oxytetracycline
MIC breakpoint: 0.2 µg/ml
Interpretation: Susceptible
(Ruangpan and Tendencia, 2004)

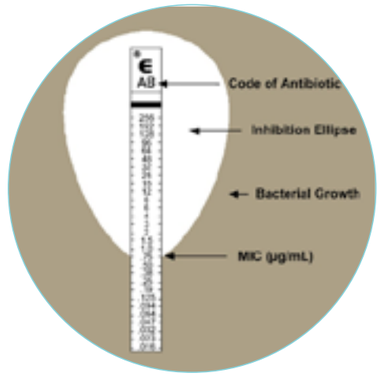
COMMERCIAL SYSTEMS

The FDA in its "Guidance and Review Criteria for Assessment of Antimicrobial Susceptibility Devices" describes the requirements to be met by manufacturers of susceptibility test systems to become "FDA-cleared". The requirements are comparable results to those of CLSI reference methods, overall performance meeting the FDA criteria and the ability of the system to be monitored in the laboratory following the recommended quality control procedures. In this chapter we will review the most widely used commercial systems.

Manual Systems

E´test®

The E-test consists of a nonporous plastic strip immobilized with a predefined gradient of a given antimicrobial agent on one side and a printed MIC scale on the other side. The stability of the gradient is maintained for up to 18 to 20 hours, which covers the critical times of a wide range of pathogens, from rapid growing aerobic bacteria to slow growing fastidious organisms, including anaerobes. When placed on an inoculated agar plate, a continuous antimicrobial gradient is established along the side of the strip. After incubating, the MIC value (µg/ml) can be read from the MIC scale printed on the strip. (Schwalbe, et al., 2007)



Advantages:

- Easy to perform and requires minimal training for optimal test performance.
- Contamination can be easily recognized.
- Minimum labour involved, compared to broth dilution methods.
- Flexible methodology (antimicrobial agent, media, incubation time and inoculum size can be adjusted depending on the microorganism tested).
- E-test can be easily set up for a small number of clinical isolates.

Limitations:

- The main limitation is its cost.

Automated Broth Microdilution Systems

Instrumentalization helps laboratories standardize end points and generally produce results faster than manual AST methods. The FDA has approved a limited amount of automated antimicrobial susceptibility systems, which provide semi-automation to full-automation depending on the system and results within short term incubations (<16 hours) to overnight incubation. System manufacturers offer different settings for their instruments in terms of panels and panel capacity, system specific features and specialized software which enable laboratories to analyze data with ease or report results faster.

The advantages of automated AST systems include reduced labour time, reproducibility, data management with analytical software, and generating results rapidly.

Effective communication of the results to clinicians and pharmacists is essential to maximize the benefits of rapid testing.

VITEK® Systems (Classic, VITEK 2®)

Instrumentalization helps laboratories standardize end points and generally produce results faster than manual AST methods. The FDA has approved a limited amount of automated antimicrobial susceptibility systems, which provide semi-automation to full-automation depending on the system and results within short term incubations (<16 hours) to overnight incubation. System manufacturers offer different settings for their instruments in terms of panels and panel capacity, system specific features and specialized software which enable laboratories to analyze data with ease or report results faster.

The advantages of automated AST systems include reduced labour time, reproducibility, data management with analytical software, and generating results rapidly.

Effective communication of the results to clinicians and pharmacists is essential to maximize the benefits of rapid testing.

MicroScan® WalkAway®

This system developed by Siemens consists of two major type of AST panels, conventional panels which read turbidimetrically after overnight incubation and rapid panels that read fluorometrically after 3.5-15 h of incubation. The panels are conventional 96-well microdilution trays which include MIC panels, MIC combination panels (some wells used for identification) and breakpoint combination panels which are for identification with a limited range of antimicrobial agent dilutions for qualitative results of susceptible, intermediate or resistant.

MicroScan comes with a data management computer and software that can be used to interpret, store and report data.

