Directorate General of Health Services
Ministry of Health and Family Welfare
Government of India

Standard Operating Procedure for Antimicrobial Resistance Surveillance

National AMR Surveillance Network (NARS-Net)

National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India
January 2023

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Acronyms	
AST	Antimicrobial Susceptibility testing
ATCC	American Type Culture Collection
$BaSO_4$	Barium Sulphate
BHI	Brain Heart Infusion
BMD	Broth Microdilution
BOD	Biological Oxygen Demand
CaMHB	Cation Adjusted Mueller Hinton Broth
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
CoA	Certificate of Analysis
DD/MM/YYYY	Date / Month / Year
EUCAST	European Committee on Antimicrobial Susceptibility Testing
H_2SO_4	Sulphuric acid
I	Intermediate
IATA-DGR	International Air Transport Association - Dangerous Goods Regulations
IQC	Internal Quality control
M/Q water	Milli Q water
MCT	Microcentrifuge tube
Mg	Milligram
mL	Milliliter
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
NARS-Net	National AMR Surveillance Network
NCDC	National Centre for Disease Control
NS	Non-Susceptible
OSBF	Other Sterile Body Fluids
QC	Quality Control
R	Resistant
S	Susceptible
SDD	Susceptible Dose-Dependent
SOP	Standard Operating Procedure
TSA	Tryptic Soya agar
TSB	Tryptic Soya Broth
UN	Unit
VAS	Vancomycin Agar Screen
µg	Microgram
µl	Microliter

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Chapter 1- Standard Operating Procedure for AMR Surveillance in Priority Bacterial Pathogens under National AMR Surveillance Network (NARS-Net)

National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India

Summary

This document describes the standards of laboratory testing to ensure consistent antimicrobial susceptibility testing (AST) procedures and systematic AMR surveillance data collection and reporting by sites participating in the National Antimicrobial Resistance (AMR) Surveillance Network (NARS-Net). This network is established under the National Programme on AMR Containment, coordinated by the National Centre for Disease Control (NCDC), New Delhi.

Accurate laboratory testing of samples of patients with infectious diseases supports the clinician in providing quality healthcare to patients. At the facility level, the AST data is generated, collected, collated, and analyzed using the standard operating procedures (SOPs) for preparing traditional and enhanced institutional antibiograms. These antibiograms are to guide clinicians in developing the institutional antibiotic policy for the rational use of antibiotics. At the National level, the AMR Surveillance data submitted from each surveillance laboratory/network is used to generate the AMR trends from different geographic regions across the country to develop the National AMR annual report to guide clinical practice and policy further.

NCDC, the designated National Coordinating Centre for AMR surveillance by the Ministry of Health and Family Welfare, reports the aggregated AMR surveillance data from the National and State level AMR Surveillance Networks to the Global Antimicrobial Resistance and Use Surveillance System (GLASS) to contribute towards a global understanding of the AMR public health threat.

This document covers guidance and references to the following:

- Priority bacterial pathogens and clinical specimens included under NARS-Net
- Standard bacterial identification and AST procedures for priority bacterial pathogens
- Quality Assurance
- AMR surveillance data management
    a. Standard AST panels for surveillance of priority bacterial pathogen
    b. Standard Reporting formats for data submission to NCDC
    c. Frequency of data submission to NCDC
- Reporting of emerging AMR Alerts
- Specialized/additional AST methods
- Storage and preservation for isolates

Pathogens and specimens included under AMR surveillance

The national AMR Surveillance Network at NCDC prioritizes the following clinically significant pathogens for AMR surveillance. The original list of seven bacterial pathogens formulated in 2017 has been updated after a consultation of experts held in May 2022:

1) *Enterococcus* species
2) *Staphylococcus aureus*
3) *Escherichia coli*
4) *Klebsiella* species
5) *Acinetobacter baumannii/ Acinetobacter calcoaceticus* complex
6) *Pseudomonas aeruginosa*
7) *Salmonella enterica* serovar Typhi and Paratyphi
8) *Shigella* species
9) *Vibrio cholerae*

Under AMR surveillance, five clinical specimens are included: blood, urine, pus aspirate, other sterile body fluids, and stool. Details on case definitions for each of the clinical specimens and the relevant organisms are as follows

Table 1.1: Clinical specimens included in AMR surveillance

Clinical Specimen	Laboratory case-definition	Priority pathogens under AMR surveillance
Blood		*Enterococcus* species *Staphylococcus aureus* *Escherichia coli* *Klebsiella* species *Acinetobacter baumannii/ Acinetobacter calcoaceticus* complex *Pseudomonas aeruginosa* *Salmonella enterica* serovar Typhi *Salmonella enterica* serovar Paratyphi
Urine[1]	Clinically significant bacteriuria*	*Enterococcus* species *Escherichia coli* *Klebsiella* species *Acinetobacter baumannii/ Acinetobacter calcoaceticus* complex *Pseudomonas aeruginosa*
Pus Aspirate[2]	Growth of pathogenic bacteria from aspirated purulent material from a closed infected site	*Enterococcus* species *Staphylococcus aureus* *Escherichia coli* *Klebsiella* species *Acinetobacter baumannii/ Acinetobacter calcoaceticus* complex *Pseudomonas aeruginosa*
Other Sterile Body Fluid[3]	Growth of pathogenic bacteria from a sterile body fluid specimen	*Enterococcus* species *Staphylococcus aureus* *Escherichia coli* *Klebsiella* species *Acinetobacter baumannii/ Acinetobacter calcoaceticus* complex *Pseudomonas aeruginosa*

Clinical Specimen	Laboratory case-definition	Priority pathogens under AMR surveillance
Stool	Isolation of pathogen from stool	*Salmonella enterica* serovar Typhi *Salmonella enterica* serovar *Paratyphi Shigella* species *Vibrio cholerae*

1. Interpret urine cultures with clinical symptoms of urinary tract infection (UTI) (such as dysuria, urinary frequency, suprapubic pain, flank pain, and fever). Avoid reporting mixed bacterial growth or samples collected from urine bags. For clean-catch (mid-stream) urine samples

   a. Bacterial growth of >**1,00,000 (>10^5)** colony forming units (CFU)/mL is suggestive of UTI

   b. Growth of 1,000 – 1,00,000 CFU/mL (10^3 – 10^5 CFU/mL) may indicate UTI in

   i. Patients with pyelonephritis, prostatitis, epididymitis, or fungal infections, or samples collected on cystoscopy or other invasive procedures

   ii. Correlate bacteriuria with the presence of pyuria (>10 pus cells/mL) and absence of squamous epithelial cells for actual infections

2. Pus aspirate includes aspiration of purulent material from a closed infected site only. Isolates from pus "swabs" and "wound swabs" should not be included in AMR surveillance reporting unless the sample collection has been done in a way to avoid surface contaminants.

3. Other sterile body fluids include cerebrospinal fluid (CSF), pleural fluid, peritoneal fluid (ascites), synovial fluid, pericardial fluid, abdominal fluid, amniotic fluid, joint fluid, knee/hip fluid, lymph node, bile, broncho-alveolar lavage (BAL), spleen, bone marrow, fluid from Bartholin's cyst, gastric fluid, gall bladder, breast milk, prostatic fluid, endocardium, semen, etc.

Sites participating in the NARS-Net are to report data on pathogens by type of clinical specimen as outlined in Table 1.2 below.

Table 1.2: Priority pathogens under AMR surveillance by specimen type

Specimen	Blood	Urine	Pus Aspirate	Other Sterile Body Fluids	Stool
S. aureus	✓	×	✓	✓	×
Enterococcus species	✓	✓	✓	✓	×
Klebsiella species	✓	✓	✓	✓	×
E. coli	✓	✓	✓	✓	×
Acinetobacter baumannii/ Acinetobacter calcoaceticus complex	✓	✓	✓	✓	×
Pseudomonas aeruginosa	✓	✓	✓	✓	×
Salmonella Typhi/Paratyphi	✓	×	×	×	✓
Shigella species	×	×	×	×	✓
Vibrio cholerae	×	×	×	×	✓

Bacterial identification and AST methods

Bacterial culture, identification, and AST will be performed according to the SOPs prescribed by the National Programme on AMR Containment. The antibiotics for which AST is done in the labs may vary from laboratory to laboratory based on local clinical prescription practices. Based on the consensus of the Committee of Experts, constituted under NARS-Net, a standard panel of antibiotics has been developed for each AMR surveillance priority pathogen. For AMR surveillance priority pathogens, AST must be performed for all the antibiotics mentioned in the surveillance SOP. These surveillance antibiotic panels also align with the automated AST systems widely used in India.

Bacterial identification

The "SOP for Culture and Identification of AMR surveillance priority pathogens" is to be used for the identification of the priority pathogens. This SOP includes identification algorithms developed by an expert group constituted under NARS-Net. Network laboratories using automated bacterial identification systems are advised to ensure stringent internal quality control (IQC) testing per the manufacturer's instructions in the user manual.

Antimicrobial susceptibility testing methods

AST of pathogens under surveillance can be done either by manual or automated methods

Manual AST methods

- **Kirby-Bauer Disc Diffusion:** Kirby-Bauer disk diffusion is the most commonly used method for performing AST. Results for disk diffusion AST should be reported as zone inhibition diameters only. Each antibiotic's zone inhibition diameter should be measurable. Overlapping zones prevent accurate measurement. Measure the diameter of the zones of complete inhibition, including the diameter of the disk. Hold the Petri plate a few inches above a black background illuminated with reflected light. AST zone diameters of vancomycin for *Enterococcus* and linezolid for *S. aureus* are to measure using transmitted light.
  Note:
  1. The disk diffusion method cannot be used to determine the susceptibility to colistin in Gram-negative bacteria
  2. *S. aureus* resistant to linezolid by disk diffusion should be confirmed by determining the MIC by broth microdilution or using an automated AST system.
- **Agar screen method:** AST for Vancomycin in *S. aureus* requires testing by vancomycin agar screen (VAS) on Brain Heart Infusion (BHI) agar containing 6 µg/ml vancomycin. Strains that show growth on 6 µg/ml BHI vancomycin plate should be confirmed by broth microdilution.(Refer to Chapter 5-SOP for Vancomycin agar screen test- *S. aureus* and *Enterococcus* species)

Broth microdilution (BMD): AST for colistin in all isolates of *E. coli, Klebsiella* species, *Acinetobacter* species, and *Pseudomonas aeruginosa* requires testing by broth microdilution. Results should be reported as MIC. (Refer to Chapter 2- SOP; Broth microdilution colistin susceptibility test for aerobic Gram-negative bacteria)

Colistin Agar dilution test: AST for colistin in all isolates of *E. coli, Klebsiella* species, and *Pseudomonas aeruginosa* can also be done using the colistin agar dilution test. Results should be reported as MIC. (Refer to SOP; Colistin agar test for colistin resistance for Enterobacterales and *Pseudomonas aeruginosa*).

Table 1.4: AST methods for testing colistin against Gram-negative organisms of the priority pathogen list

Priority Pathogen	Disk Diffusion	Colistin Agar Dilution	BMD	Automated AST system
E. coli	No	Yes	Yes	No
Klebsiella species	No	Yes	Yes	No
Pseudomonas aeruginosa	No	Yes	Yes	No
Acinetobacter baumannii/ Acinetobacter calcoaceticus complex	No	No	Yes	No

Automated AST systems

If a facility performs AST using an automated AST system, results should be reported as MIC for all pathogen-antibiotic (i.e., "bug-drug") combinations tested. If AST by BOTH manual disc diffusion and automated testing methods are performed, MIC results are preferred. AST results of colistin should not be reported if performed by an automated system.

Internal quality control

All participating laboratories are expected to perform standard IQC for AST using ATCC strains routinely. The details of IQC testing mandated by NARS-Net are explained in this SOP (Refer to SOP; IQC antimicrobial susceptibility tests using disk diffusion). This includes IQC for culture media, antibiotic discs, and automated system cartridges used for ID and AST (refer to manufacturer's instructions for the QC strains and frequency of testing).

External quality assessment

- To ensure quality of results, all participating laboratories are expected to submit isolates for each priority pathogen as per surveillance program directives to NCDC every QUARTER for confirmatory testing (Please refer to Table 6.1 of this SOP).
- Guidance on sharing the isolates with NCDC AMR-NRL for External Quality Assessment is detailed in this SOP (refer to Chapter 7-SOP: Guidance for Submission of AMR Surveillance isolate for external quality assessment and reporting emerging AMR Alerts). To store bacterial isolates, refer to Chapter 8-SOP for "Preservation of Bacterial Isolates/ Control Strains".

- Each surveillance network laboratory must enroll in Microbiology/Bacteriology EQAS conducted by the Indian Association of Medical Microbiology (IAMM), currently available from Sir Ganga Ram Hospital, New Delhi (http://www.iammeqasdelhi.com/) or Christian Medical College, Vellore, Tamil Nadu (http://www.microbiology.iammeqascmc.org/)
- Sites are expected to share the EQAS scores obtained from IAMM EQAS panels with NCDC in the EQAS score sheets shared from NCDC. For details, please refer to Chapter 7.
- Sites are expected to maintain a score of at least 80% in all the three cycles of EQAS identification/ susceptibility.

Surveillance AST panels

Pathogen-specific surveillance AST panels have been outlined in Revised AST Panel NARS-Net from Page no. 24-30

- AST for the updated list of priority bacterial pathogens – antibiotic combinations critical for public health and surveillance. This list is not exhaustive for all antibiotics that may be tested for clinical purposes.
- AST is critical to be performed and reported for ALL the listed antibiotics in the surveillance AST panel on every isolate of the pathogens and clinical sample types under surveillance. This is essential to ensure that surveillance results are a valid representation of the facilities in the network. These surveillance antibiotic panels are also in-sync with automated AST systems widely used in India.
- While the antibiotics listed in the surveillance AST panels are critical for public health purposes, microbiology laboratories may wish to perform AST for additional antibiotics per the facility's local requirement to guide clinical decision making.
- In contrast to AST surveillance reporting to NCDC, reporting of AST results in patient reports to clinicians at the facility level may be selective (cascade reporting) to promote appropriate and rational antibiotic use.
    - Example 1: For an isolate collected from urine specimen in the out-patient setting that is sensitive to all or most oral antibiotic options, the laboratory may choose to restrict clinical reporting of AST results for injectable antibiotics. These decisions can be made at the facility level in line with the local antibiotic policy or in discussion with the antimicrobial stewardship program (AMSP) committee.

