REPUBLIC OF LEBANON
MINISTRY OF PUBLIC HEALTH

World Health Organization

Standard Operating Procedures for Antimicrobial Susceptibility Testing against Commonly Encountered Bacterial species in Humans

July 2018

Printed with the support of
People's Republic of China
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Acknowledgments

This Guideline has been prepared by national experts under the guidance of WHO Lebanon Country Office team, with the overall supervision of the national AMR Committee.

Special acknowledgment goes to the Director General of the MOPH Dr. Walid Ammar for his guidance throughout the development process of this guideline.

We would like to also thank WHO Representative Dr. Gabriele Riedner, for her support throughout the whole development process of these guidelines.

Moreover, we would like to acknowledge the ample contribution of:

Ms. Rima G. El Asmar, Coordinator of Bacteriology section at AUBMC, and Ms. Lina Y. Itani, Supervisor of Clinical Microbiology at AUBMC, for drafting the guideline.

Dr. George F. Araj, Professor and Director of Clinical Microbiology at AUBMC, for his direct technical input.

Dr. Atika Berry, Head of the Preventive Medicine Department at the MOPH for facilitating the development process.

Dr. Alissar Rady, National Professional at WHO CO Lebanon, for coordinating the overall development process.

SOP for Antimicrobial Susceptibility Testing against Commonly Encountered Bacterial species in Humans

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A. Introduction and Essentials for Testing

1. Principle of Kirby-Bauer technique (K-B) SOP for Antimicrobial Susceptibility Testing

K-B testing or disk diffusion (DD) antimicrobial susceptibility testing is an In Vitro test by which antibiotic containing disks are used to determine whether a microorganism is susceptible, intermediate or resistant to a specific antibiotic.

This test uses standard disks each containing different antibiotic concentration and resulting in a zone diameter of inhibition that is interpreted according to the guidelines breakpoints of international official bodies such as the America Clinical Laboratory Standard Institute (CLSI), and the European EUCAST guidelines breakpoints, being reevaluated yearly.

Such a test and information is of a proven major help to offer reliable guide and data to the physicians for the proper use of antimicrobial agents in patients suffering from infectious pathogens.

There are several critical steps involved in performing the test that need to be closely attended to ensure credible and reliable results. These include the appropriate preparation of bacterial inoculum, media type, its adequate preparation and the necessary volume to pour in the plates, the specific antimicrobial battery to test for each type of pathogen, the optimum temperature to use for incubation of the plates, the quality control organisms to use in ensuring the potency of antimicrobial disks and the reliability of testing for generating accurate results.

2. Bacterial Inoculum preparation SOP for Bacterial Inoculum to use in Antimicrobial Susceptibility Testing

Procedure

1. Using a sterile loop, pick 2-3 well isolated fresh colonies from a pure culture plate.
2. Suspend and emulsify the colonies in a tube containing 4-5 ml of adequate broth medium such as soy bean casein digest broth, Mueller Hinton broth, or trypticase soy broth.
3. Incubate the broth tube at 35-37 °C for approximately 1 hour to enhance logarithmic growth phase and produce moderate cloudiness resulting in 0.5 McFarland (MF) turbidity (check below). If time doesn't permit for incubation, more colonies can be added to the broth. If increased turbidity, broth culture can be diluted with sterile saline or broth to obtain a turbidity equivalent to 0.5 MF turbidity standard. Note that for slow growing microorganisms such as *S. pneumoniae*, *Haemophilus* spp, and *N. gonorrheae*, a direct saline or broth inoculums from an overnight culture plate is the method of choice as their grow in broth could fail.
4. McFarland (MF) standard can be purchased ready or prepared in lab. It is used as a turbidity standard in the preparation of microorganism suspensions for performing antimicrobial susceptibility testing, A 0.5 MF turbidity is the optimal requirement for susceptibility testing. It provides an optical density comparable to the density of a bacterial suspension $1.5x10^8$ colony forming units per ml (CFU/ml). Sulfuric acid (99.5 ml – 0.18 M) and Barium Chloride (0.5 ml – 0.048M) are used to prepare 100 ml of 0.5 MF standard turbidity using aseptic technique.
   Prepared tubes are stored at 2-25°C for up to 6 months. Tubes that show any sign of contamination, discoloration, or deterioration should be discarded.

3. SOP of Media Preparation for Antimicrobial Susceptibility Testing

Mueller Hinton (MH) is the standard medium for the Bauer-Kirby antimicrobial susceptibility method. Different types of agar (e.g. MH, MH blood (MHB), Haemophilus testing medium (HTM) and Vancomycin (Va) agar plates) are used to perform antibiotic susceptibility testing for different species of bacteria. In media preparation, several factors are attended to for ensuring adequate performance characteristics regarding bacterial growth needs, such as:

- The effects of differences in concentration of divalent cations Mg++ and Ca++. These effects are shown as MIC variations with aminoglycosides against *Pseudomonas aeruginosa* and tetracycline against staphylococci.
- Variation in thymine and thymidine content, which affect sulphonamide and trimethoprim MIC values.
- Differences in the characteristics of the agar used in the medium, especially diffusion properties.

  It is worth noting that all these variables are taking care of in the commercially available media.

The following are Steps for Preparation of Mueller Hinton agar and other Media:

A. Preparation of Mueller Hinton (MH) agar Plates

1. Mueller Hinton media are prepared according to manufacturer's instructions
   - Distilled water is added to the dehydrated/ powder medium to dissolve it.
   - Mixture is boiled to dissolve completely
   - Medium is then sterilized by autoclaving at 121°C for 15 minutes

   Readymade media can be also purchased
2. Plates are placed on a leveled horizontal surface
3. Media is poured in petri dishes to result a 4mm depth of agar, for 14 cm plates approximately 60 to 70 ml of agar is poured and for 9 cm plates 25-30 ml.
4. Plates are covered directly with lid and left at room temperature to harden allowing extra moisture to evaporate.
5. Agar medium PH should be adjusted between 7.2 and 7.4 at room temperature and checked after gelling using a PH electrode by letting a small amount of agar gel around the PH electrode. If needed adjust PH using 1NNaOH or HCl before autoclaving.
6. Susceptibility agar plates can be used directly after cooling or stored in plastic bags at 2-8 °C for 7 days.
7. **Note** that before using refrigerated plates should be brought to room temperature for 10-30 minutes allowing extra moisture out.