Becton Dickinson Phoenix™

BD Phoenix is a fully automated susceptibility testing system; which uses chromogenic and fluorogenic substrates in the same panel. This system holds up to 100 panels that contain 136 wells, using standardized inoculums, panels are manually inoculated and placed in the instrument for incubation and reading. The panels are read every 20 minutes until testing is completed. Phoenix also comes with a software that stores data and includes a system for reviewing results.

Sensititre™ ARIS 2X System

The Sensititre system by Thermo Scientific uses standard 96-well microdilution panels, which are inoculated by the Sensititre Autoinoculator, and is capable of handling 64 panels. Bacterial growth in each panel is detected from the fluorescent intensity monitored over 18-24 hours post incubation. (Syal et al., 2017)
Sensititre MIC plates can be customized for use with FDA, CLSI or EUCAST recommendations along with a full customization of the plates tailored to each laboratory's needs. This method is a micro-version of the regular broth dilution method and it provides qualitative and quantitative MIC results. Results can be read manually by visual reading of growth or automatically on an auto-reader using fluorescence.

Advantages and Disadvantages of Automated Systems

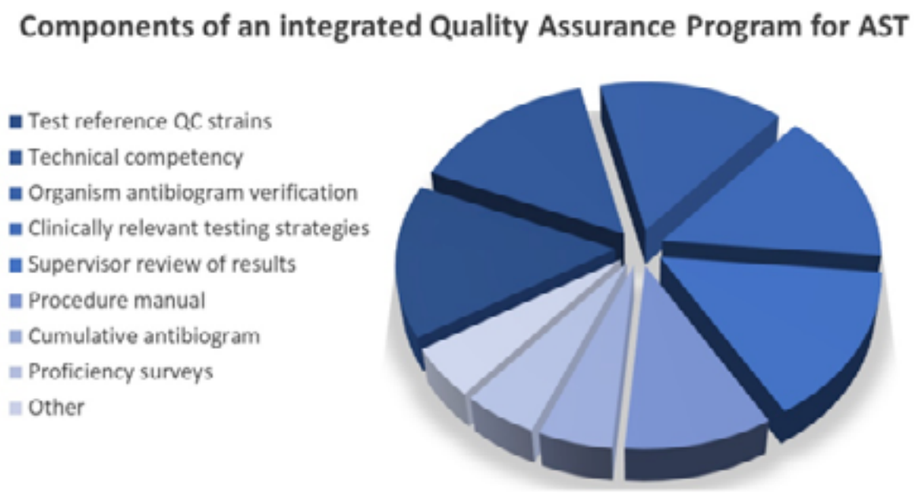
Advantages include labour savings, reproducibility, data management with expert software analysis and results in a shorter period of time. There are limited data showing financial and clinical benefits in association with the rapid provision of AST results. The time required to complete AST testing may eventually be reduced further with the application of molecular techniques. Additional research, increased automation, and lower cost are needed to make this molecular technology available for clinical laboratories. Effective communication of the results to clinicians and pharmacists is essential to realize the potential benefits of rapid testing. Communication may be enhanced by software packages that interface with medication records and alert clinicians or pharmacists when adjustments in antimicrobial therapy are needed. (Jorgensen, et al., 2015)

Disadvantages of automated systems lie mostly on a higher cost in terms of equipment and materials than with manual methods. Predetermined antimicrobial panels and the inability to test all clinically relevant bacteria or antimicrobial agents also pose a great shortcoming for these systems.