Mandatory clinical and epidemiological details of isolates reported to NCDC

The demographic and clinical data fields must be reported to NCDC by each NARS-Net site as mentioned in the table below. All the variables are mandatory for data entry, and attempts should be made to collect the details of the patients, including the district of their residence. All attempts should be made to retrieve and enter demographic data for each unique patient's isolate.

Table 1.5: Details of clinical and demographic data fields mandatory to be reported in WHONET to NCDC

S. No.	Data Variable	Description	Example/ Guidance
1	Hospital Code	Currently, each hospital is assigned an alpha-numeric hospital code. A configured WHONET file is sent to each NARS-Net site with the unique hospital code generated by NCDC/GoI.	BJMC Ahmedabad: BJMC-AHM SMS Jaipur: SMS-JAIPUR
2	Patient ID	Patient's unique Hospital ID	Refer to the Guidance manual on AMR Data reporting using WHONET. While reporting data to NARSNET, the Patient ID from the hospital shall be "Encrypted" using WHONET."
3	Specimen ID	Laboratory ID assigned to the specific clinical sample.	For example, if culture/AST is performed on multiple clinical samples from the same patients, multiple laboratory specimen IDs may be associated with a single Patient ID.
4	State	State of patient's residence WHONET codes* for each state of India are configured in the "State," which could be visible in the specific laboratory configuration	Few examples as below: Assam: "AS" Chandigarh: "CH" Kerala: "KL" Telangana: "TS" Puducherry: "PY" Odisha: "OR"
5	District	District of patient's residence	
6	Village/Locality (Optional)	Village of patient's residence for rural patients The locality of patient's residence for urban patients	City/ Town or locality of the patient origin.

S. No.	Data Variable	Description	Example/ Guidance
7	Age	Age of the Patient	-For ages 12 months and older. Age in years rounded to the nearest whole year (1, 2, 3…) -For infants one month to 11 months of age, enter the age in months (1m, 2m, 3m,….11m) -For neonates more than one week and less than 28 days, enter (1w, 2w,3w,4w) -For neonates less than one week (1-6 days of age, enter age in days. The date of birth should be taken as one day (1d, 2d, 3d,…6d)
8	Sex	Male, Female, Other, Unknown	Male: m Female: f Other: o Unknown: u
9	Date of Admission	In DD/MM/YYYY format, the date the patient was admitted to the reporting health facility. Every attempt should be made to record the date of admission of all patients. If the date of admission is unavailable, leave this field blank, but do NOT remove the field from your reporting format.	If the date of admission of the patient is 25th December 2022, then the date of admission is entered as 25/12/2022
10	Patient Location	Location of the patient: Ward number, OPD number, ICU type in the hospital where the clinical specimen was collected from patients.	If the patient is admitted to Medicine ward no.12, the "Location" is ward 12. If the sample was referred from Urology OPD No 22, then the location is OPD 22 and Select Urology in the Department

S. No.	Data Variable	Description	Example/ Guidance
11	Location Type	-Location Type of the Patient: classify if the specimen is taken from a patient seen in the intensive care unit (ICU), inpatient department (in), or outpatient department (out). -Standard WHONET codes* shall be used to generate location type-wise antibiograms while entering these details. -Record the hospital location of the patient and the department under which the patient was admitted from whom the specimen is collected.	- If the patient is admitted to the ICU of the surgery department, then "icu" is entered in the location type. -If the patient visited the OPD Gynae department and the urine specimen is sent for testing: then the location type is 'out.'
12	Department	-The clinical department in which the clinician admits or examines the patient during the sample referral must be specified. -Standard WHONET codes* will be used while entering these details to generate department-wise antibiograms. -Every attempt should be made to record the hospital location of the patient and the department under which the patient was admitted from whom the specimen is collected.	In the latest version of Lab specific WHONET configuration, almost all the clinical departments are added and use these codes for entering department details such as: -Medicine: "Med" -Surgery: "Sur" -Gynecology: "Gyn" -Pediatric: "Ped"
13	Specimen collection date	In DD/MM/YYYY format, the date the specific clinical sample was collected from the patient.	If the date of specimen collection of the patient is 20th October 2022, then the specimen collection date is entered as 20/10/2022

S. No.	Data Variable	Description	Example/ Guidance
14	Specimen type	Classify the type of clinical sample—blood, urine, pus aspirate, other sterile body fluid, stool.	Use the correct WHONET code* for each specimen type while entering data in WHONET
15	Pathogen isolated	Classify priority pathogen isolated: • *Enterococcus species* • *S. aureus* • *E. coli* • *Klebsiella species* • *Acinetobacter baumannii / Acinetobacter calcoaceticus complex* • *Pseudomonas aeruginosa* • *Salmonella enterica* serovar Typhi • *Salmonella enterica* serovar Paratyphi • *Shigella* species • *Vibrio cholerae*	Standard WHONET codes* for each pathogen will be used while entering the pathogen isolated. For example: for entering *Enterococcus faecalis*, use code "efa" while generating an antibiogram for *Enterococcus* species, use capital "ENT," which includes all the *Enterococcus* species. Refer to an extended list of species of AMR Surveillance priority pathogens page no. 31-32
16	Save the isolate for EQAS/Alert sent to NCDC	All the Alerts/EQAS isolates, which are to be sent to NCDC quarterly for EQAS, must be stored and mention "yes" in this field	If the isolate is stored to be sent to NCDC for EQAS or Alert, then enter "YES" if saved and "NO" if not saved.
17	AST results	AST Results to be reported in the formats for each antibiotic designated in the surveillance AST panel for each organism/ specimen type	- Disc Diffusion: Zone inhibition diameter (in mm) - Broth microdilution (for colistin and vancomycin): MIC value (in µg/mL) - Automated AST results: MIC value (in µg/mL) - Agar Dilution (for colistin): MIC value (≤ 1 µg/mL - ≥ 4 µg/mL)

*Refer to the Guidance manual on AMR Data Reporting using WHONET for using the standard WHONET codes for State, Department, Location Types, Specimen Type and pathogen isolated

Format for data submission to NCDC

Data should be submitted to NCDC in a WHONET data file

- NCDC will share a Reporting Configuration WHONET file with each participating surveillance network site. Sites not using WHONET already should use this NCDC Reporting Configuration WHONET file for entering and reporting AMR surveillance data. The labs already using WHONET for their routine lab data reporting need to run the NCDC reporting configuration against their lab data file.
- The NCDC Reporting Configuration WHONET file includes mandatory data fields for entering demographic details of each patient isolate and specific bug-drug combinations used for National AMR surveillance.
- Data entry can be directly done into WHONET using the NCDC reporting configuration. Without deleting/modifying the existing variables, sites may add additional variables in this configuration, including additional antibiotics used as per their local requirements. This data can be directly exported to NCDC later using the data export guidance manual provided by NCDC.
- The institutions using Laboratory Information Management System (LIMS) to enter bacterial identification and AST details may use the BacLink option of WHONET to extract and report their data to NCDC.
- The same BacLink option can also be used for laboratories managing their data in Microsoft excel. To avoid repeated data entry of already available data in excel or LIMS.
- NCDC will provide technical support to sites to configure their systems for data importation from automated/hospital information system/Excel to WHONET on a case-to-case basis through in-person or online support.
- NCDC recommends that all AMR surveillance sites migrate to WHONET- 2022 or a later version.

Data cleaning and deduplication

All the surveillance network sites must review their data weekly and perform quick analysis every month. The nodal officer of the AMR surveillance program at the network laboratory should coordinate with the NCDC to discuss the monthly data and address the data quality issues. NCDC shall provide data validation, consistency check, and feedback within 15 days of submitting the quarterly data in the prescribed WHONET format.

De-duplication of priority pathogen isolate data

During the data analysis at NCDC, data de-duplication shall be done on isolate-level data received from network laboratories using WHONET. Deduplication is done to achieve one isolate per patient. When several clinical specimens are collected during patient management at the reporting facility, duplicate findings for the same patient shall be excluded (de-duplication) for surveillance purposes. For each surveillance period, only one AST result is included, i.e. the result of the first isolate reported from the same patient during the hospital stay for each patient per surveyed specimen type and surveyed pathogen.

- **Example 1:** If two blood cultures from the same patient yield growth of *E. coli*, only the first result is included in the data analysis and report; if the growth of *E. coli* is detected in one culture and of *K. pneumoniae* in the other, both results shall be analyzed and reported.
- **Example 2:** If there is the growth of *E. coli* in one blood culture and one urinary culture from the same patient, both specimen types are analyzed and reported. Repeated negative results for the same specimen type in the same patient should also be de-duplicated at the facility level.

Data de-duplication is primarily performed using WHONET at the facility level before creating antibiograms; NCDC will provide technical support to sites on this process.

Frequency of data submission to NCDC

AMR surveillance data for every month is to be submitted to NCDC in WHONET file latest by the 5th working day of the next month. The link officers at NCDC will assist the site AMR Nodal Officer and Data Manager through virtual meetings in cleaning of monthly WHONET data files.
Subsequently revised corrected WHONET data files are to be submitted by the site at the earliest and not later than five working days.

Alerts for reporting resistance patterns of public health concern

- Detection of the following resistance patterns should prompt an ALERT for notification to NCDC
  - o Colistin resistant *E. coli*, *Klebsiella* species, *Acinetobacter baumannii/Acinetobacter calcoaceticus* complex, or *Pseudomonas aeruginosa*.
  - o Vancomycin intermediate susceptibility (VISA) (I: MIC 4-8 µg/mL) or vancomycin resistance (VRSA) in *S. aureus* (R: MIC ≥ 16 µg/mL)
  - o Linezolid resistant Gram-positive cocci

    Staphylococcus aureus: R: MIC ≥ 8 µg/mL, Zone ≤ 20 mm

    Enterococcus species: R: MIC ≥ 8 µg/mL, Zone ≤ 20 mm
  - o Ceftriaxone or Azithromycin-resistant *Salmonella enterica* serovar Typhi and Paratyphi
  - o Ceftriaxone intermediate sensitive *Salmonella enterica* serovar Typhi and Paratyphi
  - o Imipenem resistant *Salmonella enterica* serovar Typhi and Paratyphi
  - o Any other significant drug-resistant pathogen
- When the above-listed resistance patterns are detected, an alert should be sent to NCDC within **ONE WEEK** using the duly filled "Alert Form for Emerging Resistance Patterns" **.**
- The soft copy of the form should be submitted via email to amrsurveillance@gmail.com and the hard copy should be sent along with the alert isolate sent to NCDC following the triple layer packaging (refer to SOP; Guidance for submission of AMR Surveillance isolate for External Quality Assessment and Reporting Emerging AMR Alerts)
- The suspected AMR pattern should be reported to NCDC along with the resistant isolate for confirmation within a week; NCDC will confirm the ID and AST profile and provide feedback within two weeks.

Revised AST Panel NARS-Net

***Enterococcus* species**

Antibiotic Panels	Disk Concentration	Automated Method	Blood	Pus	OSBF	Urine
Ampicillin[$]	10 µg	-	✓	✓	✓	✓
Gentamycin (high level)	120 µg	+	✓	✓	✓	✓
Erythromycin	15 µg	+	✓	✓	✓	
Vancomycin	30 µg	+	✓	✓	✓	✓
Linezolid**	30 µg	+	✓	✓	✓	✓
Doxycycline[1]	30 µg	-	✓	✓	✓	
Ciprofloxacin	5 µg	+				✓
Nitrofurantoin	300 µg	+				✓
Fosfomycin	200 µg	-				✓
Tetracycline[1] (A)[#]	MIC	+	✓	✓	✓	✓
Teicoplanin[##]	30 µg	+	✓	✓	✓	✓
Benzyl penicillin (A)	MIC	+	✓	✓	✓	✓

*** For Vancomycin AST against Enterococci, either the disk diffusion method (Read results after 24 hours) o r Vancomycin screen agar or Automated AST systems may be used.** Vancomycin zone should be examined using transmitted light AST for Vancomycin in all isolates of *Enterococcus* spp. (Except *E. gallinarum and E. casseliflavus*) in which growth has been detected on vancomycin screen agar (6 µg/ml) requires AST by Broth Microdilution (BMD).

**Organisms resistant to linezolid by disk diffusion or automated systems should be confirmed by BMD

Tetracycline to be tested (Instead of Doxycycline) when the primary testing method is by Automated Systems. [1]Doxycycline and Tetracycline are kept only for surveillance purposes. Routine clinical reporting of Tetracycline and Doxycycline for Enterococci is not recommended.

##*E. gallinarum* and *E. casseliflavus* are intrinsically resistant to Vancomycin, please do AST for Teicoplanin and report

(A) denotes this drug is available in the automated AST systems. Need not be tested routinely by disk diffusion method unless indicated

[$]Please extrapolate the results of Benzylpenicillin for ampicillin sensitivity

"Organisms that are susceptible to Tetracycline are also considered susceptible to Doxycycline and Minocycline. However, some organisms that are intermediate or resistant to Tetracycline may be susceptible to Doxycycline, minocycline, or both"(CLSI M100, 32nd Edition).

For testing and reporting of E. faecalis urinary tract infection isolates only. The 200-µg Fosfomycin disk contains 50 µg glucose-6-phosphate" (CLSI M100, 32nd Edition).