B. Preparation of Mueller Hinton Sheep Blood (MHSB) agar Plates

Prepare the MHSB following the MH steps except to add 5% Sheep blood after autoclaving and cooling the media to around 56 °C. Keep mixing till the sheep blood is homogenized with the agar. Subsequently, do the pouring in plates as noted above.

C. Preparation of Haemophilus Test Media (HTM) agar Plates

HTM is commercially available readymade lyophilized media.Follow the Manufacturer instruction in its preparation, essentially proceeding as noted for the preparation of MH agar media above.

D. Quality Control (QC) for Prepared Media.

1. Few plates (3-5) of the prepared media should be incubated at 37°C to ensure sterility.
2. QC microorganisms should be tested on each prepared media lot to ensure appropriate growth.
3. Use ATCC strains of *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213), *S. pneumoniae* (ATCC 49619), and *Haemophilus influenzae* (ATCC 49247) for QC testing.

Table. Bacterial species vs. susceptibility agar plates to use

Microorganism Vs. susceptibility agar plate			
Microorganism	**MH**	**MHB**	**HTM**
Staphylococcus species	x		
Beta hemolytic Streptococcus species		x	
Streptococcus pneumoniae		x	
Streptococcus viridians group		x	
Enterococcus species	x		
Enterobacteriaceae species	x		
Pseudomonas aeruginosa	x		
Acinetobacter species	x		
Stenotrophomonas maltophilia	x		
Burkholderia cepacia	x		
Bacillus species	x		
Listeria species		x	
Diphtheroids species		x	
Haemophilus species			x
Moraxella species		x	

4. SOP for Plates Inoculation in Antimicrobial Susceptibility Testing

Procedure

1. Adequate media plates are brought to room temperature approximately 15 minutes before testing.
2. Within 15 minutes after adjusting the inoculums turbidity, a sterile cotton swab is dipped into the inoculums tube, then rotated up against the inside of the tube to remove excess broth.
3. Then entire surface of the Mueller Hinton agar plate is streaked evenly using the swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. The inoculums are left to dry for 3-5 minutes but not longer than 15 minutes with lid closed.
5. The appropriate antibiotic disks for the pathogen under testing (Check Tables for antimicrobial batteries to use for specific pathogens) are then placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser. With the sterile forceps the disks are pressed gently on the agar to ensure contact. Always ensure forceps sterility to avoid carryover of bacteria or antibiotic. Never remove a placed disk as the contained antibiotic diffuses immediately after contact.
6. About 8 disks are placed in the outer ring and two disks in the center of the plate. Disks should be placed evenly with 24mm minimum distance.
7. Antibiotic disks that produce large inhibition zones should be placed in the outer circle and smaller ones in the inner circle to avoid overlapping zones.
8. Plates are covered, inverted and incubated immediately or within 15 minutes at 35°C or 37°C with or without CO2 according to the microorganism tested.
9. Plates are ready to read after 18-24 hrs, to determine and interpret inhibition zone diameters.

5. SOP for Measurement and Interpretation of Inhibition Zones in Antimicrobial Susceptibility Testing

1. A Vernier caliper or a ruler is used to measure the inhibition zone around the antibiotic disks.
2. The zone diameter (including the 6 mm disk) is measured against a dark back ground or measuring the zones against the side of roof top light source so as not to miss mutants. If growth doesn't permit reading the whole diameter, the radius can be measured from the center of the disk to the edge of the inhibition zone and multiplied by two.

Note that if Muller Hinton blood agar is used it is best to measure the diameter from the top of the plate with the lid removed.

3. When zone diameter is irregular, choose the most representative part of the zone to measure.
4. The inhibition zone can be judged by naked eye, but in some cases accurate interpretation should be considered. Such cases include:
   a) The swarming of *Proteus* species into the areas of inhibition, this veil **should be ignored** and the zone interpreted at the edge of the clearly outlined inhibition zone.
   b) Sulfonamide inhibition zones might include a slight growth (haze) because some organisms grow through several generations before sulfonamides take effect and because Mueller Hinton agar contains thymidine which inhibits sulfonamide activity. This also **should be ignored** and the inhibition zone measured at the edge of the clearly outlined inhibition zone.

c) Mutants growing inside the inhibition zone **should be considered** as they indicate emerging resistance within the microorganism and the inhibition zone should be measured from the edge of the growing mutants.

Note that technician should first confirm that these are mutants of the same microorganism and not a contaminant by re-identifying these colonies.

d) Zone diameters should always be read after 18-24 hours of incubation, and in Staphylococci should be read not before 24hrs for proper detection of Methicillin resistance.

e) Inhibition zones are recorded to the nearest 0.1 mm.

5. Extrapolate the zone diameter from the breakpoint zones for S, I or R specified by the International Guideline, e.g. CLSI.
6. Caution should be exercised and emphasized to provide the proper results in the overall final report. For example when Methicillin resistance is noted in Staphylococci, the penicillin's should be extrapolated as R, though the zone show S to them. Also one should be aware to check for the intrinsic resistance in some pathogens. A table of Pathogens with Intrinsic Resistance for different antimicrobial agents is provided.

B. Gram Negative Bacteria

1. *Acinetobacter* species - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar (MH)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from MacConkey Agar plate.
3. **Streaking**
   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 37°C, for 20-24 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Acinetobacter* species table on next page).