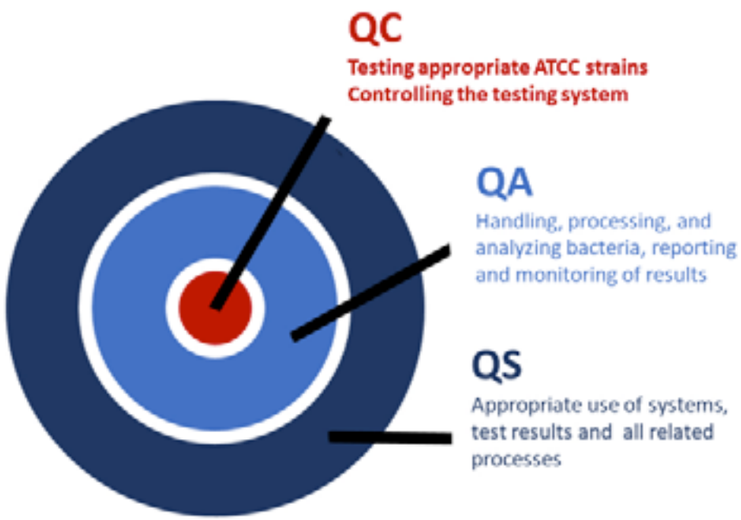
Quality Control

In AST, quality control (QC) includes the procedures to monitor the performance of a test system to ensure reliable results. This is achieved by multiple tasks which focus on monitoring precision and accuracy of susceptibility test procedures, performance of reagents used in the tests and performance of the personnel carrying out the tests and reading the results.

Quality assurance (QA) programs help ensure that all testing materials and processes are providing reliable results. The activities in a QA program include monitoring and evaluating processes, taking corrective actions when needed, record keeping, calibration and maintenance of equipment, training and proficiency testing.



(Cavalieri et al., 2005)



Relationship between the Quality System (QS), Quality Assurance (QA) and Quality Control (QC). (Cavalieri et al., 2005)

The percentages reflect the amount of effort needed by a laboratory to achieve reliable results. There are specific QC requirements for AST in addition to the Clinical Laboratory Improvement Amendments (CLIA) of 1998, which are:

- Making sure to check each new batch of media and each lot of antimicrobial disks before (or simultaneously) initial use, using approved reference strains.
- Verifying that disk diffusion zones and MIC for reference strains are within the established limits before notifying AST results.
- Using appropriate control strains to make sure the test results are accurate.
- Testing control strains weekly, making sure CLSI daily quality standards are met, or performing daily quality control as outlined by CLSI quality standards.

QC Strains

CLSI recommends the use of ATCC® strains for quality control of AST, which are performed to ensure that the tests are working appropriately.

Acceptable Ranges

Acceptable ranges for veterinary QC strains are listed in CLSI VET01 manual and in CLSI M100. These tables are updated annually and provide new changes at the beginning of each manual.

Storage

Each laboratory must have QC strains that suit their needs. The storage for these microorganisms must be properly done to preserve its qualities.

For long-term storage of stock cultures use either:

1. Suitable stabilizing medium (like Tryptic soy broth with 10-15% glycerol or defibrinated sheep blood preferably at -60°C).
2. Store lyophilized.
3. Obtain commercial freeze-dried (lyophilized) stock cultures.

For stock cultures used once a month (or more frequently):

1. Subculture from a permanent stock culture (frozen or lyophilized) to plated media.
2. Subculture 4-5 isolated colonies from plated media to an agar slant and incubate overnight.
3. Store non-fastidious strains on Trypticase soy agar slants and fastidious strains on chocolate agar slants at 2-8°C.

Two days prior to QC testing:

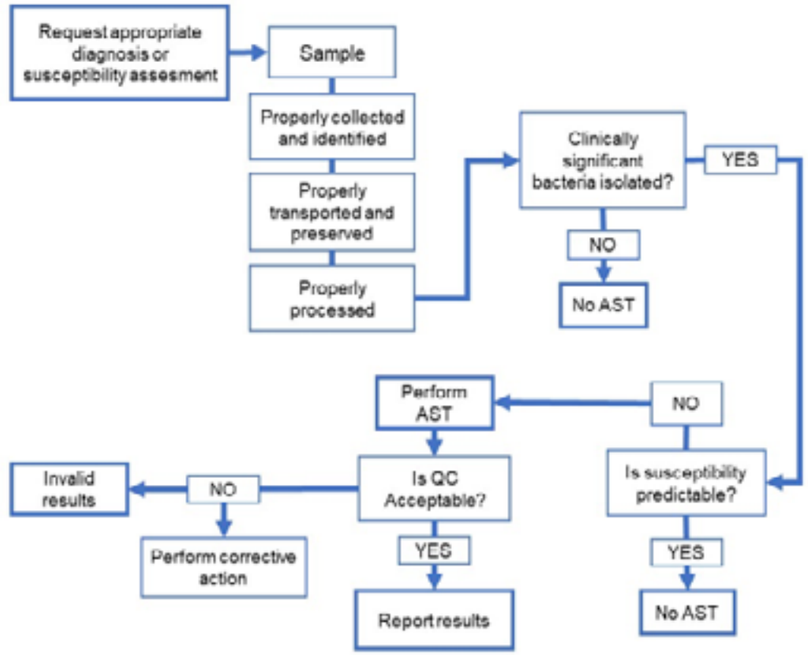
1. Subculture growth from the agar slant to plated media and incubate overnight.
2. Select 4-5 isolated colonies from the plate for QC testing and test with the same method used for sample isolates.

Frequency of Testing

QC strains should be tested each day that susceptibility tests are performed on food samples or samples of animal origin.
Note: Corrective action must be taken if more than 1 in 20 daily QC results for a given drug/organism combination is out of range.
If satisfactory there is performance of daily disk diffusion or MIC QC test by following CLSI standards, and the results are within CLSI ranges and clearly documented in laboratory QC records, you can switch to a weekly QC schedule and test your QC strains once per week.

Quality Systems

The QS approach views the QA as a workflow which involves the whole laboratory and all its processes. The following workflow chart shows a sample path for decision-making during AST as suggested by CLSI.



Sample workflow chart for laboratories. (Cavalieri et al., 2005).

CLSI indicates that the essentials for QS are the organization, personnel, equipment, purchasing process/ inventory, process control, records, occurrence management, internal assessment, process improvement, service and satisfaction as they relate to the workflow.

Annexes

1. MATERIALS

Annex 1.1
Materials and Equipment Used for Disk Diffusion Method.

- Mueller-Hinton Agar (or other suitable agar for the desired bacteria to test)
- Petri dishes
- Pure isolated bacterial cultures
- Antibiotic paper disks
- Quality control organisms
- Distilled water (or deionized water)
- Tryptic soy broth (or 0.9% saline solution)
- 0.5 McFarland Standard
- Cotton swabs
- Forceps (or antibiotic disk dispenser)
- Airtight plastic containers (or plastic bags)
- Inoculation loop
- Autoclave
- pH Meter
- Thermometer
- Incubator
- Refrigerator
- Spectrophotometer

Annex 1.2
Materials and Equipment Used for Agar and Broth Microdilution Methods.

- Mueller-Hinton Agar (or other suitable agar for the desired bacteria to test)
- Powdered antimicrobial agent (and suitable solvent as indicated by manufacturer)
- Pure isolated bacterial cultures
- Quality control organisms
- 0.5 McFarland standard
- Petri dishes
- Microtiter plates
- Multichannel micropipette
- Weighing scale
- Autoclave
- pH Meter
- Thermometer
- Spectrophotometer
- Incubator
- Refrigerator