Staphylococcus aureus

Antibiotic Panels		Disk Concentration	Automated Method	Blood	Pus	OSBF
Gentamicin		10 µg	+	✓	✓	✓
Ciprofloxacin		5 µg	+	✓	✓	✓
Erythromycin		15 µg	+	✓	✓	✓
Trimethoprim-Sulfamethoxazole		1.25/23.75 µg	+	✓	✓	✓
Cefoxitin[#]		30 µg	+	✓	✓	✓
Clindamycin		2 µg	+	✓	✓	✓
Vancomycin	6 µg/ml	Screen Agar*	+	✓	✓	✓
	MIC	BMD/Auto		✓	✓	✓
Linezolid**		30 µg	+	✓	✓	✓
Doxycycline[1]		30 µg	-	✓	✓	✓
Tetracycline[1] (A)		MIC	+	✓	✓	✓
Teicoplanin (A)		MIC	+	✓	✓	✓

#Report Cefoxitin susceptible as MSSA and Cefoxitin resistance as MRSA

* If positive growth has been detected on vancomycin screen agar (6 µg/ml), please perform AST by Broth Microdilution or Automated method.

**Linezolid zone should be examined using transmitted light. Organisms resistant by disk diffusion or automated systems should be confirmed by BMD

[1]Tetracycline is to be tested (Instead of Doxycycline) by centres only when the primary testing method is automated AST systems.

(A)denotes that this drug is available in automated systems. Need not be tested routinely by the disk diffusion method unless indicated.

[1]Doxycycline and Tetracycline are kept only for surveillance purposes. Routine clinical reporting of Tetracycline and Doxycycline for *Staphylococcus aureus* is not recommended.

"Organisms that are susceptible to Tetracycline are also considered susceptible to Doxycycline and Minocycline. However, some organisms that are intermediate or resistant to Tetracycline may be susceptible to Doxycycline, minocycline, or both"(CLSI M100, 32nd Edition).

Escherichia coli

Antibiotic Panels	Disk Concentration	Automated Method	Blood	Pus	OSBF	Urine
Ampicillin	10 µg	-	✓	✓	✓	✓
Amoxicillin-clavulanate	20/10 µg	+	✓	✓	✓	✓
Gentamicin	10 µg	+	✓	✓	✓	✓
Amikacin	30 µg	+	✓	✓	✓	✓
Cefuroxime	30 µg	+	✓	✓	✓	✓
Cefotaxime	30 µg	-	✓	✓	✓	✓
Ceftriaxone	30 µg	+	✓	✓	✓	✓
Cefepime	30 µg	+	✓	✓	✓	✓
Piperacillin-tazobactam	100/10 µg	+	✓	✓	✓	✓
Trimethoprim/Sulfamethoxazole	1.25/23.75 µg	+	✓	✓	✓	✓
Imipenem	10 µg	+	✓	✓	✓	✓
Ertapenem	10 µg	+	✓	✓	✓	✓
Meropenem	10 µg	+	✓	✓	✓	✓
Nitrofurantoin	300 µg	-				✓
Fosfomycin	200 µg	+				✓
Colistin*	MIC	-	✓	✓	✓	✓
Ciprofloxacin	5 µg	+	✓	✓	✓	✓
Doxycycline** (Optional)	30 µg	+		✓	✓	

*** AST for Colistin should be performed by Agar Dilution at 0µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml or by using the reference Broth Microdilution (BMD) method.**

****Doxycycline is a reserve antibiotic included only for Pus and OSBF**

**Doxycycline is kept only for surveillance purposes. Routine clinical reporting of Doxycycline is discouraged.

"The 200-µg Fosfomycin disk contains 50 µg glucose-6-phosphate"(CLSI M100, 32nd Edition).

***Klebsiella* species**

Antibiotic Panels	Disk Concentration	Automated Method	Blood	Pus	OSBF	Urine
Amoxicillin-clavulanate	20/10 µg	+	✓	✓	✓	✓
Gentamicin	10 µg	+	✓	✓	✓	✓
Amikacin	30 µg	+	✓	✓	✓	✓
Cefuroxime	30 µg	+	✓	✓	✓	✓
Cefotaxime	30 µg	-	✓	✓	✓	✓
Ceftriaxone	30 µg	+	✓	✓	✓	✓
Cefepime	30 µg	+	✓	✓	✓	✓
Piperacillin-tazobactam	100/10 µg	+	✓	✓	✓	✓
Trimethoprim/Sulfamethoxazole	1.25/23.75 µg	+	✓	✓	✓	✓
Imipenem	10 µg	+	✓	✓	✓	✓
Ertapenem	10 µg	+	✓	✓	✓	✓
Meropenem	10 µg	+	✓	✓	✓	✓
Nitrofurantoin	300 µg	-				✓
Colistin*	MIC	-	✓	✓	✓	✓
Ciprofloxacin	5 µg	+	✓	✓	✓	✓
Doxycycline** (Optional)	30 µg	+		✓	✓	

*AST for Colistin should be performed by Agar Dilution at 0µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, or BMD
**Doxycycline is included only for Pus aspirate and OSBF

***Acinetobacter baumannii/ Acinetobacter calcoaceticus* complex**

Antibiotic Panels	Disk Concentration	Automated Method	Blood	Pus	OSBF	Urine
Gentamicin	10 µg	+	✓	✓	✓	✓
Amikacin	30 µg	+	✓	✓	✓	✓
Ampicillin Sulbactam	10 µg /10µg	-	✓	✓	✓	✓
Ceftazidime	30 µg	+	✓	✓	✓	✓
Piperacillin Tazobactam	100/10 µg	+	✓	✓	✓	✓
Imipenem	10 µg	+	✓	✓	✓	✓
Meropenem	10 µg	+	✓	✓	✓	✓
Trimethoprim+ Sulfamethoxazole	1.25/23.75 µg	+	✓	✓	✓	✓
Tetracycline	30 µg	-				✓
Minocycline	30 µg	+	✓	✓	✓	✓
Colistin*	MIC	+	✓	✓	✓	✓
Ciprofloxacin	5 µg	+	✓	✓	✓	✓

*AST for Colistin should be performed by Broth Microdilution

Pseudomonas aeruginosa

Antibiotic Panels	Disk Concentration	Automated Method	Blood	Pus	OSBF	Urine
Ceftazidime	30 µg	+	✓	✓	✓	✓
Piperacillin Tazobactam	100/10 µg	+	✓	✓	✓	✓
Gentamicin	10 µg	+	✓	✓	✓	✓
Amikacin	30 µg	+	✓	✓	✓	✓
Aztreonam	30 µg	+	✓	✓	✓	✓
Imipenem	10 µg	+	✓	✓	✓	✓
Meropenem	10 µg	+	✓	✓	✓	✓
Ciprofloxacin	5 µg	+	✓	✓	✓	✓
Colistin*	MIC	-	✓	✓	✓	✓
Netilmicin	30 µg	+	✓	✓	✓	✓

*AST for Colistin should be performed by agar dilution at 0 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml or Broth Microdilution

***Salmonella enterica* serovar Typhi and Paratyphi**

Antibiotic Panels	Disk Concentration	Automated Method	Blood	Stool
Ampicillin	10 µg	+	✓	✓
Ciprofloxacin	5 µg	+	✓	✓
Trimethoprim + Sulfamethoxazole	1.25/23.75 µg	+	✓ ✓	✓ ✓
Chloramphenicol	30 µg	+	✓	✓
Cefixime	30 µg	-	✓	✓
Ceftriaxone	30 µg	+	✓	✓
Azithromycin*	15 µg	-	✓	
Imipenem**	10 µg	+	✓	✓
Pefloxacin#	5 µg	-	✓	✓

** For *Salmonella* Typhi and Paratyphi blood Isolates, **perform AST for imipenem if resistance/ intermediate sensitivity is observed for 3rd generation cephalosporin.**

#Pefloxacin is not to be reported to clinicians unless there is resistance to ciprofloxacin

"AST for Azithromycin should be performed only on isolates of Salmonella* Typhi.** ***Azithromycin breakpoints are based on a dosage regimen of 500 mg administered daily"(CLSI M100, 32nd Edition)

"A third generation cephalosporin should be tested and reported, and if requested, chloramphenicol and azithromycin may be tested and reported"(CLSI M100, 32nd Edition)

"For Salmonella spp., aminoglycosides, first- and second-generation cephalosporins, and cephamycins may appear active in vitro but are not effective clinically and should not be reported as susceptible. When fecal isolates of Salmonella spp., are tested, only ampicillin, a fluoroquinolone, and trimethoprim-sulfamethoxazole should be reported routinely" "Routine susceptibility testing is not indicated for nontyphoidal Salmonella spp. isolated from intestinal sources. In contrast, susceptibility testing is indicated for all Shigella isolates" (CLSI M100, 32nd Edition)

Shigella species

Antibiotic Panels	Disk Concentration	Stool
Ampicillin	10 µg	✓
Ciprofloxacin	5 µg	✓
Trimethoprim + Sulfamethoxazole	1.25/23.75 µg	✓
Ceftriaxone	30 µg	✓
Azithromycin*	15 µg	✓
Chloramphenicol	30 µg	✓

Azithromycin disk diffusion zones can be hazy and difficult to measure, especially S. sonnei. A MIC-based AST method is recommended if an isolate has a zone of inhibition that is difficult to measure. The media source may affect the clarity of the endpoints for disk diffusion tests".(CLSI M100, 32nd Edition)

"For Shigella spp. aminoglycosides, first- and second generation cephalosporins, and cephamycins may appear active invitro but are not effective clinically and should not be reported as susceptible"(CLSI M100. 32nd Edition)

"Data regarding whether amoxicillin should be used to treat shigellosis are conflicting. When reporting ampicillin results, state that treatment of shigellosis with amoxicillin might not be comparable to ampicillin, with poorer efficacy". (CLSI M100, 32nd Edition)

Vibrio cholerae

Antibiotic Panels	Disk Concentration	Stool
Ampicillin	10 µg	✓
Azithromycin	15 µg	✓
Chloramphenicol	30 µg	✓
Trimethoprim + Sulphamethoxazole	1.25/23.75 µg	✓
Tetracycline	30 µg	✓
Doxycycline (Optional)	30 µg	✓

"For V. cholerae, isolates susceptible to tetracycline are also susceptible to doxycycline" (CLSI M45)

The expanded list of pathogens and their codes for data entry in WHONET

Priority Pathogen	Extended species list of each pathogen	WHONET Code
Enterococcus species	All *Enterococcus* species	ENT
	Enterococcus species	ent
	E. asini	ean
	E. avium	eav
	E. casseliflavus	eca
	E. cecorum	ecc
	E. columbae	ecb
	E. dispar	eds
	E. durans	edu
	E. faecalis	efa
	E. faecium	efm
	E. flavescens	efv
	E. gallinarum	ega
	E. gilvus	egv
	E. hirae	enh
	E. malodoratus	ema
	E. mundtii	emn
	E. pallens	epa
	E. porcinus	evi
	E. raffinosus	era
	E. saccharolyticus	esh
	E. seriolicida	ese
	E. solitaries	eso
	E. sulfureus	esu
	E. villorum	evi
Staphylococcus aureus	*Staphylococcus aureus ss. aureus*	sau
Escherichia coli	*Escherichia coli*	eco
***Klebsiella* species**	All *Klebsiella* species	KL-
	Klebsiella species	kl-
	K. aerogenes	eae
	K. granulomatis	cgr
	K. mobilis	eae
	K. mrnithinolytica	kor
	K. oxytoca	kox
	K. ozaenae	koz

Priority Pathogen	Extended species list of each pathogen	WHONET Code
	K. planticola	kpl
	K. pneumoniae	kpn
	K. pneumoniae ss. ozaenae	koz
	K. pneumoniae ss. pneumoniae	kpn
	K. pneumoniae ss. rhinoscleromatis	krn
	K. quasipneumoniae	kqp
	K. rhinoscleromatis	krn
	K. terrigena	kte
	K. trevisanii	kpl
	K. variicola	kvi
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	pae
Acinetobacter baumannii/ Acinetobacter calcoaceticus complex	All *Acinetobacter species*	AC-
	Acinetobacter calcoaceticus baumannii complex	abx
	A. baumannii	aba
	A. calcoaceticus	aca
	A. pittii	apt
	A. nosocomialis	ano
***Salmonella enterica* serovar Typhi**	*Salmonella enterica* serovar Typhi	sat
***Salmonella enterica* serovar Paratyphi**	*Salmonella* Paratyphi	pty
	Salmonella Paratyphi A	saa
	Salmonella Paratyphi B	sab
***Shigella* species**	*All Shigella* species	SHI
	Shigella species	shi
	S. boydii	shc
	S. dysenteriae	sha
	S. flexneri	shb
	S. sonnei	shd
Vibrio cholerae	*Vibrio cholera*	vic

Unique laboratory codes for AMR data reporting to NCDC using WHONET

Sl No	Current Lab Code	State	NCDC AMR Network Laboratory	Lab code for NCDC Reporting
1	**BJMC-AHM**	Gujarat	BJ Medical College, Ahmedabad	**N01**
2	**BJMC-PUNE**	Maharashtra	BJ Medical College, Pune	**N02**
3	**GMCH-CHG**	Chandigarh	Govt. Medical College, Chandigarh	**N03**
4	**GSVM-KAN**	Uttar Pradesh	GSVM Medical College, Kanpur	**N04**
5	**LHMC-DEL**	Delhi	Lady Hardinge Medical College, New Delhi	**N05**
6	**MMCRI-MYSR**	Karnataka	Mysore Medical College and Research Institute, Mysore	**N06**
7	**SMS-JAIPUR**	Rajasthan	Sawai Man Singh Medical College, Jaipur	**N07**
8	**SAFDAR-DEL**	Delhi	VMMC and associated Safdarjung Hospital, New Delhi	**N08**
9	**GMC-TRV**	Kerala	Government Medical College, Thiruvananthapuram	**N09**
10	**KAPV-TRY**	Tamil Nadu	KAPV Government Medical College, Tiruchirappalli	**N10**
11	**GMC-GAUHAT**	Assam	Gauhati Medical College and Hospital, Guwahati	**N11**
12	**NEIGRIHMS**	Meghalaya	North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIMS), Shillong	**N12**
13	**MGMMC-INDO**	Madhya Pradesh	Mahatma Gandhi Memorial Medical College, Indore	**N13**
14	**IGMC-SHIML**	Himachal Pradesh	Indira Gandhi Medical College, Shimla	**N14**
15	**GMC-AUG**	Maharashtra	Government Medical College, Aurangabad,	**N15**
16	**OSMC-HYD**	Telangana	Osmania Medical College Hyderabad	**N16**
17	**GMC-GUNT**	Andhra Pradesh	Guntur Medical College, Guntur	**N17**
18	**AGMC-AGA**	Tripura	Agartala Govt. Medical College & GBP Hospital, Agartala	**N18**
19	**SCBMC-CUT**	Odisha	SCB Medical College, Cuttack	**N19**
20	**GMCH-JMU**	Jammu & Kashmir	Government Medical College and Hospital, Jammu	**N20**