Note: In case of Multi Drug Resistant (MDR) strains of *Acinetobacter baumanii* test the isolates against Tigecycline, Colistin, and Minocycline antibiotics and interpret the results as mentioned in the table below.

***Acinetobacter* species**

Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category

***Acinetobacter* species**				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Piperacillin/tazobactam	(100/10 μg)	≤17	18-20	≥21
Ceftazidime	(30μg)	≤14	15-17	≥18
Cefepime	(30 μg)	≤14	15-17	≥18
Imipenem	(10 μg)	≤18	19-21	≥22
Gentamicin	(10 μg)	≤12	13-14	≥15
Amikacin	(30 μg)	≤14	15-16	≥17
Tetracycline	(30 μg)	≤11	12-14	≥15
Ciprofloxacin	(5 μg)	≤15	16-20	≥21
Trimethoprim/Sulfamethoxazole	(1.25/23.75μg)	≤10	11-15	≥16
Colistin	(10 μg) *	≤12	13	≥14
Tigecycline	(15 μg)	≤12	13-15	≥16
Minocycline	(30 μg)	≤12	13-15	≥16

* For Colistin Breakpoints are interpreted according to IIAA 2008;31.434

2. *Burkholderia cepacia* - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar (MH)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from MacConkey Agar plate.
3. **Streaking**
   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 37°C, for 20-24 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Burkholderia* species table on next page).

***Burkholderia* species-** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

Burkholderia cepacia				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Ceftazidime	(30 µg)	≤17	18-20	≥21
Meropenem	(10 µg)	≤15	16-19	≥20
Minocycline	(30 µg)	≤14	15-18	≥19
Trimethoprim/Sulfamethoxazole	(1.25/23.75µg)	≤10	11-15	≥16

3. *Campylobacter* species - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Chocolate agar plate (Choc)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Chocolate or Campylobacter Agar plate.
3. **Streaking**
   Streak the entire surface of the Choc agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 35°C, CO2, for 20-24 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Campylobacter* species table on next page).

***Campylobacter* species -** Antimicrobial disks (content), and E- test together with interpretive zone diameter for susceptible, intermediate and resistant category.

***Campylobacter* species**							
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**			**MIC (ug/ml)**		
		R	**I**	**S**	**S**	**I**	**R**
Erythromycin	(15 μg)	6			≤8	16	≥32
Ciprofloxacin	(5 μg)	6			≤1	2	≥4

Appearance of any zone of inhibition (DD) would require MIC determination for accurate categorization of susceptibility.

4. *CRE & ESBL-SOP for Enterobacteriaceae spp.*

It is crucial from a clinical, treatment and infection control aspects to recognize and detect CRE and ESBL isolates encountered among Enterobacteriaceae species isolates. Thus, it is important to detect such isolates according to the following approaches:

1. **Carbapenem Resistant *Enterobacteriaceae*** [(CRE), e.g. in *Klebsiella pneumoniae, K. oxytoca*, *Escherichia coli* and *Proteus mirabilis, Enterobacter* spp., *Citrobacter* spp.] are primarily determined from the inhibition zone of Ertapenem (10 µg) susceptibility screening disk, showing ≤ 21 mm in the battery of antimicrobials included for Enterobacteriaceae testing. Once detected, additional MIC testing for Ertapenem, Imipenem and Meropenem is required. The latter is carried out using a pure fresh bacterial suspension, same as the disk diffusion technique. MH plate is streaked and the E-test strips are placed on the Mueller Hinton agar, 2 strips can be placed/accommodated on each agar plate, and incubated for 18-24 hrs at 37°C under aerobic conditions. While reading/determining the breakpoints (at the intersection of the inhibition zones), take into consideration the presence of mutant colonies. Interpretation should be done including the last mutant within the zone of inhibition. Inhibition zones are interpreted according to the following breakpoints:

Antibiotic	S	I	R
Imipenem	≤1	2	≥4
Ertapenem	≤0.5	1	≥2
Meropenem	≤1	2	≥4

In addition, Colistin and Tigecycline disks are both added in the testing for CRE isolates, as these are very important for treatment purposes.

Antibiotic		R	I	S
Colistin	**(10µg)**	≤11		≥14
Tigecycline	**(15 µg)**	≤12	13-15	≥16

2. **Extended-spectrum β-lactamases (ESBL)** are β-lactamases capable of conferring bacterial resistance to the penicillins; first-, second- and third-generation cephalosporins; and Aztreonam (but not the cephamycins or carbapenems e.g. Cefoxitin) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid.

   CLSI provide guidelines for the detection of ESBLs in *Klebsiella pneumoniae*, *K. oxytoca*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella* spp. *Shigella s*pp. Common to all ESBL-detection methods is the general principle that the activity of extended-spectrum cephalosporins against ESBL-producing organisms will be enhanced by the presence of clavulanic acid
   In suspected ESBL isolates we check the main antibiotics before reporting, and it should be showing R or I for Aztreonam, Ceftazidime, Cefotaxime, and or Ceftriaxone But shows S to Cefoxitin and Imipenem, irrespective of the remaining antibiotics. This has been validated and reflects a cost effective and accurate method in the determination of ESBL.
   Double disk diffusion assay (e.g. containing antimicrobial and Beta lactamase inhibitor) are commercially available for routine or confirmatory use as well.

5. *Enterobacteriaceae* species (*E. coli, Klebsiella* spp., *Proteus* spp., *Salmonella* spp., *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp. etc.) SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar (MH)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from MacConkey Agar plate.
3. **Streaking**
   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 37°C, for 18-20 hrs
6. **Reading (Alert/Causion to detect CRE and ESBL isolates)**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Enterobacteriaceae* species table on next page).