Annex 2. Disk Diffusion Troubleshooting Guide

Result	Possible Cause(s)	Suggested Solution
Zones too small	Inoculum too heavy	Use McFarland standard or calibrator to carefully measure inoculum density and perform colony counts.
	Agar too thick	Measure agar depth carefully.
	Disk expired or inactive	Use new lot of disks or unopened cartridge
	Inoculated plates left too long prior to application of disks	Apply disks within 15 minutes.
	Wrong medium for organism	Follow CLSI guidelines for appropriate choice of media, perform quality control.
Zones too large	Inoculum too light	Use McFarland standard or calibrator to carefully measure inoculum density and perform colony counts.
	Agar too thin	Measure agar depth carefully.
	Poor growth (too fastidious, wrong media, not fresh isolate)	Check all variables.
Single disk out of control	Improper storage of disks	Maintain majority of disk stock at -20°C, only keep maximum of 1 week supply at 4°C (be cautious of β-lactams, clavulanic acid containing disks and imipenem).
	Media pH too low or too high	Particularly affects tetracycline, macrolides and clindamycin (CO2 incubation can decrease pH).
	Cation concentrations too low	Especially affects aminoglycosides and P. aeruginosa.
	Transcription or reading error	Reread or reset the test.
Colonies within zone	Mixed population	Re isolate or Gram stain the colonies.
Deformation of zone	Disks too close to each other	Place fewer disks on plate (especially with very susceptible organisms).

(Schwalbe, et al., 2007).

Annex 3. CLSI MIC Breakpoint and Zone Diameter Interpretive Criteria for Cattle, Poultry and Swine

Cattle

Antimicrobial Agent	MIC Breakpoint (µg/ml)			Disk Content	Zone Diameter (mm)		
	S	I	R		S	I	R
Aminoglycosides							
Spectinomycin • *Mannheimia haemolytica* • *Pasteurella multocida* • *Histophilus somni*	≤ 32	64	≥ 128	100 µg	≥ 14	11-13	≤ 10
Penicillins							
Penicillin G • *Mannheimia haemolytica* • *Pausterella multocida* • *Histophilus somni*	≤0.25	0.5	≥1.0	—	—	—	—
Penicillin G – Novobiocin • *Staphylococcus aureus* • *Streptococcus agalactiae* • *Streptococcus dysgalactiae* • *Streptococcus uberis*	≤ ½	2/4	≥ 4/8	10 units/30 µg	≥ 18	15-17	≤ 14
Cephalosporins							
Ceftiofur • *Escherichia coli* • *Staphylococcus aureus* • *Streptococcus agalactiae* • *Streptococcus dysgalactiae* • *Streptococcus uberis*	≤2	4	≥8	30 µg	≥ 21	18–20	≤ 17
• *Mannheimia haemolytica* • *Pasteurella multocida* • *Histophilus somni*	≤ 2	4	≥ 8	30 µg	≥ 21	18-20	≤ 17
Fluoroquinolones							
Danofloxacin • *Mannheimia haemolytica* • *Pasteurella multocida*	≤0.25	—	—	5 µg	≥22	—	—
Enrofloxacin • *Mannheimia haemolytica* • *Pasteurella multocida* • *Histophilus somni*	≤0.25	0.5-1	≥2	5µg	≥21	17-20	≤16
Lincosamides							
Pirlimycin • *Staphylococcus aureus* • *Streptococcus agalactiae* • *Streptococcus dysgalactiae* • *Streptococcus uberis*	≤2	—	≥4	2µg	≥13	—	≤12
Macrolides							
Tildipirosin • *Mannheimia haemolytica*	≤4	8	≥16	60 µg	≥20	17–19	≤16
• *Pasteurella multocida*	≤8	16	≥32	60 µg	≥21	18-20	≤17
• *Histophilus somni*	≤8	16	≥32	60 µg	≥17	14–16	≤13
Tulathromycin • *Mannheimia haemolytica* • Pasteurella multocida • *Histophilus somni*	≤16	32	≥64	30 µg	≥18	15–17	≤14
Phenicols							
Florfenicol • *Mannheimia haemolytica*	≤ 2	4	≥ 8	30 µg	≥ 19	15-18	≤ 14
• *Histophilus somni*	≤16	32	≥64	30 µg	≥18	15–17	≤14
Tetracyclines							
Tetracycline • *Enterobacteriaceae*	≤4	8	≥16	30 µg	≥15	12–14	≤11
• *Mannheimia haemolytica*	≤2	4	≥8	—	—	—	—