Sl No	Current Lab Code	State	NCDC AMR Network Laboratory	Lab code for NCDC Reporting
21	**BDSPGI-RTK**	Haryana	Pt. BDS Post Graduate Institute of Medical Sciences, Rohtak	**N21**
22	**RIMS-RAN**	Jharkhand	Rajendra Institute of Medical Sciences, Ranchi	**N22**
23	**IGIMS-PATN**	Bihar	Indira Gandhi Institute of Medical Science, Patna	**N23**
24	**GMCH-HALDW**	Uttarakhand	Government Medical College, Haldwani	**N24**
25	**JLNMC-CHAT**	Chhattisgarh	Pt. Jawahar Lal Nehru Medical College, Raipur	**N25**
26	**GMC-BHOPAL**	Madhya Pradesh	Gandhi Medical College, Bhopal	**N26**
27	**STM-KOLKTA**	West Bengal	Calcutta School of Tropical Medicine, Kolkata	**N27**
28	**GMERS-VALS**	Gujarat	GMERS Medical College and Hospital, Valsad	**N28**
29	**LLRM-MERIT**	Uttar Pradesh	Lala Lajpat Rai Memorial Medical College, Meerut	**N29**
30	**CMC-COIMB**	Tamil Nadu	Coimbatore Medical College, Coimbatore	**N30**
31	**MAMC-DEL**	Delhi	Maulana Azad Medical College, Delhi	**N31**
32	**SPMCH-BIK**	Rajasthan	Sardar Patel Medical College & Hospital, Bikaner	**N32**
33	**KIMS-HUBLI**	Karnataka	Karnataka Institute of Medical Sciences, Hubli	**N33**
34	**IGMRI-PUDU**	Puducherry	Indira Gandhi Medical College & Research Institute, Puducherry	**N34**
35	**NAMO-SILVA**	Daman & Diu	Namo Medical Education and Research Institute, Silvassa	**N35**
36	**GMC-GOA**	Goa	Goa Medical College and Hospital, Goa	**N36**
37	**STNM-SIKIM**	Sikkim	STNM Hospital, Gangtok	**N37**
38	**GMC-PAT**	Punjab	Government Medical College, Patiala	**N38**
39	**ZMC-ZORAM**	Mizoram	Zoram Medical College, Mizoram	**N39**
40	**ANIIMS-PB**	Andaman and Nicobar Islands	Andaman and Nicobar Institute of Medical Sciences, Port Blair	**N40**

Antibiotic codes for surveillance data entry in WHONET

Antibiotic	WHONET code	Measurement
Amikacin 30 µg	AMK	Disk
Amoxicillin-clavulanate 20/10 µg	AMC	Disk
Ampicillin 10 µg	AMP	Disk
Ampicillin Sulbactam 10 µg/ 10 µg	SAM	Disk
Azithromycin 15 µg	AZM	Disk
Aztreonam 30 µg	ATM	Disk
Benzyl Penicillin	P	MIC
Cefepime 30 µg	FEP	Disk
Cefixime 30µg	CFM	Disk
Cefotaxime 30 µg	CTX	Disk
Cefoxitin 30 µg	FOX	Disk
Ceftazidime 30 µg	CAZ	Disk
Ceftriaxone 30 µg	CRO	Disk
Cefuroxime 30 µg	CXM	Disk
Chloramphenicol 30 µg	CHL	Disk
Ciprofloxacin 5 µg	CIP	Disk
Clindamycin 2 µg	CLI	Disk
Doxycycline 30 µg	DOX	Disk
Erythromycin 15µg	ERY	Disk
Ertapenem 10 µg	ETP	Disk
Fosfomycin 200 µg	FOS	Disk
Gentamicin 10 µg	GEN	Disk
Gentamicin High 120 µg	GEH	Disk
Imipenem 10 µg	IMP	Disk
Linezolid 30 µg	LNZ	Disk
Meropenem 10 µg	MEM	Disk
Minocycline 30 µg	MNO	Disk
Netilmicin 30 µg	NET	Disk
Nitrofurantoin 300 µg	NIT	Disk
Piperacillin/Tazobactam 100/10 µg	TZP	Disk
Tetracycline 30 µg	TCY	Disk
Tobramycin 10 µg	TOB	Disk
Teicoplanin 30µg	TEC	Disk
Trimethoprim/ Sulfamethoxazole 1.25/23.75 µg	SXT	Disk
Vancomycin 30µg	VAN	Disk
Colistin	COL	MIC
Fosfomycin	FOS	MIC
Teicoplanin	TEC	MIC
Vancomycin	VAN	MIC

Chapter 2- Standard Operating Procedure: Broth-microdilution Colistin Susceptibility Test for Aerobic Gram-Negative Bacteria

National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India

Objectives & Scope

This SOP describes the standard broth microdilution method used to determine the in vitro susceptibility to Colistin for aerobic Gram-negative bacterial isolates in Clinical Microbiology laboratory. It addresses the preparation of Cation adjusted Mueller Hinton Broth (CAMHB) and colistin stock solution for dilution tests, testing conditions (including inoculum preparation, use of selective quality control (QC) strains, incubation time, and temperature), determining minimal inhibitory concentration (MIC), result analysis using approved breakpoints and QC procedures.

Background

As per the latest recommendations by CLSI and EUCAST, broth microdilution method should be used to quantitatively measure colistin's in vitro activity against aerobic Gram-negative bacterial isolate. The method described herein is intended primarily for testing aerobic Gram-negative isolates that grow well after overnight incubation in un-supplemented CAMHB.

Broth microdilution antimicrobial susceptibility testing

This SOP is prepared for in vitro broth microdilution AST of colistin sulphate for clinical reporting of MIC values as recommended by CLSI guidelines: M07- A11 and M100-32nd Edition 2022.

Medium: Cation-adjusted Mueller- Hinton Broth (CAMHB)

- Use CAMHB for routine broth dilution susceptibility testing of rapidly growing aerobic gram-negative bacteria (*E. coli, Klebsiella* species, *Pseudomonas aeruginosa* and *Acinetobacter* species)
- Check each batch of MHB and ensure that the final pH is between 7.2 to 7.4. Select the ATCC strains for QC that most closely resemble the patients' isolates being tested.
- Perform MIC with each batch of MHB using a standard set of QC organisms (Table-2.3).
- If a new lot of MHB does not yield the expected MIC values, investigate the cation content along with other variables and components of the test. (Refer to CLSI M07-A11 for the preparation of cation-adjusted MHB).

Preparation of Drug Stock Solution of Colistin

- The formulation of reference standard powder used for AST is Colistin sulphate (30,000 units/mg or 30 UN/µg) as per CLSI Standard.
- The colistin powder available in the market has different potencies
- Before preparing the stock solution, please check the lot number and Certificate of Analysis (CAS/ CoA) the supplier provided separately for each lot.
- Please ensure the lot number as mentioned in the colistin sulphate vial and the CAS are the same, as the potency might differ for each lot number

Box 2.1

- ≥ 15000 UN/mg mentioned in colistin vial doesn't mean the potency is 15000 UN/mg
- Each lot number of colistin vial supplied by manufacturer have different potency of UN/ mg
- If CAS is not provided with the colistin sulphate vial, request the vendor for the certificate of analysis for the Lot# of colistin purchased.

Potency calculation:

- The pure agent (reference) of colistin sulphate has a potency of 30,000 UN/mg, which is 30 UN/ µg. The first step is to determine the potency of the colistin sulphate powder to be used for MBD. For this, divide the given potency results in UN/mg (as given in the CoA of the colistin powder used for BMD) by 30 UN/µg. This will give the potency of the colistin powder in µg/mg (micro-gram/mg). (CLSI M07-A11).
- The second step is to prepare the primary stock solution so that the final concentration of active colistin sulphate (calculated against referenced pure salt) is 1mg/ml. Label this as **Primary stock Colistin.**
- Use the above prepared primary stock solution (1000 µg/ml) to make the desired concentration of working stock solution.

Box 2.2: Example 1

- If the potency of Colistin powder available in colistin sulphate salt mentioned in the CAS/ CoA Batch number SLBZ 2145 is 23299 UN/mg.
- Then potency with reference to pure agent is $\frac{23299 \text{ UN/mg}}{30 \text{ UN/ µg}} = 776$ µg/mg
- To prepare stock solution of 1000 µg/ml (1mg/ml) weigh 10 mg of this colistin sulphate powder with potency of 776 µg/mg and add 7.76 ml of autoclaved distilled water.

7760 µg /7.76 ml= 1mg/ml

Box 2.3: Example 2

- If the potency of Colistin powder available in colistin sulphate salt mentioned in the CAS is 22974 UN/mg).
- Then potency with reference to pure agent is $\frac{22974 \text{ UN/mg}}{30 \text{ UN/ µg}} = 765$ µg/mg
- To prepare stock solution of 1000 µg/ml (1mg/ml) weigh 10 mg of this colistin sulphate powder with potency of 765 µg/mg and add to 7.65 ml of autoclaved distilled water.

7650 µg /7.65 ml= 1 mg/ml

Storage of primary stock solution of Colistin

Dispense small volumes of the primary stock solutions (approx. 100 µl – 200 µl as per requirement) into sterile 1.5 – 2 ml cryovials/cryotubes; carefully label and seal them and store them at –20°C. Never store them in a self-defrosting freezer.

Note:

- Do not refreeze the stocks; repeated freeze-thaw cycles accelerate the degradation of antimicrobial agents, particularly polymyxins.
- Stock solutions of antibiotics can be stored at –20°C or less for one to two months without significant loss of activity. In all cases, directions provided by the drug manufacturer must be considered in addition to these general recommendations.

Preparation of working stock solution of Colistin

Take out from the freezer the required number of primary stock solution tubes and prepare working stock solution of 4 x final drug concentration. To achieve a final concentration of 16 µg/ml concentration, prepare a working stock solution of 64 µg/ml in a sterile microcentrifuge tube (MCT). To achieve this, add 64 µl from the primary stock solution to 936 µl of autoclaved MHB in another MCT.

As an alternative, to avoid wastage, 64 µl of primary stock solution can also be stored and used as such for each run. Label this MCT as Working Stock Colistin 64 µg/ml

Box 2.4

For making 64 µg/ml (4x working stock solution) from primary stock solution (1000 µg/ml)

C1 V1	=	C2 V2
1000 µg/ml V1	=	64 µg/ml x 1000 µl
V1	=	64 µl

*Volume of the working stock solution can be increased depending on number of strains to be tested.

Preparation of dilutions of Colistin

Add 500 µl from the 64 µg/ml working stock solution to 500 µl MHB in MCT and perform twofold serial dilutions (in 9 MCTs containing 500 µl MHB) as shown in Fig. 2.1 to get drug concentrations as 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml and so on

Fig. 2.1: Preparation of Colistin dilutions

	(1)	1:2 (2)	1:2 (3)	1:2 (4)	1:2 (5)	1:2 (6)	1:2 (7)	1:2 (8)	1:2 (9)	1:2 (10)	GC (11)	MC (12)
MCT	936 sterile MHB + 64µgl working stock solution	500 sterile MHB	500	500	500	500	500	500	500	500	Only MHB + Inoc-ulum	Only MHB
Colistin conc. (µg/ml) in MCT	64	32	16	8	4	2	1	0.5	0.25	0.125		
From MCTs take 25 µgl and add to all wells in corresponding microtitre plate column containing 50µgl CAMHB (25 µl of inoculum is added in column 1-11, see text below)												
Final conc. in micro-titer plate wells	16	8	4	2	1	0.5	0.25	0.25	0.06	0.03		

Preparation of 96 well-round bottom microtiter plate

Addition of dilutions of Colistin

- **Step 1:** Add 50 µl of MHB broth to all wells of columns 1 to 10, 75 µl in column 11, and 100 µl in column 12 of the microtiter plate.
- **Step 2:** Add 25 µl of colistin dilution 64 µg/ml to column 1, 32 µg/ml to column 2, and so on till 0.125 µg/ml in column 10 (Fig. 2.1) of the microtiter plate. Column 11 will be growth control containing only media and bacterial inoculum, while column 12 will be media control containing only media 100 µl. Each well of the microtiter plate should finally contain a total volume of 100 µl after adding inoculum.

Inoculum Preparation

Culture of Test/ QC Strains

- Make new subcultures from glycerol stocks/agar stabs on Tryptic Soya Agar (TSA) or any other non-selective media on a day before putting up the BMD test and incubate at 35-37°C in a BOD incubator for 18 to 24 h.

- Put up the ATCC QC strains in each BMD plate, the QC strain is to be selected depending on test strains, refer to Table 2.1 below.
- Refer to the IQC SOP provided by NCDC for the selection, maintenance, and testing of QC strains

Table 2.1: Routine ATCC QC Strains for Colistin AST by Broth Microdilution

Organism	QC number	Tested for
Escherichia coli	ATCC25922	For testing Colistin against *Enterobacteriaceae*
Pseudomonas aeruginosa	ATCC27853	For testing Colistin against *P. aeruginosa* and *Acinetobacter* species

Inoculum Preparation

- Prepare a standardized inoculum of 0.5 McFarland using the direct colony suspension method. Take 3-5 well-isolated colonies of the same morphological type from the 18 – 24 hour culture plate and make saline suspensions in tubes containing sterile saline.
- Mix the inoculum well to make a homogenous suspension and adjust to 0.5 McFarland standard.
- Adjust the turbidity equivalent to 0.5 McFarland turbidity standard (approx. $1.5x10^8$ CFU/ml).