***Enterobacteriaceae* species -** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

***Enterobacteriaceae* spp.**				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Ampicillin	(10 µg)	≤13	14-16	≥17
Amoxicillin/clavulanic acid	(20/10 µg)	≤13	14-17	≥18
Piperacillin/tazobactam	(100/10 µg)	≤17	18-20	≥21
Cefuroxime	(30 µg)	≤14	15-17	≥18
Cefepime	(30 µg)	≤18	19-24*	≥25
Cefoxitin	(30 µg)	≤14	15-17	≥18
Cefotaxime	(30 µg)	≤22	23-25	≥26
Ceftazidime	(30 µg)	≤17	18-20	≥21
Cefixime	(5 µg)$^{+}$	≤15	16-18	≥19
Imipenem	(10 µg)	≤19	20-22	≥23
Gentamicin	(10 µg)	≤12	13-14	≥15
Amikacin	(30 µg)	≤14	15-16	≥17
Ciprofloxacin	(5 µg)	≤15	16-20	≥21
Trimethoprim/Sulfamethoxazole	(1.25/23.75µg)	≤10	11-15	≥16
Nitrofurantoin^{+}	(300 µg)	≤14	15-16	≥17
Aztreonam	(30 µg)	≤17	18-20	≥21
Ertapenem	(10 µg)	≤18	19-21	≥22
Fosfomycin^{+}	(200 µg)	≤12	13-15	≥16
Tigecycline	(15 µg)	≤12	13-15	≥16
Tetracycline	(30 µg)	≤11	12-14	≥15

($^{+}$) antibiotic added for urine isolates only

***** "Cefepime" New interpretation: S-DD (susceptible dose dependent) breakpoint

Note: Extrapolate:

- Levofloxacin, Ofloxacin & Norfloxacin from Ciprofloxacin
- Cefamandole from Cefuroxime
- Imipenem from Ertapenem

6. *Haemophilus* species - SOP for Antimicrobial Susceptibility Testing

1. **Medium**

   Haemophilus Test Medium (HTM)

2. **Inoculum**

   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Chocolate Agar plate

3. **Streaking**

   Streak the entire surface of the HTM agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.

4. **Adding the disks**

   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser

5. **Incubation**

   Incubate at 35°C, 5% CO2 for 16-18 hrs

6. **Reading**

   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Haemophilus* species table on next page).

Note: Always perform Beta lactamase testing on all *Haemophilus* species isolates and correlate with Ampicillin, taking into consideration the presence of BLNAR strains (Beta Lactamase Negative Ampicillin Resistant).

Also serotyping *H. influenzae*, as tybe b or not b, is of clinical and epidemiologic importance to help vaccination policy in the country.

***Haemophilus* species** - Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category

***Haemophilus* species**				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Ampicillin	(10 µg)	≤18	19-21	≥22
Amoxicillin/clavulanic acid	(20/10 µg)	≤19		≥20
Ceftriaxone	(30 µg)	≤25		≥26
Cefuroxime	(30 µg)	≤16	17-19	≥20
Levofloxacin	(5 µg)	≤16		≥17
Trimethoprim/Sulfamethoxazole	(1.25/23.75µg)	≤10	11-15	≥16

7. *Moraxella catarrhalis* - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar with 5% sheep blood
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.
3. **Streaking**
   Streak the entire surface of the MHSB agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 35°C, 5% CO2 for 20-24 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Moraxella catarrhalis* table on next page).

Note: Beta-lactamase testing should be done and reported on any recovered isolate.

***Moraxella* species -** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category

Moraxella catarrhalis				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Amoxicillin/clavulanic acid	(20/10 µg)	≤19		≥20
Trimethoprim/Sulfamethoxazole	(1.25/23.75µg)	≤10	11-15	≥16
Cefuroxime	(30 µg)	≤16	17-19	≥20
Levofloxacin	(5 µg)	≤16		≥17
Ciprofloxacin	(5 µg)	≤20		≥21

***** Interpreted based on *Haemophilus* spp.

8. *Neisseria gonorrheae* - SOP for Antimicrobial Susceptibility Testing

For Neisseria gonorrheae, Beta lactamase testing is done in lab, while full antimicrobial testing is sent to a reference lab. This is so because specialized media and condition are needed for its testing.

9. *Non-Enterobacteriaceae* species - SOP for Antimicrobial Susceptibility Testing

This group of *Non-Enterobacteriaceae* include**:**

- Pseudomonas species other than *Pseudomonas aeruginosa* and nonfastidious, glucose non-fermenting gram negative bacilli other than *Acinetobacter* species, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*.
- For this group disk diffusion testing is currently not recommended as the method hasn't been systematically studied yet. Testing is therefore performed by E-test method (MIC).

Test procedure

1. **Medium**
   Mueller Hinton agar (MH)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from MacConkey Agar plate (or BAP)
3. **Streaking**
   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.

4. **Adding the disks**
   The appropriate antibiotic test strips / E-test (listed in Table) are placed on the surface of the streaked agar plate (2 strips on each MH plate) using a sterile forceps
5. **Incubation**
   Incubate at 37°C, for 18-20 hrs
6. **Reading**
   Read the inhibition zone and interpret as (S, I, R) according to the CLSI standards (check Non-Entero bacteriaceae species table on next page).

Non-Enterobacteriaceae species – MIC interpretive criteria for susceptible, intermediate and resistant category

***Non-Enterobacteriaceae* species**			
Antimicrobial	**MIC (µg/ml)**		
	S	**I**	**R**
Piperacillin/tazobactam	≤ 16/4	32/4 – 64/4	≥ 128/4
Ceftazidime	≤ 8	16	≥ 32
Cefepime	≤ 8	16	≥ 32
Cefotaxime	≤8	16 -32	≥ 64
Ceftriaxone	≤8	16 -32	≥ 64
Aztreonam	≤8	16	≥32
Imipenem	≤ 4	8	≥ 16
Gentamicin	≤ 4	8	≥ 16
Amikacin	≤ 16	32	≥ 64
Ciprofloxacin	≤ 1	2	≥ 4
Trimethoprim/Sulfamethoxazole	≤ 2/38		≥ 4/76
Colistin	≤ 2	4	≥ 8

10. *Pseudomonas aeruginosa* - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar (MH)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from MacConkey Agar plate.
3. **Streaking**
   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 37°C , for 18-20 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Pseudomonas aeruginosa* table on next page).