Poultry

Antimicrobial Agent	MIC Breakpoint (µg/ml)			Disk Content	Zone Diameter (mm)		
	S	I	R		S	I	R
Enrofloxacin • *Escherichia coli*	≤0.25	0.5–1	≥2	5 µg	≥23	17–22	≤16

Swine

Antimicrobial Agent	MIC Breakpoint (µg/ml)			Disk Content	Zone Diameter (mm)		
	S	I	R		S	I	R
Penicillins							
Penicillin G • *Streptococcus sues* • *Pasteurella multocida*	≤0.25	0.5	≥1	—	—	—	—
Cephalosporins							
Ceftiofur • *Pasteurella multocida* • *Actinobacillus pleuropneumoniae*	≤2	4	≥8	30 µg	≥21	18–20	≤17
Fluoroquinolones							
Enrofloxacin • *Streptococcus sues*	≤0.5	1	≥2	—	—	—	—
• *Pasteurella multocida* • *Actinobacillus pleuropneumoniae*	≤0.25	0.5	≥1	5 µg	≥23	19–22	≤18
Macrolides							
Tildipirosin • *Bordetella bronchiseptica*	≤8	—	—	60 µg	≥18	—	—
• *Pasteurella multocida*	≤4	—	—	60 µg	≥19	—	—
• *Actinobacillus pleuropneumoniae*	≤16	—	—	—	—	—	—
Tilmicosin • *Pasteurella multocida* • *Actinobacillus pleuropneumoniae*	≤16	—	≥32	15 µg	≥11	—	≤10
Tulathromycin • *Bordetella bronchiseptica* • *Pasteurella multocida*	≤16	32	≥64	30 µg	≥18	15–17	≤14
• *Actinobacillus pleuropneumoniae*	≤64	—	—	30 µg	≥10	—	—
Phenicols							
Florfenicol • *Salmonella 32ntérica* subsp. *32ntérica* serovar Choleraesuis	≤4	8	≥16	—	—	—	—
• *Streptococcus sues* • *Bordetella bronchiseptica* • *Actinobacillus pleuropneumoniae*	≤2	4	≥8	30 µg	≥22	19–21	≤18
Pleuromutilins							
Tiamulin • *Actinobacillus pleuropneumoniae*	≤16	≥32	30 µg	≥9	—	—	—
Tetracyclines							
Tetracycline • *Streptococcus sues* • *Actinobacillus pleuropneumoniae*	≤0.5	≥2	—	—	—	—	—

(CLSI, 2015)

Annex 4. Summary of Current AST technologies

AST Technology	Summary of Method	Time of AST	Real MIC	FDA Approved
Solid media cultures				
Agar Dilution Assay	Bacteria inoculated on agar plates with antibiotic discs of different concentrations	16-24 Hours	Yes/No	Yes
Disk Diffusion	Bacteria inoculated on agar plates with a single antibiotic disk	16-24 Hours	Yes/No	
E-test	Bacteria inoculated on agar plates with a graded antibiotic concentration strips	16-24 Hours	Yes	Yes
Liquid media cultures				
Broth Dilution Assay	Bacteria inoculated in liquid media with different antibiotics to monitor growth	12-24 Hours	Yes	Yes
Automated Instruments				
MicroScan® WalkAway®	Measure bacterial growth in the presence of antibiotics by recording bacterial turbidity using a photometer	4.5-18 Hours	Yes	Yes
Vitek®/Vitek-2®	Measure bacterial growth in the presence of antibiotics by recording bacterial turbidity using a photometer	6-11 Hours	Yes	Yes
BD Phoenix™	Record bacterial growth in the presence of antibiotics by recording bacterial turbidity and colorimetric changes	9-15 Hours	Yes	Yes
Sensititre™	Record bacterial growth with antibiotics by measuring fluorescence	18-24 Hours	Yes	Yes

(Syal, *et al.* 2017)

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