Note:

- To standardize the inoculum density, use a $BaSO_4$ turbidity standard equivalent to 0.5 McFarland standards or its optical equivalent.
- If Densitometer is available, use that to adjust the inoculum to 0.5 McFarland standard
- Do not use broth or agar slants for inoculum preparation for MIC (to ensure the purity of culture).

Inoculation in Microtiter Plates

- Dilute the 0.5 McFarland suspension 1:75 times by adding 10 µl to 740 µl of autoclaved MHB medium. From this diluted suspension, take 25 µl and add to each of the wells in columns 1 to 11 already containing 75 µl (50 µl MHB + 25 µl antibiotic) to yield a bacterial concentration of approximately $5 x10^4$ CFU/well.
- Incubate the microtiter plates at 35 ± 2°C for 16 to 24 h in an ambient air incubator within 15 minutes of adding the inoculum.
- To prevent drying, seal each microdilution plate or cover it with a tight-fitting plastic cover or stack 4 trays in a plastic bag before incubating
- Incubation times may differ for each organism for example, 16–20 hours for Enterobacteriaceae and *Pseudomonas aeruginosa*; 20-24 hours for *Acinetobacter* species for testing colistin.
- Perform a purity check of the inoculum suspension by subculturing a 10 µl aliquot onto a non-selective agar plate for simultaneous incubation.

Note:

- Do not add inoculum in media control well (column 12)
- Inoculations must be done within 15 minutes of inoculum preparation.
- To maintain the same incubation temperature for all cultures, do not stack microdilution trays more than 4 high.

Colony counts of inoculum suspensions

Laboratories are recommended to perform colony counts on inoculum suspension at least quarterly to ensure that the concentration of the final inoculum routinely obtained closely approximates 5 X 10^5 CFU/ml.

- Perform colony count of a few randomly selected cultures.
- Take 10 µl from the growth-control well immediately after inoculation and dilute it in 10 ml of sterile saline (1:1000 dilution).
- After mixing, spread 100 µl over the surface of an agar medium like Tryptic soy agar or nutrient agar.
- Incubate the plates at 35±2°C for 16 to 20 hours in a BOD incubator. The presence of approximately 50 colonies indicates an inoculum density of 5×10^5 CFU/ml.

Determining broth microdilution end points

- Read the MIC of Colistin as the lowest colistin concentration that completely inhibits the organism's growth in the microdilution wells as detected by the unaided eye.
- Compare the amount of growth in the wells containing the antibiotic with the amount of growth in the growth control well (column 11) in each set of tests when determining the growth end points.
- For a test to be considered valid, acceptable growth (definite turbidity or button) must occur in the growth-control well

Recording of the Results

Enter the results in the raw data sheet as shown below.

Date: **Lot no. of Antibiotic powder:**

Antibiotic tested: **Lot no. of MHB:**

Name of the Performer:

Organism tested	Column	Antibiotic conc. (µg/ml)												
		1	2	3	4	5	6	7	8	9	10	11	12	
	Specimen ID no.	16	8	4	2	1	0.5	0.25	0.125	0.06	0.03	GC	MC	MIC (µg/ml)
E.coli ATCC25922	QC strain	-	-	-	-	-	-	+	+	+	+	+	-	0.5
Test Strain 1		-	-	-	-	+	+	+	+	+	+	+	-	2
Test Strain 2		-	-	-	-	-	-	+	+	+	+	+	-	0.5
Test Strain 3		-	-	-	-	-	+	+	+	+	+	+	-	1
Test Strain 4		-	-	+	+	+	+	+	+	+	+	+	-	8
Test Strain 5		-	-	-	-	-	-	+	+	+	+	+	-	0.5
Test Strain 6		-	-	-	-	-	-	-	-	+	+	+	-	0.125
In-house/ QC Positive Control	In-house	-	+	+	+	+	+	+	+	+	+	+	-	16

+ is equivalent to growth, - is equivalent to no growth; GC: Growth Control; MC: Media Control

Note: Every raw data sheet should be signed by the performer and countersigned by the verifier.

Rejection criteria

- Reject the experiment if the MIC of colistin against the QC strains does not fall in the acceptable MIC range (M100-S32, 2022).
- Also, reject the results if contamination in the broth media control or any significant deterioration of an antimicrobial agent is reflected in the results of susceptibility testing using QC strains.

Reporting of minimal inhibitory concentration results

The MIC results may be reported directly to clinicians for patient care purposes. To ensure that anti-microbial susceptibility test results on patients' isolates are accurate, make sure:

- Results with QC strains are within the acceptable range.
- Growth is satisfactory.
- The test is not contaminated (mixed).
- The overall susceptibility profile is consistent with the expected results for colistin
- Atypical resistance, if present, must be confirmed.

End-Point Interpretation Control

- Monitor end-point interpretation periodically to minimize variation in the interpretation of MIC end points among observers.
- All laboratory personnel performing BMD should independently read a set of dilution tests.
- Record the results and compare them to those obtained by an experienced reader. All readers should agree within ± 1 twofold dilution.
- Refer to Table 2.4 (Page 49) for interpretive categories and MIC Breakpoints (µg/ml) for colistin against *E. coli, Klebsiella* species, *P. aeruginosa* and *Acinetobacter* species as per CLSI document and Table 2.5 for MIC breakpoints as per EUCAST document. (Page 50)

Quality control in MIC AST by broth microdilution

Reference Strains for Quality Control

- Use QC strains from a recognized source (e.g., ATCC).
- Include respective routine QC strains as mentioned in Table 2.1 whenever performing broth microdilution
- Refer to Table 2.3 on page no. 49 for the expected MIC QC range for QC strains against colistin
- Record the MIC of QC strains along with the MIC of test isolates.

Storage and Maintenance of Quality Control Strains/Clinical isolates

For storage and maintenance of QC strains, refer to IQC SOP provided by NCDC.

- Make glycerol stocks of strains (10 – 15% glycerol in TSB) and keep them at -20^{O}C.
- The day before MIC testing, subculture fresh on an agar plate (overnight incubation) to obtain isolated colonies for inoculum suspension preparation

References

- CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for bacteria that Grow Aerobically; —Eleventh Edition. CLSI document M07-A11. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
- CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Thirty-Second edition. CLSI document M100-A32 edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.
- European Committee on Antimicrobial Susceptibility Testing -EUCAST clinical breakpoints and guidance; Tables v.13.0, 1-1-2023

Definitions

Antimicrobial susceptibility tests interpretive category – a classification based on an in *vitro* response of an organism to an antimicrobial agent at levels corresponding to blood or tissue levels attainable with usually prescribed doses of that agent

- **Susceptible (S)** isolates are inhibited by the usually achievable concentrations of antimicrobial agents and infection is expected to respond when the recommended dosage is used for the site of infection.
- **Intermediate (I)** isolates have antimicrobial agent MICs that usually approach attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates with usually recommended doses. It implies

a) clinical efficacy in body sites where the drugs are physiologically concentrated (e.g. quinolones in urine) or

b) at sites where the drug is not explicitly concentrated, clinical efficacy at a higher-than-normal drug dosage (e.g. β-lactams).

- **Resistant (R)** isolates are not inhibited by the usually achievable concentrations of the agent or demonstrate zone diameters that fall in the range where specific microbial resistance mechanisms (e.g., β-lactamases) are likely, and infection is not expected to respond to treatment with the highest recommended doses.
- **Non-susceptible (NS)** organisms have only a susceptible interpretive category, not an intermediate or resistant one. A susceptible-only interpretive category may be applied to new antimicrobial agents for whom no resistant isolates have been encountered when initial interpretive criteria are determined. Isolates that test with a MIC above the susceptible interpretive breakpoint are considered non-susceptible.
- **SDD – Susceptible Dose-Dependent** Category in which the susceptibility of antibiotics against an organism depends on the treatment regimen dosage.

Minimal inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

Preparation of barium sulfate turbidity standard

Table 2.2: McFarland Turbidity Standards

Barium Sulfate Turbidity Standard	Volume (ml) of:		Turbidity equivalent to cell density of *Escherichia coli* broth culture (10^8/ml)
	Solution A, 0.048 mol of $BaCl_2$/litre	Solution B, 0.18 mol of H_2SO_4/ litre	
0.5	0.05	9.95	1.5
1	0.1	9.9	3
2	0.2	9.8	6
3	0.3	9.7	9
4	0.4	9.6	12
5	0.5	9.5	15
6	0.6	9.4	18
7	0.7	9.3	21
8	0.8	9.2	24

Quality control range

Table 2.3: MIC QC Ranges for QC Strains and Select Antimicrobial Agents

Organism	MIC QC Ranges (µg/ml)
	Colistin
E. coli ATCC 25922	0.25–2
P. aeruginosa ATCC 27853	0.5 – 4

Table 2.4: Interpretive categories and MIC Breakpoints (µg/ml) for colistin against *E. coli*, *Klebsiella sp.*, *P. aeruginosa,* and *Acinetobacter* species as per CLSI document

Organism	MIC Breakpoints (µg/ml)		
	S	I	R
E. coli	-	≤2	≥4
Klebsiella sp	-	≤2	≥4
P. aeruginosa	-	≤2	≥4
Acinetobacter species	-	≤2	≥4

The MICs obtained from testing colistin predict MICs for polymyxin B.

Table 2.5: Interpretive categories and MIC Breakpoints (µg/ml) for colistin against *E. coli*, *Klebsiella sp.*, *P. aeruginosa* and *Acinetobacter* species as per EUCAST document

Organism	MIC Breakpoints (µg/ml)		
	S	I	R
E. coli	≤2	-	>2
Klebsiella species	≤2	-	>2
P. aeruginosa	≤4	-	>4
Acinetobacter species	≤2	-	>2

Table 2.6. Organisms that are intrinsically resistant to Colistin

Morganella morganii
Proteus spp. (mirabilis, penneri & vulgaris)
Providencia spp. (rettgerii & stuartii)
Serratia marcescens
Burkholderia cepacia complex

Chapter 3- Standard Operating Procedure: Broth-microdilution Vancomycin Susceptibility Test - Aerobic Gram-Positive Cocci

National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India

Objective & Scope

This SOP describes the standard broth microdilution method used to determine the in vitro susceptibility to vancomycin for aerobic Gram-positive cocci isolated in the Clinical Microbiology laboratory. It addresses the preparation of Cation adjusted Mueller Hinton Broth (CAMHB) and vancomycin stock solution for dilution tests, testing conditions (including inoculum preparation, use of selective quality control (QC) strains, incubation time, and temperature), determining minimal inhibitory concentration (MIC), result analysis using approved breakpoints and Quality Control procedures.

Background

As per the latest recommendations by CLSI and EUCAST, the broth microdilution method should be used to quantitatively measure vancomycin's in vitro activity against aerobic Gram-positive cocci, the method described herein is intended primarily for testing aerobic Gram-positive cocci that grow well after overnight incubation in un-supplemented CAMHB.

Broth microdilution susceptibility testing

This SOP is prepared for in-vitro broth microdilution susceptibility testing of vancomycin hydrochloride for clinical reporting of MIC values as recommended by CLSI guidelines: M07- A11 and M100-32nd Edition 2022.

Medium: Cation adjusted Mueller- Hinton Broth (CA-MHB)

- Use CAMHB for routine broth dilution susceptibility testing of rapidly growing aerobic gram-positive cocci (*Staphylococcus aureus* and *Enterococcus* species)
- Check each batch of CAMHB and ensure the final pH is between 7.2 and 7.4. Select the ATCC strains for QC that most closely resemble the patients' isolates being tested.
- Perform MIC with each batch of broth using a standard set of QC organisms **(Table-3.3,** Page no. 61**).**
- If a new lot of CAMHB does not yield the expected MICs, investigate the cation content along with other variables and components of the test. (Refer to CLSI M07-A11 for the preparation of cation-adjusted MHB).

Preparation of drug stock solution of vancomycin

- The formulation of reference standard powder used for antimicrobial susceptibility testing (AST) is vancomycin hydrochloride as per CLSI Standard.
- The vancomycin hydrochloride powder available in the market has different potencies.
- Before preparing the stock solution, please check the lot number and Certificate of Analysis (CAS/ CoA) the supplier provided separately for each lot.
- Please ensure that the lot # as mentioned in the vancomycin hydrochloride vial and ,the CAS are the same, as the potency might differ for each lot number.
- If CAS is not provided with the vancomycin hydrochloride vial, request the vendor for the certificate of analysis for the Lot# of vancomycin hydrochloride powder purchased. Potency calculation:

- Check the potency of vancomycin hydrochloride written on the vial or the CAS provided by the vendor.
- The first step is to prepare the primary stock solution so that the final concentration of active vancomycin hydrochloride (calculated against referenced pure salt) is 1mg/ml. **Label** this as **Primary stock Vancomycin.**

Box-3.1: Example 1

- If the potency of vancomycin powder available in vancomycin hydrochloride salt mentioned in the CAS/ CoA is 950 µg/mg.
- To prepare primary stock solution of 1000 µg/ml (1mg/ml) weigh 10 mg of this vancomycin hydrochloride powder with potency of 950 µg/mg and add 9.5 ml of autoclaved distilled water. This is the 1 mg/ml primary stock solution

Box- 3.2: Example 2

- If the potency of vancomycin powder available in vancomycin hydrochloride salt mentioned in the CAS/ CoA is 975 µg/mg.
- To prepare stock solution of 1000 µg/ml (1mg/ml) weigh 10 mg of this vancomycin hydrochloride powder with potency of 975 µg/mg and add 9.75 ml of autoclaved distilled water. This is the 1 mg/ml primary stock solution.

Use the above prepared **primary stock** solution (1000 µg/ml) to make the desired concentration of working stock solution.