***Pseudomonas aeruginosa* -** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category

Pseudomonas aeruginosa				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Piperacillin/tazobactam	(100/10 µg)	≤14	15-20	≥21
Ceftazidime	(30 µg)	≤14	15-17	≥18
Cefepime	(30 µg)	≤14	15-17	≥18
Imipenem	(10 µg)	≤15	16-18	≥19
Aztreonam	(30 µg)	≤15	16-21	≥22
Gentamicin	(10 µg)	≤12	13-14	≥15
Amikacin	(30 µg)	≤14	15-16	≥17
Ciprofloxacin	(5 µg)	≤15	16-20	≥21
Colistin *	(10 µg)	≤10		≥11

***Note**: For resistant strains of *Pseudomonas aeruginosa* test the isolates against Colistin.
Breakpoints are interpreted according to IIAA 2008;31.434

11. *Salmonella* spp., and *Shigella* spp. - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar (MH)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from MacConkey Agar plate.
3. **Streaking**
   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 37°C, for 18-20 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Salmonella* and *Shigella* species table on next page).

***Salmonella and Shigella* species-** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

***Salmonella* and *Shigella* species**				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Ampicillin	(10 µg)	≤13	14-16	≥17
Trimethoprim/Sulfamethoxazole	(1.25/23.75µg)	≤10	11-15	≥16
Ciprofloxacin	(5 µg)	≤20	21-30	≥31
Ceftazidime	(30 µg)	≤17	18-20	≥21
Cefotaxime	(30 µg)	≤22	23-25	≥26
Ceftriaxone	(30 µg)	≤19	20-22	≥23
Pefloxacin	(5 µg)*	≤23		≥24

Note:** **Pefloxacin** antimicrobial disk is performed on ***Salmonella species only.
In case of Pefloxacin resistance it should always be noted that" treatment with Fluoroquinolones might be compromised" irrespective of the sensitivity of Ciprofloxacin. This is so because Pefloxacin is a surrogate test for Ciprofloxacin and, thus, the latter might be resistant in vivo even if it is sensitive in vitro.

12. *Stenotrophomonas maltophilia* - SOP for Antimicrobial Susceptibility Testing

1. **Medium**

   Mueller Hinton agar (MH)
2. **Inoculum**

   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from MacConkey Agar plate.
3. **Streaking**

   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**

   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**

   Incubate at 37°C, for 20-24 hrs
6. **Reading**

   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Stenotrophomonas maltophilia* table below).

***Stenotrophomonas maltophilia* -** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

Stenotrophomonas maltophilia				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Minocycline	(30 µg)	≤14	15-18	≥19
Levofloxacin	(5 µg)	≤13	14-16	≥17
Trimethoprim/Sulfamethoxazole	(1.25/23.75µg)	≤10	11-15	≥16

C. Gram Positive Bacteria

1. *Bacillus* species - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar (MH)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.
3. **Streaking**
   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 37°C, for 20-24 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Bacillus* species table on next page).

***Bacillus* species-** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

Bacillus* species				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Penicillin	(10 µg)	≤28		≥29
Amoxicillin/clavulanic acid	(20/10µg)	≤19		≥20
Chloramphenicol	(30 µg)	≤12	13-17	≥18
Ciprofloxacin	(5 µg)	≤15	16-20	≥21
Clindamycin	(2 µg)	≤14	15-20	≥21
Erythromycin	(15 µg)	≤13	14-22	≥23
Imipenem	(10 µg)	≤13	14-15	≥16
Teicoplanin	(30 µg)	≤10	11-13	≥14

* Interpreted based on *Staphylococcus* spp.

2. Beta hemolytic streptococcus [*S. pyogenes* (group A), *S. agalactiae* (group B), and groups (C, G, F)] - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar with 5% sheep blood (MHSB)
2. **Inoculum**
   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.
3. **Streaking**
   Streak the entire surface of the MHSB agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser
5. **Incubation**
   Incubate at 35°C, 5% CO2 for 20-24 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check Beta hemolytic Strep table on next page).

Also check for D-phenomena (D-shape testing) as reported under the SOP for Staphylococci.

Caution: if any strain shows resistance to Penicillin, repeat the testing and send to a reference Lab through the Ministry for confirmation.

Beta Hemolytic Streptococcus species- Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

Beta hemolytic Streptococcus				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Penicillin	(10 µg)	≤23		≥24
Cefepime	(30 µg)	≤23		≥24
Vancomycin	(30 µg)	≤16		≥17
Erythromycin	(15 µg)	≤15	16-20	≥21
Clindamycin	(2 µg)	≤15	16-18	≥19

3. Diphtheroids species - SOP for Antimicrobial Susceptibility Testing

1. **Medium**

   Mueller Hinton agar with 5% sheep blood

2. **Inoculum**

   Direct colony suspension, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.

3. **Streaking**

   Streak the entire surface of the MHSB agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.

4. **Adding the disks**

   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser

5. **Incubation**

   Incubate at 37°C, for 20-24 hrs

6. **Reading**

   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check Diphtheroids species table on next page).