Storage of primary stock solution of vancomycin

Dispense small volumes of the primary stock solutions (approx. 100 µl – 200 µl as per requirement) into sterile 1.5 – 2 ml cryovials/cryotubes; carefully label and seal. Store at –20°C temperature and never in a self-defrosting freezer.

Note:

- Do not refreeze the stocks; repeated freeze-thaw cycles accelerate the degradation of antimicrobial agents.
- Stock solutions of antibiotics can be stored at –20°C or less for one to two months without significant loss of activity. In all cases, directions provided by the drug manufacturer must be considered in addition to these general recommendations.

Preparation of working stock solution of vancomycin

Take the required number of primary stock solution tubes from the freezer and prepare a working stock solution of 4 X final drug concentration. To achieve a final concentration of 32 µg/ml, prepare a working stock solution of 128 µg/ml in a sterile micro centrifuge tube (MCT). Add 128 µl from the

primary stock solution to 872 µl of autoclaved MHB in another MCT. As an alternative to avoid wastage, smaller volumes of primary stock solutions can be stored and used. Label this MCT as Working Stock vancomycin 128 µg/ml.

Box-3.3

For making 128 µg/ml (4x working stock solution) from primary stock solution (1000 µg/ml)

C1 V1 = C2 V2

1000 µg/ml V1 = 128 µg/ml x 1000 µl

V1 = 128 µl

*Volume of the working stock solution can be increased depending on number of strains to be tested.

Preparation of dilutions of vancomycin

- Add 500 µl from the 128 µg/ml working stock solution to 500 µl MHB in MCT and perform twofold serial dilutions (in 9 MCTs containing 500 µl MHB) as shown in Fig. 3.1 to get drug concentrations as 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml and so on.

Fig. 3.1: Preparation of vancomycin dilutions

	(1)	1:2 (2)	1:2 (3)	1:2 (4)	1:2 (5)	1:2 (6)	1:2 (7)	1:2 (8)	1:2 (9)	1:2 (10)	GC (11)	MC (12)
MCT	872 sterile MHB + 128µl working stock solution	500 sterile MHB	500	500	500	500	500	500	500	500	Only MHB + Inoc-ulum	Only MHB
Vancomy-cin conc. (µg/ml) in MCT	128	64	32	16	8	4	2	1	0.5	0.25		
From MCTs, take 25µl and add to all wells in the corresponding microtitre plate column containing 50µl CaMHB. (25 µl of inoculum is added in Columns 1-11, see text below)												
Final conc. in micro-titer plate wells	32	16	8	4	2	1	0.5	0.25	0.125	0.06		

Preparation of 96 well, round bottom microtiter plate

Addition of dilutions of vancomycin

- **Step 1:** Add 50 µl of MHB broth to all wells of columns 1 to 10, add 75 µl in column 11, and 100 µl in column 12 of the microtitre plate.
- **Step 2:** Add 25 µl of vancomycin dilution 128 µg/ml to column 1, 64 µg/ml to column 2 and so on till 0.25 µg/ml in column 10 (Fig. 3.1) of the microtiter plate. Column 11 will be growth control containing only media and bacterial inoculum while column 12 will be media control containing only media 100 µl. Each microtiter plate's well should finally contain a total volume of 100 µl.

Inoculum Preparation

Culture of Test/ QC Strains

- Make fresh subcultures from glycerol stocks/ agar stabs on Tryptic Soya Agar (TSA) or any other non-selective media on a day before putting up the BMD test and incubate at 35-37°C in BOD incubator for 18 to 24 h.
- Put up the ATCC QC strains in each BMD plate. The QC strain is to be selected depending on test strains; refer to Table 3.1 below.
- Refer to the IQC SOP provided by NCDC to select, maintain, and test QC strains.

Table 3.1: Routine ATCC QC Strains for vancomycin AST by Broth Microdilution

Organism	QC number	Tested for
Staphylococcus aureus	ATCC29213	For testing vancomycin against S. *aureus*
Enterococcus faecalis	ATCC29212	For testing vancomycin against *Enterococcus species*

Inoculum Preparation

- Prepare a standardized inoculum of 0.5 McFarland using the direct colony suspension method. Take 3-5 well-isolated colonies of the same morphological type from the 18-24-hour culture plate and suspend saline in sterile tubes.
- Mix the inoculum well to make a homogenous suspension and adjust to 0.5 McFarland standard.
- Adjust the turbidity equivalent to 0.5 McFarland turbidity standard (approx. $1.5x10^8$ CFU/ml).

Note:

- To standardize the inoculum density, use a $BaSO_4$ turbidity standard equivalent to 0.5 McFarland standards or its optical equivalent.
- If Densitometer is available, it can be used to adjust inoculum to 0.5 McFarland standard
- Do not use broth or agar slants for inoculum preparation for MIC (to ensure the purity of culture).

Inoculation in Microtitre Plates

- Dilute 0.5 McFarland suspension 1:75 times by adding 10 µl to 740 µl of autoclaved MHB medium. From this diluted suspension, take 25 µl and add to each of the wells in columns 1 to 11 already containing 75 µl (50 µl MHB + 25 µl antibiotic) to yield a bacterial concentration of approximately 5 x10^4 CFU/well.
- Incubate the microtitre plates at 35± 2°C for 24 h in an ambient air incubator within 15 minutes of adding the inoculum.
- To prevent drying, seal each microdilution plate or cover with a tight-fitting plastic cover or stack 4 trays in a plastic bag before incubating
- Perform a purity check of the inoculum suspension by sub-culturing a 10 µl aliquot onto a non- selective agar plate for simultaneous incubation.

Note:

- Do not add inoculum in media control well (column 12)
- Inoculations must be done within 15 minutes of inoculum preparation.
- To maintain the same incubation temperature for all cultures, do not stack microdilution trays more than 4 high.

Colony Counts of Inoculum Suspensions

Laboratories are recommended to perform colony counts of inoculum suspension at least quarterly to ensure that the final inoculum concentration routinely obtained closely approximates 5 X 10^5 CFU/ml.

- Perform colony count of few randomly selected cultures.
- Take 10 µl from the growth-control well immediately after inoculation and dilute it in 10 ml of sterile saline (1:1000 dilutions).
- After mixing, spread 100 µl over the surface of agar medium like TSA or nutrient agar.
- Incubate the plates at 35±2°C for 24 h in BOD incubator. The presence of approximately 50 colonies indicates an inoculum density of 5×10^5 CFU/ml.

Determining Broth Microdilution End Points

- Read the MIC of vancomycin as the lowest concentration of vancomycin that completely inhibits the growth of the organism in the microdilution wells as detected by the unaided eye.
- Compare the amount of growth in the wells containing the antibiotic with the amount of growth in the growth control well (column 11) in each set of tests when determining the growth end points.
- For a test to be valid, acceptable growth (definite turbidity or button) must occur in the growth-control well.

Recording of the Results

Enter the results in the raw data sheet as shown.

Date: **Lot no. of Antibiotic powder:**

Antibiotic tested: **Lot no. of MHB:**

Name of the Performer:

Organism tested	Column	Antibiotic concentration. (µg/ml)												
		1	2	3	4	5	6	7	8	9	10	11	12	
	Specimen ID no.	32	16	8	4	2	1	0.5	0.25	0.125	0.06	GC	MC	MIC (µg/ml)
S. aureus ATCC 29213	QC strain	-	-	-	-	-	-	+	+	+	+	+	-	1
Test Strain 1		-	-	-	-	+	+	+	+	+	+	+	-	4
Test Strain 2		-	-	-	-	-	-	+	+	+	+	+	-	1
Test Strain 3		-	-	-	-	-	+	+	+	+	+	+	-	2
Test Strain 4		-	-	+	+	+	+	+	+	+	+	+	-	16
Test Strain 5		-	-	-	-	-	-	+	+	+	+	+	-	1
Test Strain 6		-	-	-	-	-	-	-	-	+	+	+	-	0.25
In-house/ QC Positive Control	In-house	-	+	+	+	+	+	+	+	+	+	+	-	32

+ is equivalent to growth; - is equivalent to no growth; GC: Growth Control; MC: Media Control

Note: Every raw data should be signed by the performer and countersigned by the verifier.

Rejection criteria

- Reject the experiment if the MIC of vancomycin against the QC strains does not fall in the acceptable MIC range (M100-S32, 2022).
- Also, reject the study if contamination in the broth media control or any significant deterioration of an antimicrobial agent is reflected in the results of susceptibility testing using QC strains.

Reporting of minimal inhibitory concentration (MIC) results

The MIC results may be reported directly to clinicians for patient care purposes. To ensure that antimicrobial susceptibility test results on patients' isolates are accurate, make certain:

- Results with QC strains are within the acceptable range.
- Growth is satisfactory.
- Test is not contaminated (mixed).
- The overall susceptibility profile is consistent with expected results for vancomycin.

- Atypical resistance, if present, must be confirmed.

Refer to table 3.4 on page 61 for interpretive categories and MIC Breakpoints (µg/ml) for *Staphylococcus aureus* and *Enterococcus* species against vancomycin

End-Point interpretation control

- Monitor end-point interpretation periodically to minimize variation in the interpretation of MIC end points among observers.
- All lab personnel performing BMD should independently read a set of dilution tests.
- Record the results and compare them to those obtained by an experienced reader. All readers should agree within ± 1 twofold dilution.

Quality control in MIC AST by broth microdilution

Reference Strains for Quality Control

- Use QC strains from a recognized source (e.g., ATCC).
- Include respective routine QC strains as mentioned in Table 3.1 whenever performing BMD
- Refer to Table 3.3 for the expected MIC QC range for QC strains against vancomycin.
- Record the MIC of QC strains along with the MIC of test isolates.

Storage and Maintenance of Quality Control Strains/Clinical isolates

For storage and maintenance of QC strains, refer to IQC SOP provided by NCDC.

- Make glycerol stocks of strains (10 – 15% glycerol in TSB) and keep at -20^{O}C.
- The day before MIC testing, subculture fresh on an agar plate (overnight incubation) to obtain isolated colonies for inoculum suspension preparation.

References

- CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for bacteria that Grow Aerobically; —Eleventh Edition*. CLSI document M07-A11. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
- CLSI. *Performance Standards for Antimicrobial Susceptibility Testing; Thirty-Second edition*. CLSI document M100-A32 edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.

Definitions

Antimicrobial susceptibility tests interpretive category – A classification based on in vitro response of an organism to an antimicrobial agent at levels corresponding to blood or tissue levels attainable with usually prescribed doses of that agent

- **Susceptible (S)** isolates are inhibited by the usually achievable concentrations of antimicrobial agents, and infection is expected to respond when the recommended dosage is used for the site of infection.
- **Intermediate (I)** isolates have antimicrobial agent MICs that usually approach attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates with normally recommended doses. It implies

c) clinical efficacy in body sites where the drugs are physiologically concentrated (e.g., quinolones in urine) or

d) at sites where the drug is not specifically concentrated, clinical efficacy is higher than normal drug dosages (e.g. β-lactams).

- **Resistant (R)** isolates are not inhibited by the usually achievable concentrations of the agent and/or that demonstrate zone diameters that fall in the range where specific microbial resistance mechanisms (e.g., β-lactamases) are likely and infection is not expected to respond to treatment with highest recommended doses.

Minimal inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

Preparation of barium sulfate turbidity standard

Table 3.2: McFarland Turbidity Standards

Solution A, 0.048 mole of $BaCl_2$/litre	Solution B, 0.18 mole of H_2SO_4/litre		Turbidity equivalent to cell density of *Escherichia coli* broth culture (10^8/ml)
0.5	0.05	9.95	1.5
1	0.1	9.9	3
2	0.2	9.8	6
3	0.3	9.7	9
4	0.4	9.6	12
5	0.5	9.5	15
6	0.6	9.4	18
7	0.7	9.3	21
8	0.8	9.2	24

Quality control range

Table 3.3: MIC QC Ranges for QC Strains and Select Antimicrobial Agents

Antimicrobial Agent	MIC QC Ranges (µg/mL)	
	Staphylococcus aureus ATCC 29213	*Enterococcus faecalis* ATCC 29212
Vancomycin	0.5–2	1 – 4

Table 3.4: Interpretive categories and MIC Breakpoints (µg/ml) for *Staphylococcus aureus* and *Enterococcus* species against vancomycin

Antimicrobial Agent	Interpretive Categories and MIC breakpoints, µg/ml					
	Staphylococcus aureus			*Enterococcus spp*		
	S	I	R	S	I	R
Vancomycin	≤2	4-8	≥16	≤4	8-16	≥32

Chapter 4- Standard Operating Procedure: Colistin Agar Test for colistin resistance for Enterobacterales and *Pseudomonas aeruginosa*

National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India

Objectives & Scope

This SOP describes the agar dilution technique, a qualitative method to determine the in vitro susceptibility to Colistin, and is intended primarily for testing Enterobacterales and *Pseudomonas aeruginosa* in the Clinical Microbiology laboratory. It addresses the preparation of Mueller Hinton agar (MHA) plates containing different concentrations of colistin sulphate and colistin sulphate stock solution for agar dilution tests, testing conditions (including inoculum preparation, use of selective quality control (QC) strains, incubation time, and temperature), determining minimal inhibitory concentration (MIC), result analysis using approved breakpoints and Quality Control procedures.

Background

As per the latest recommendations by CLSI, broth microdilution (BMD), broth disk elution and agar dilution MIC methods are acceptable for colistin susceptibility testing. For broth microdilution testing of colistin, refer to SOP for colistin susceptibility using broth microdilution test for aerobic gram-negative bacteria. Disk diffusion and gradient diffusion methods should not be performed. Colistin and polymyxin B are considered equivalent agents, so MICs obtained from testing colistin predict MICs to polymyxin B and vice versa.

Agar dilution susceptibility testing

This SOP is prepared for determining colistin resistance using the colistin agar test for Enterobacterales and *P. aeruginosa* as recommended by CLSI guidelines: M07- A11 and M100-32nd Edition 2022.