Diphtheroids species - Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category

Diphtheroids species				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Penicillin	(10 µg)	≤28		≥29
Amoxicillin/clavulanic acid	(20/10 µg)	≤19		≥20
Clindamycin	(2 µg)	≤14	15-20	≥21
Erythromycin	(15 µg)	≤13	14-22	≥23
Gentamicin	(10 µg)	≤12	13-14	≥15
Teicoplanin	(30 µg)	≤10	11-13	≥14

* Interpreted based on *Staphylococcus* spp.

4. *Enterococcus* species - SOP for Antimicrobial Susceptibility Testing

1. **Medium**

   Mueller Hinton agar (MH)

2. **Inoculum**

   Direct colony suspension in trypticase soy broth, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.

3. **Streaking**

   Streak the entire surface of the (MH) agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.

4. **Adding the disk**

   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser.

5. **Incubation**

   Incubate at 35°C, for 20-24 hrs

6. **Reading**

   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Enterococcus* spp. table on next page).

7. ***Enterococcus* spp in blood isolates and testing for HLAR.**
For *Enterococcus* spp recovered from blood culture it is crucial to test for High Level Aminoglycoside Resistant (HLAR) using Gentamicin (120ug) discs and Streptomycin (300ug) discs on MH agar same as for the usual disk antimicrobial testing. Zone diameters are interpreted according to the following breakpoints:

***Enterococcus* species**	**HLA Interpretive criteria (mm)**		
	S	**I**	**R**
Streptomycin (300 µg)	≥10	7-9	≤6
Gentamicin (120 µg)	≥10	7-9	≤6

8. **Vancomycin Resistant *Enterococcus* spp** (VRE) that are suspected by disk diffusion is confirmed by Vancomycin and Teicoplanin E-tests. MIC testing is done on MH agar using same technique of disk diffusion method. The results are interpreted as follows:

***Enterococcus* species**	**MIC Interpretive criteria (µg/ml)**		
Antimicrobial	**S**	**I**	**R**
Vancomycin	≤4	8-16	≥32
Teicoplanin	≤8	16	≥32

***Enterococcus* species-** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

***Enterococcus* species**				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Ampicillin	(10 µg)	≤16		≥17
Vancomycin	(30 µg)	≤14	15-16	≥17
Teicoplanin	(30 µg)	≤10	11-13	≥14
Erythromycin	(15 µg)	≤13	14-22	≥23
Tetracycline^{+}	(30 µg)	≤14	15-18	≥19
Ciprofloxacin^{+}	(5 µg)	≤15	16-20	≥21
Nitrofurantoin^{+}	(300 µg)	≤14	15-16	≥17
Fosfomycin^{+}	(200 µg)	≤12	13-15	≥16

($^{+}$) added for urine isolate

4. *Listeria* species - SOP for Antimicrobial Susceptibility Testing

1. **Medium**

   Mueller Hinton agar with 5% sheep blood (MHSB)

2. **Inoculum**

   Direct colony suspension in trypticase soy broth, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.

3. **Streaking**

   Streak the entire surface of the (MHSB) agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.

4. **Adding the disks**

   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser.

5. **Incubation**

   Incubate at 35°C, 5% CO2 for 20-24 hrs

6. **Reading**

   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Listeria* species table on next page).

***Listeria* species -** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category

***Listeria* species ***				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		S	**I**	**R**
Ampicillin	(10 µg)	≥29		≤28

* Interpreted based on *Staphylococcus* spp.

5. *Staphylococcus* species - SOP for Antimicrobial Susceptibility Testing

1. **Medium**

   Mueller Hinton (MH) agar

2. **Inoculum**

   Direct colony suspension in tyrpticase soy broth, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.

3. **Streaking**

   Streak the entire surface of the MH agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.

4. **Adding the disks**

   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser.

5. **Incubation**

   Incubate at 35°C, for 20-24 hrs

6. **Reading**

   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Staphylococcus* species table on next page).

7. **MRSA**

   Methicillin resistant in *S. aureus* or coagulase negative Staphylococcus is interpreted by extrapolating Oxacillin from Cefoxitin disk diffusion, for the latter being a surrogate marker. Check table for breakpoints.

8. **GISA**

   Glycopeptide Intermediate *S. aureus (*Gisa) is screened for by inoculating (1drop) of the bacterial broth suspension (previously prepared for susceptibility testing) on a prepared MH agar with 4% NaCl and containing 6ug/ml Vancomycin (See media preparation). Any present growth is confirmed by E-Testing.

9. **D-SHAPE TESTING**

   D-phenomena is tested by always placing Clindamycin and Erythromysin disks next to each other during antibiotic testing (24mm distance). D-shape zone inhibition forms when resistant Erythromycin (no inhibition zone) induces resistance on the sensitive Clindamycin disk forming a D shape. So Clindamycin is reported as resistant to the tested microorganism.

***Staphylococcus* species-** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

***Staphylococcus* species**				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Cefoxitin	**(30 µg)**	***S. aureus* & *S.lugdunensis* ≤21 CNS ≤24**		***S. aureus* & *S. lugdunensis* ≥22 CNS ≥25**
Erythromycin	(15 µg)	≤13	14-22	≥23
Nitrofurantoin^{+}	(300 µg)	≤14	15-16	≥17
Clindamycin	(2 µg)	≤14	15-20	≥21
Sulfamethoxazole/Trimethoprim	(1.25/23.75 µg)	≤10	11-15	≥16
Teicoplanin	(30 µg)	≤10	11-13	≥14
Tetracycline	(30 µg)	≤14	15-18	≥19
Rifampin	(5 µg)	≤16	17-19	≥20
Ciprofloxacin	(5 µg)	≤15	16-20	≥21
Gentamicin	(10 µg)	≤12	13-14	≥15
Novobiocin^{+}	(5 µg)	≤14		≥15

($^{+}$) added for urine isolates

6. *Streptococcus* species (*Streptococcus viridans* group) - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar with 5% sheep blood (MHSB)
2. **Inoculum**
   Direct colony suspension in trypticase soy broth, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.
3. **Streaking**
   Streak the entire surface of the (MHSB) agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser.
   Note: if Penicillin testing is required, it should be done by MIC determination method, as the DD is not reliable.
5. **Incubation**
   Incubate at 35°C, 5%CO2 for 20-24 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Streptococcus* species/ *viridans* group table on next page).