Medium: Mueller- Hinton Agar (MHA)

- Use Mueller-Hinton Agar (MHA) for agar dilution susceptibility testing of rapidly growing aerobic Enterobacterales and *Pseudomonas aeruginosa*.
- This agar dilution method for testing colistin sulphate was evaluated for Acinetobacter spp. by CLSI and yielded inaccurate results.
- Prepare fresh MHA on the day the colistin dilutions are added to agar plates
- Check the pH of each batch of MHA and ensure that the final pH is between 7.2 to 7.4.
- Select the ATCC strains for QC that most closely resemble the patients' isolates being tested.
- Perform MIC with each batch of MHA plate containing colistin sulphate dilutions using a standard set of QC organisms.
- If a new lot of MHA does not yield the expected MICs, investigate the other variables and components of the test.

Preparation of drug stock solution of Colistin and potency calculation

To prepare a 1 mg/ml primary stock solution of colistin and its potency calculation, refer to Chapter 2, page 40.

Potency Calculation:

For the potency calculation of colistin sulphate, refer to the potency calculation of SOP for broth microdilution colistin susceptibility test for aerobic gram-negative bacteria.

Use the above prepared primary stock solution (1000 µg/ml) to make the desired concentration of working stock solution.

Storage of primary stock solution of colistin

For the storage of the primary stock solution of colistin, refer to SOP for colistin susceptibility test using broth microdilution for aerobic gram-negative bacteria.

Preparation of MHAplates with different concentrations of Colistin

- 100 ml of Mueller-Hinton agar was prepared in 4 flasks (250 ml flasks) and autoclaved at 121°C for 15 min.
- Prepare 4 µg/ml colistin agar plate from primary stock 1mg/ml of colistin sulphate using $C_1V_1=C_2V_2$ formula as below.

Box- 4.1

To prepare 4 µg/ml (in 100ml MHA) from original stock solution (1000 µg/ml) of colistin

C1 V1	=	C2 V2
1000 µg/ml V1	=	4 µg/ml x 100 ml
V1	=	0.4 ml or 400 µl

*Add 400 µl of 1mg/ml stock solution in 100 ml of autoclaved MHA. Volume of the working stock solution can be increased depending on number of plates to be prepared as per the requirement

- For preparing 4 µg/ml colistin agar plates, add 400 µl of colistin to the molten 100 ml MHA flask that has equilibrated in a water bath to 45 to 50°C.
- Mix the flask thoroughly and pour the mixture into 100 mm Petri plates (labeled as 4 µg/ml) on a level surface to produce an agar depth of 3-4 mm. Approx. 5 plates are made from 100 ml MHA containing colistin.
- Similarly, for preparing 2 µg/ml, and 1 µg/ml colistin agar plates, add 200 µl and 100 µl of 1 mg/ml colistin to the molten 100 ml MHA flask that has equilibrated in a water bath to 45 to 50°C. Mix the flask thoroughly and pour the mixture into 100 mm Petri plates (labeled as 2 µg/ml and 1 µg/ml) on a level surface to produce an agar depth of 3-4 mm.

- For the preparation of 0 µg/ml colistin agar plates, pour MHA into 100 mm plates and label them as 0 µg/ml)
- Note: Pour the plates quickly after mixing to prevent cooling and partial solidification in the agar plate.
- Let the agar solidify at room temperature, and either use plates immediately or store them in sealed plastic bags in the dark at 2-8°C for up to 15 days
- MHA plates with the final concentration of colistin as 1 µg/mL, 2 µg/mL, and 4 µg/mL are ready to use.
- Let plates stored at 2 to 8°C equilibrate to room temperature before use. Ensure that the agar plate is dry before inoculating the plates.

Inoculum preparation and inoculation

Culture of Test/ QC Strains

- Make fresh subcultures from glycerol stocks/slants/ agar plates on Tryptic Soya Agar (TSA) or any other non-selective media a day before putting up the agar dilution test incubate at 35-37°C in a BOD incubator for 18 to 24 h.
- Put up the quality control (QC) strains with each test; the QC strains to be used are:

Table 4.1: Routine ATCC QC Strains for Colistin Agar Test

Organism	QC number	MIC range for Colistin	Strain Characteristic
Escherichia coli	ATCC BAA-3170	≤1 - >4 µg/ml, with a target of 2 µg/ml)	Positive Control (mcr-1)
P. aeruginosa	ATCC 27853	1 – 4 µg/ml	Negative control
Escherichia coli	ATCC 25922	0.25 – 2 µg/ml	Negative control

Refer to the IQC SOP provided by NCDC for the selection, maintenance, and testing of QC strains

Inoculum Preparation

- Using a loop or swab, pick 3–5 well-isolated colonies from a fresh (18–24 hours) nonselective agar plate and transfer to sterile saline (4–5 mL).
- Mix the inoculum well to make a homogenous suspension and Adjust turbidity to the equivalent of a 0.5 McFarland turbidity standard.
- Adjust the turbidity equivalent to 0.5 McFarland turbidity standard (approx. $1.5x10^8$ CFU/ml).

Note: To standardize the inoculum density, use a $BaSO_4$ turbidity standard equivalent to 0.5 McFarland standards or its optical equivalent.

- If Densitometer is available, can use that to adjust inoculum to 0.5 McFarland standard.
- Dilute the standardized 0.5 McFarland inoculum 1:10 in saline.

Inoculation and incubation of agar plates

- Divide each colistin agar plate of different concentrations into 10 parts, with a marker to test up to 10 isolates per plate. Label each part with the appropriate isolate number (see Figure 4.1).

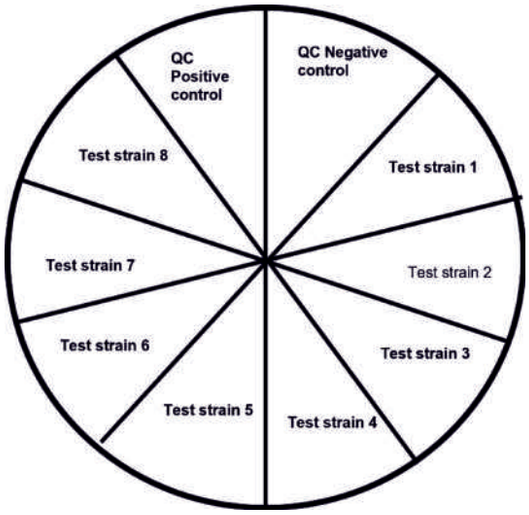


Figure 4.1 Template for testing 10 strains on each colistin agar plate

- Using a pipette or a 10-µL loop, streak 10 µL of the 1:10 dilution of each test strain and QC strain onto each colistin agar plate as per the labeling on the plates.
- Incubate the colistin agar and purity plates at 33 to 35°C for 16 to 20 h in an ambient air incubator within 15 minutes of adding the inoculum.
- Perform a purity check of the inoculum suspension by subculturing a 10 µl aliquot onto a blood agar plate for simultaneous incubation.

Colony counts of inoculum suspensions

Refer to Chapter 2, Page 44

Results and Interpretation

- Examine the purity plate to ensure the inoculum is pure.
- Examine the growth control plate, i.e., 0 µg/ml colistin agar plate, which must demonstrate confluent growth of all the test strains and QC strains for the test to be valid.
- Examine the colistin plates carefully with transmitted light for the colony or light film of growth.
- Read the MIC as the lowest colistin agar plate concentration that completely inhibits the growth of the test isolate (e.g., even 1 colony would be considered growth).
- Result can be recorded as + or -, + is equivalent to growth, - is equivalent to no growth. (Figure 4.2)

Note: Every raw data should be signed by the performer and countersigned by the verifier.

For *Enterobacterales* and

≤ 2 µg/mL = Intermediate
>4 µg/ml = Resistant

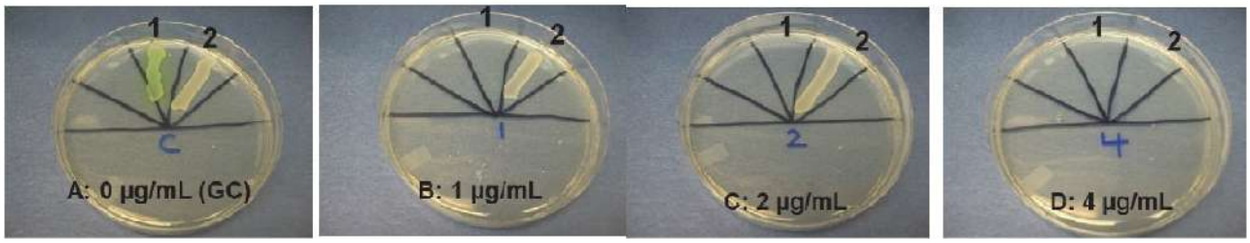


Figure 4.2 Colistin Agar Test. The plates must be carefully examined with transmitted light for confluent growth, individual colonies, or light film of growth to determine the MIC. Colistin agar test results for routine QC strain *P. aeruginosa* ATCC® 27853 at position 1 with a MIC ≤ 1 µg/mL and for additional QC strain *E. coli* ATCC® BAA-3170 ™ (formerly AR bank # 349 mcr-1 at position 2 with a MIC 4 µg/mL. The plates shown contain 0 µg/mL (control) (A), 1 µg/mL (B), 2 µg/mL (C), and 4 µg/mL (D) colistin.

Additional testing and reporting

Repeat the test if there is an inconsistent growth pattern (e.g., no growth in 2 µg/mL but growth at 1 µg/mL and 4 µg/mL).

An inconsistent growth pattern may occur as a result of:

- Contamination at higher dilutions
- Hetero resistance
- Improper concentrations of antimicrobial agent in the colistin agar plates
- Error inoculating the plates

Limitations

During this SOP preparation in January 2023, CLSI reported that they had not evaluated polymyxin B testing methods, and the procedures mentioned for colistin should not be adapted to polymyxin B.

References

- CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Thirty Second edition. CLSI document M100-A32 edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.
- CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for bacteria that Grow Aerobically; —Eleventh Edition. CLSI document M07-A11. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.

Quality control ranges

Table 4.2: MIC QC Ranges for QC Strains and Select Antimicrobial Agents

Antimicrobial Agent	MIC QC Ranges (µg/ml)	
	E. coli ATCC 25922	*P. aeruginosa* ATCC 27853
Colistin	0.25–2	0.5 – 4
Polymyxin B	0.25–2	0.5 – 2

Table 4.3: Interpretive categories and MIC Breakpoints (µg/ml) for colistin against *E. coli, Klebsiella* species, and *P. aeruginosa*

Organism	MIC (µg/ml)			
	S	SDD	I	R
E. coli	-	-	≤2	≥4
Klebsiella species	-	-	≤2	≥4
P. aeruginosa	-	-	≤2	≥4

The MICs obtained from testing colistin predict MICs for polymyxin B.

Chapter 5 - Standard Operating Procedure: Vancomycin Agar Screen Test - *Staphylococcus aureus and Enterococcus species*

National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India

Objectives and Scope:

Vancomycin Agar Screen test is used to screen *Staphylococcus aureus* strains and *Enterococci* for resistance to vancomycin. The isolates that give a positive result on vancomycin agar screen test must be tested for MIC determination using the broth microdilution method to determine vancomycin MIC.

Preparation of vancomycin hydrochloride stock solution

- The formulation of vancomycin available in the market is vancomycin hydrochloride.
- Check the potency of vancomycin hydrochloride written on the vial or the Certificate of Analysis (CoA) provided by the vendor along with the vial.
- If the potency of vancomycin hydrochloride is 950 µg/mg, in 1mg (1000 µg) powder of vancomycin hydrochloride, 950 µg is the active vancomycin powder.
- The first step is to prepare the 1 mg/ml stock solution of vancomycin hydrochloride, for this weigh 1 mg of powder in a 2 ml sterile centrifuge tube.
- Now add 950 µl of autoclaved distilled water in 1 mg weighed powder to make 1 mg/ml (1000 µg/ml) stock solution of vancomycin. Label each vial of aliquoted 1 mg/ml stock solution as "Working Stock Vancomycin."

Box-5.1: Example 1

- If the potency of vancomycin powder available in vancomycin hydrochloride salt mentioned in the CoA is 950 µg/mg.
- To prepare the stock solution of 1000 µg/ml (1mg/ml) weigh 1 mg of this vancomycin hydrochloride powder with potency of 950 µg/mg and add 950 µl of autoclaved distilled water. This is the 1mg/ml stock solution of vancomcyin.

Storage of stock solution of vancomycin

- Working stock solutions of higher volumes can be prepared as per the requirement, and further aliquots of smaller volumes for one-time use can be prepared and stored in deep freezers for a longer time.
- Dispense small volumes of the stock solutions as required into sterile 1.5 – 2 ml cryovials/ cryotubes.

Box 5.2

For 100 ml media preparation, 600 µl from 1 mg/ml stock solution of vancomcyin is required for preparation of 6 µg/ml VAS plate. Therefore, aliquots of 600 µl can be prepared and stored in deep freezers.

- Carefully label each cryovial and seal it properly. Store at −70°C or less, but never at a temperature more than −20°C and never in a self-defrosting freezer.

Preparation of vancomycin agar screen plates

- The medium used for vancomycin agar screen is Brain Heart Infusion (BHI) agar supplemented with 6 µg/ml vancomycin.
- To prepare 100 ml of BHI agar plates containing 6 µg/ml vancomycin, take out from the deep freezer 1 vial of aliquoted 600 µl of the vancomycin stock with a concentration of 1 mg/ml and add to the 100 ml of autoclaved BHI agar media.

Box 5.3: Calculation for preparation of 6 µg/ml VAS plate

For making 6 µg/ml vancomycin BHI agar plate from 1 mg /ml stock solution (1000 µg/ml)

C1 V1 = C2 V2

1000 µg/ml V1 = 6 µg/ml x 100 ml

V1 = 600 µl

Note:

- Add the antibiotic stock while the autoclaved media temperature is 45 -50°C.
- Immediately after adding antibiotic stock solution, slowly mix it and pour approx. 5 plates.