***Streptococcus* species / *Streptococcus viridans* group-** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

***Streptococcus* species / *Streptococcus viridans* group**				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Ceftriaxone	(30 µg)	≤24	25-26	≥27
Cefepime	(30 µg)	≤21	22-23	≥24
Vancomycin	(30 µg)	≤16		≥17
Erythromycin	(15 µg)	≤15	16-20	≥21
Clindamycin	(2 µg)	≤15	16-18	≥19
Levofloxacin	(5 µg)	≤13	14-16	≥17

7. *Streptococcus pneumoniae* - SOP for Antimicrobial Susceptibility Testing

1. **Medium**
   Mueller Hinton agar with 5% sheep blood (MHSB)
2. **Inoculum**
   Direct colony suspension trypticase soy broth, equivalent to 0.5 McFarland standard, using fresh colonies (18-24hrs) from Sheep Blood Agar plate.
3. **Streaking**
   Streak the entire surface of the (MHSB) agar plate using a sterile cotton swab and in three different directions (turn plate 60 degrees each time) to ensure even distribution of the inoculums.
4. **Adding the disks**
   The appropriate antibiotic disks (listed in Table) are placed on the surface of the streaked agar plate using a sterile forceps or an antibiotic dispenser.
5. **Incubation**
   Incubate at 35°C, 5%CO2 for 20-24 hrs
6. **Reading**
   Read zone diameter of inhibition zone and interpret as (S, I, R) according to the CLSI standards (check *Streptococcus pneumoniae* table on next page).

7. Penicillin resistant strains of *S. pneumoniae* can be detected by using oxacillin disk (1ug) and extrapolating penicillin, ampicillin and amoxicillin-clavulinic acid from it. In case of resistance to oxacillin, MIC for penicillin and ceftriaxone should be determined for confirmation, and for importance in case of pneumonia, bacteremia and CNS infection.
8. Also check for D-phenomena (D-shape testing), as reported under the SOP for **Staphylococci**.

***Streptococcus pneumoniae* -** Antimicrobial disks (content) and interpretive zone diameter for susceptible, intermediate and resistant category.

S. pneumoniae				
Antimicrobial	**(Disk content)**	**Zone diameter (mm)**		
		R	**I**	**S**
Oxacillin	(1 µg)	≤19		≥20
Erythromycin	(15 µg)	≤15	16-20	≥21
Vancomycin	(30 µg)	≤16		≥17
Tetracycline	(30 µg)	≤24	25-27	≥28
Levofloxacin	(5 µg)	≤13	14-16	≥17
Sulfamethoxazole/Trimethoprim	(1.25/23.75 µg)	≤15	16-18	≥19
Clindamycin	(2 µg)	≤15	16-18	≥19

D.QC and Storage

1. SOP - Quality Control - for Antimicrobial Susceptibility Testing

Quality control (QC) should be done weekly on all antibiotics, and whenever a new lot is received in the lab. Data should be documented and filed as a proof of compliance.

Reference quality control strains (ATCC) that should be available in the lab for use in QC include: *S. aureus* (ATCC 29213), *E.coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853). These are the ones that should be tested for any new disk batches and weekly to ensure expected results accuracy. The generated results should be compared to the CLSI standard QC Tables breakpoint category for each of the tested antimicrobial with the QC isolate to ensure the valid reliability of the testing (Refer to Appendix)

Control strains should be stored at -20°C or below if available (up to -80°C) in a suitable stabilizer (50% fetal calf serum in broth or defibrinated sheep blood), or in a lypholized state without the risk of altering their antibiotic susceptibility. Also trypticase soy broth with 15% glycerol is a suitable medium for storage.

2. Antimicrobial Disks Storage - SOP for Antimicrobial Susceptibility Testing

Storage of antimicrobials

1. Antimicrobial disk stock packages should be kept frozen at -20°C and anhydrous condition ensured at all times.
2. On hand/working supply disks must be maintained at refrigerator temperature (2-8 °C) for up to 1 week only, also at anhydrous conditions.
3. Refrigerated antimicrobial disk containers should be brought to room temperature and allowed to equilibrate to room temperature before being opened and used.
4. If disk dispensers are used, make sure that they are well fitted with a tight cover and the appropriate antibiotic cartridges filled in. Dispensers carrying antibiotic cartridges should always be refrigerated and kept away from moisture.
5. Only antimicrobial lots that have passed QC can be used for testing
6. Technicians should always pay attention to the manufacturer's expiration date printed on the antimicrobial disk cartridges and any expired item should be immediately discarded.

E. Beta lactamase testing

SOP for Antimicrobial Susceptibility Testing

Cefinase disk is commercially available to test the production of Beta lactamse enzyme by a microorganism. This enzyme provides a multi resistance to Beta lactam antibiotics such as penicillins and cephalosporins by cleaving the Beta lactam ring. Most clinically important producers of this enzyme are *Staphylococcus s*pp., *Haemophilus* spp., *Moraxella catarrhalis*, *Bacteroides* spp.,and *Neisseria gonorrheae*

The chromogenic testing for Beta lactamase production is performed as follows:

1. Bring the nitrocefin disk to room temperature
2. Place a drop of distilled water on the disk
3. Add some of the desired colonies for testing on the nitrocefin disk
4. In 1 to 5 minutes a change in disk color from yellow to red indicates positivity.

Strains with a positive result are thus reported as Beta- lastamase producers and resistant to Penicillin.

F. Intrinsic resistance

SOP for Antimicrobial Susceptibility Testing

Intrinsic resistance is the innate (natural-not acquired) ability of a microorganism to resist the activity of a particular antibiotic through its inherent structural or functional characteristics. The bacteria allows its tolerance (resistance) of a particular drug or antimicrobial class independent of previous antibiotic exposure and is not caused by horizontal gene transfer.

To avoid reporting false susceptible results for such pathogens, the CLSI yearly provides data about important intrinsic resistance patterns of bacteria as shown in the table on next page.

Microorganisms' with intrinsic resistance vs. antimicrobials

Microorganism	Antimicrobial										
	Ampicillin	Amoxicillin/ clavulanic\ acid	Cefoxitin	Cefuroxime	Tetracycline	Tigecycline	Nitrofurantoin	Colistin	Trimetho-prim/ Sulfa-methoxazole	Novobiocin	Vancomycin
Staphylococcus saprophyticus										R	
Enterococcus gallinarum/ E.casseliflavus											R
Pseudomonas aeruginosa									R		
Klebsiella pneumoniae	R										
Enterobacter aerogenes	R	R	R	R							
Enterobacter cloacae	R	R	R	R							
Citrobacter freundii	R	R	R	R							
Citrobacter koseri	R										
Hafnia alvei	R	R	R								
Morganella morganii	R	R		R		R	R	R			
Proteus vulgaris	R			R	R	R	R	R			
Serratia marcescens	R	R	R	R			R	R			
Providencia stuartii/ P.rettgeri	R	R			R	R	R	R			
Yersinia enterocolitica	R	R									

G. References

1. Turnidge J.D. 2015 . Susceptibility test Methods: General Considerations in Jorgensen J.H. etal. Manual of Clinical Microbiology 11th ed. 2015, pages 1246-1252
2. Jorgensen J.H. and Turnidge J.D. 2015. Susceptibility Test Methods: Dilution and Disk Diffusion Methods in Jorgensen J.H. etal. Manual of Clinical Microbiology 11th ed. 2015, pages 1253 – 1273
3. Karlowsky J.A. and Richter S.S. 2015. Antimicrobial susceptibility Testing Systems in Jorgensen J.H. etal. Manual of Clinical Microbiology 11th ed. 2015, pages 1274 -1285
4. Limbago B.M. and Swenson J.M. 2015. Special Phenotypic Methods for Detecting Antibacterial Resistance in Jorgensen J.H. etal. Manual of Clinical Microbiology 11th ed. 2015, pages 1286 – 1313
5. Humphries R.M. and Hindler J.A. 2015. Susceptibility Test Methods: Fastidious Bacteria in Jorgensen J.H. etal. Manual of Clinical Microbiology 11th ed. 2015, pages 1314 – 1341
6. For more info check the following websites:

   http://www.bd.com/europe/regulatory/Assets/IFU/HB/CE/SD/SD-BD.pdf

   http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0337&c=UK&lang=EN

   https://www.ncbi.nlm.nih.gov/

   http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/

   https://clsi.org/media/1647/m51a_sample.pdf

H. Appendix

As listed in the Quality Control SOP; the following are disk diffusion quality control ranges for nonfastidious organisms extracted from the CLSI-M100 27th edition. (Check table below)

Quality Control Acceptable Disk Diffusion Susceptibility Test Ranges for Assessing Antimicrobial Agents (upon receipt and weekly) Against ATCC Non-Fastidious Organisms using Un-supplemented Mueller Hinton Medium

Antimicrobial Agent	Disk Content	Disk Diffusion QC Ranges (mm)		
		Escherichia coli ATCC 25922	*Staphylococcus aureus* ATCC 25923	*Pseudomonas aeruginosa* ATCC 27853
Amikacin	30 µg	19-26	20-26	18-26
Amoxicillin-clavulanate	20/10 µg	18-24	28-36	-
Ampicillin	10 µg	15-22	27-35	-
Aztreonam	30 µg	28-36	-	23-29
Cefepime	30 µg	31-37	23-29	25-31
Cefixime	5 µg	23-27	-	-
Cefotaxime	30 µg	29-35	25-31	18-22
Cefoxitin	30 µg	23-29	23-29	-
Ceftazidime	30 µg	25-32	16-20	22-29
Ceftriaxone	30 µg	29-35	22-28	17-23
Cefuroxime	30 µg	20-26	27-35	-
Chloramphenicol	30 µg	21-27	19-26	-

Antimicrobial Agent	Disk Content	Disk Diffusion QC Ranges (mm)		
		***Escherichia coli* ATCC 25922**	***Staphylococcus aureus* ATCC 25923**	***Pseudomonas aeruginosa* ATCC 27853**
Ciprofloxacin	5 µg	30-40	22-30	25-33
Clindamycin	2 µg	-	24-30	-
Colistin	10 µg	11-17	-	11-17
Ertapenem	10 µg	29-36	24-31	13-21
Erythromycin	15 µg	-	22-30	-
Fosfomycin	200 µg	22-30	25-33	-
Gentamicin	10 µg	19-26	19-27	17-23
Imipenem	10 µg	26-32	-	20-28
Levofloxacin	5 µg	29-37	25-30	19-26
Minocycline	30 µg	19-25	25-30	-
Nitrofurantoin	300 µg	20-25	18-22	-
Oxacillin	1 µg	-	18-24	-
Pefloxacin	5 µg	25-33	-	-
Penicillin	10 units	-	26-37	-
Piperacillin-tazobactam	100/10 µg	24-30	27-36	25-33
Rifampin	5 µg	8-10	26-34	-
Teicoplanin	30 µg	-	15-21	-
Tetracycline	30 µg	18-25	24-30	-
Tigecycline	15 µg	20-27	20-25	9-13
Trimethoprim-sulfamethoxazole	1.25/23.75µg	23-29	24-32	-