Inoculum preparation

- Make fresh subcultures from glycerol stocks/slants/ agar plates on Tryptic Soya Agar (TSA) or any other non-selective media on a day before putting up the VAS test and incubate at 35-37°C in a BOD incubator for 18 to 24 h.
- Put up the ATCC QC strains with each VAS plate; refer to QC Section, page 76.
- Use a fresh culture of the strain to be tested and prepare a suspension of the tested strain equivalent to 0.5 McFarland standard.
- Prepare a standardized inoculum of 0.5 McFarland using the direct colony suspension method. Take 3-5 well-isolated colonies of the same morphological type from the 18-24 hour culture plate and make saline suspensions in tubes containing sterile saline. Adjust the turbidity equivalent to 0.5 McFarland turbidity standard (approx. $1.5x10^8$ CFU/ml).

Note:

- To standardize the inoculum density, use a Barium Sulphate ($BaSO_4$) turbidity standard equivalent to 0.5 McFarland standards or its optical equivalent.
- If Densitometer is available, adjust the inoculum to 0.5 McFarland standard
- Mix the inoculum well to make a homogenous suspension and adjust to 0.5 McFarland standard.

Plate inoculation

- Inoculate the suspension using a micropipette to spot a 10 µl drop (final concentration. 10^6 CFU/ml) on the surface of the BHI agar plate containing 6 µg/ml vancomycin.
- *Alternatively*, a swab may be dipped in the McFarland suspension, the excess liquid expressed and used to inoculate the VAS plate.
- Make a square grid template in figure 1 to spot approximately 13 – 14 test strains and 2 QC strains in one plate.
- Incubation conditions: Incubate the plates at 35±2°C for 24 h in ambient air.

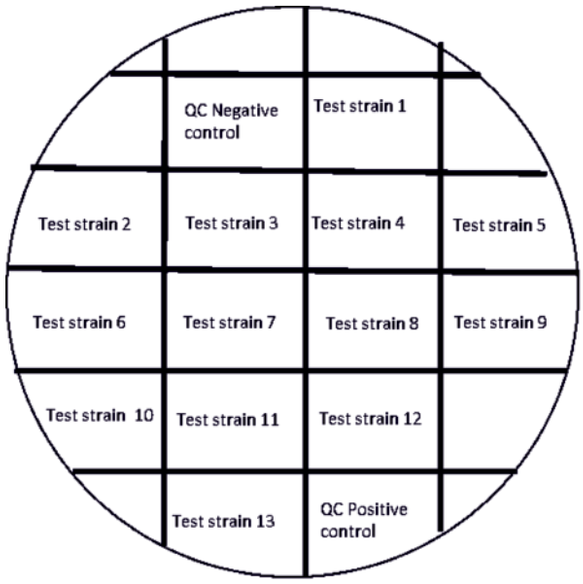


Figure 5.1: Vancomycin Agar Screen Square Grid Template

Interpretation and reporting

- Examine carefully with transmitted light for > 1 colony or light growth film.
- The presence of more than one colony of the strain or light film of growth is interpreted as reduced susceptibility to vancomycin.
- >1 colony = presumptive reduced susceptibility to vancomycin.
- If there is no growth on the screen agar plate, the S. aureus isolate is considered sensitive. If the *S. aureus* isolates grow at 6 µg/ml VAS plate, VRSA should be suspected, and BMD should be performed to determine vancomycin MIC. Results should be reported to the AMR-NRL at NCDC/laboratory identified by NCDC.

Quality control strains

Negative control strain:

- *Enterococcus faecalis* ATCC 29212 – susceptible or No growth

Note:

- Test negative QC strain with each new lot/shipment of testing materials
- Weekly if the test is performed at least once a week and criteria for converting from daily to weekly QC testing have been met.
- Daily, if the test is performed less than once per week and /or if criteria for converting from daily to weekly QC testing have not been met.

Positive control strain

- *Enterococcus faecalis* ATCC 51299 - resistant or greater than one colony
- In-house positive control strain of E. faecalis

Note:

- Test positive (resistant) QC strain at minimum with each new lot/shipment of testing materials

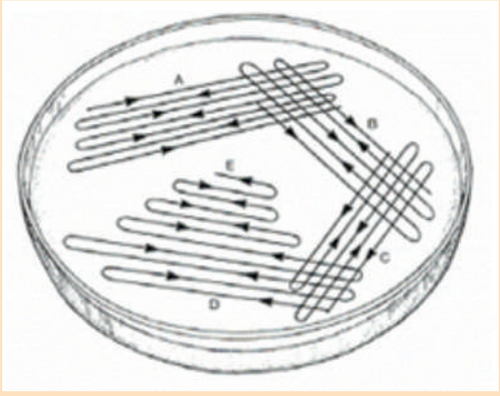
Limitations of the procedure

E. gallinarum/casseliflavus is intrinsically resistant due to the VanC gene, which may not be expressed when testing on the vancomycin screen plate.

References

CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Thirty-Second edition. CLSI document M100-A32 edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.

Chapter 6 - Standard Operating Procedure: Internal Quality Control for Disc Diffusion Antimicrobial Susceptibility Test



National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India

Introduction

Internal Quality control (IQC) is essential for monitoring the day-to-day consistency of the analytical testing phase and helps to determine the results' reliability. All participating NCDC AMR surveillance network laboratories must conduct the IQC in all testing processes. This SOP chapter guides IQC for disk diffusion antimicrobial susceptibility tests (AST) in a hospital's Microbiology/Clinical Microbiology laboratory.

Objective & Scope

This SOP gives an overview of the purpose of the IQC for Disc Diffusion Antimicrobial Susceptibility Tests:

- Ensure all laboratory testing materials, including media and antibiotic disks, are correctly maintained.
- Description of the selection, maintenance, and testing of QC strains
- The recommended frequency for performing QC testing includes the required testing to reduce QC frequency from daily to weekly.
- Suggestions for troubleshooting out-of-range QC results
- Factors to consider before reporting patient results when out-of-range QC results are observed
- Guidance for confirming noteworthy or uncommon results encountered when testing patient isolates

Selection of strains for quality control

- Procure strains from a recognized source (e.g., ATCC, NCTC) for QC of antimicrobial susceptibility tests to ensure the test system is working properly
- Select and include routine QC strains as recommended in the CLSI document
- CLSI-recommended QC strains should produce results within the expected range as referenced in Tables 6.1 to 6.2, Pages 90-91.
- Use the recommended testing conditions for the respective organism groups referenced in Table 6.4, Page 93.
- Select QC strains that most closely resemble the organism isolated from the clinical specimen.

- For commercial test systems, follow the manufacturer's recommendations for all QC procedures, including the QC strains recommended by the manufacturer

Maintenance and testing of quality control strains

- For long-term storage, maintain stock cultures at −20°C or below in 10% to 15% glycerol in Tryptic soy broth (TSB). Alternatively, strains can be lyophilized. A temperature lower than −60°C or liquid nitrogen is preferred for long-term storage as some QC strains, particularly those with plasmid-mediated resistance (e.g., *E. coli* ATCC 35218), have been shown to lose the plasmid when stored at temperatures higher than −60°C.
- Revive the original frozen or freeze-dried ATCC strain as instructed in the kit insert, culture on a Tryptic soy agar plate, and incubate. Label this as F1 culture plate. Follow the steps mentioned in Figure 6.1.
- From the F1 culture plate, each QC strain makes at least 12/52 F1 (either 12 monthly or 52 weekly) TSB glycerol stock vials, which last for one year. Label these as F1/month or F1/ week (e.g., F1/Jan; F1/Feb…..F1/Dec; F1/week1; F1/week2…….F1/week52) QC strain glycerol stock and store them at −20°C or below.
- For each month/week, use particular F1-month/week stock. E.g., for January, use F1/Jan; for February, use F1/Feb and so on; in case of weekly stock vials, use F1/week1 for week1 and F1/week52 for the last week of the calendar year.
- Subculture the glycerol stock on TSA plate/slant to get F2 weekly working QC strain for 1 week. This step is to be repeated every week. After one-month next F1/ month vial or after a week next F1/week vial is to be revived.
- Store F2 weekly working QC strain plate/slant at 2 to 8°C or as appropriate for the organism type
- Since fresh subcultures (e.g., overnight incubation) will be used for inoculum preparation for AST, subculture the F2 weekly working QC strain to obtain isolated colonies - the F3 QC strain.
- Discard F2 weekly working QC strain after one week. Repeat the step starting with F1/ month or F1/week QC strain glycerol stock for getting the next F2 weekly working QC strain for week 2, and so on.

Note:

- If QC strain appears to be contaminated or QC results are questionable, reviving a new F1 QC strain glycerol stock may be necessary.
- Test QC strains using the same materials and methods that are used to test clinical isolates.

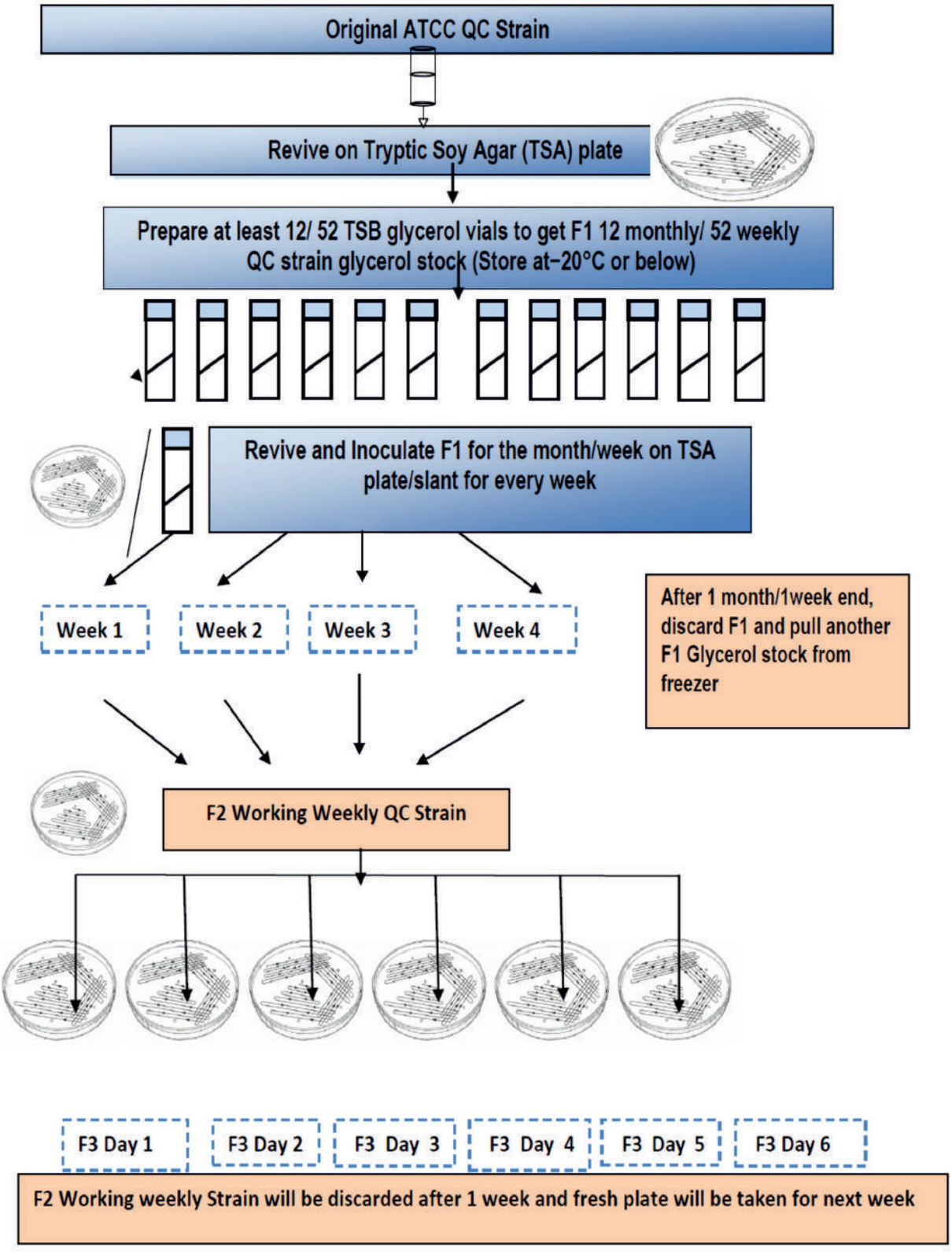


Figure 6.1: Maintenance of QC strains in a bacteriology lab

Quality control (QC) results documentation - zone diameter

- Document results from all disk diffusion QC tests on a QC log sheet (Sample of QC log sheet in Table 6.6 at Page 95).
- Acceptable zone diameter QC ranges for the QC test (single-drug/single-organism combination) for select antibiotics are listed in Tables 6.1 and 6.2 at Pages 90-91.

  Note: For acceptable zone diameter QC ranges for other antibiotics and β-lactam combinations, refer to CLSI M100-32nd Ed, tables 4 A1- A 2, Pages 178 - 183.
- Results for QC strains must fall within CLSI-specified ranges for them to be acceptable.

Frequency of Quality Control Testing

Two plans are available to demonstrate satisfactory performance with daily QC testing before going to weekly QC testing.

1. **20- or 30-day plan**
2. **15-replicate (3× 5 day) plan.**

Either plan allows a laboratory to perform weekly QC testing once satisfactory performance with daily testing of QC strains is documented.

QC conversion plan

Performance Criteria for Reducing the Frequency of Quality Control testing to Weekly

The 20- or 30-Day Plan

- Test all applicable QC strains for 20 or 30 consecutive test days and document results.
- When no more than 1 out of 20 or 3 out of 30 consecutive tests are outside the acceptable limit, performance shall be considered satisfactory to proceed to weekly testing. If not, take corrective action.

The 15-Replicate (3× 5 Day) Plan

- Test three replicates of each applicable QC strain using individual inoculum preparation for five consecutive test days and document results.
- After completing the 15-replicate (3 × 5 day) plan, the lab can undergo weekly QC testing.
- If completing the 15-replicate (3 × 5 day) plan is unsuccessful, take corrective action as appropriate, and continue daily QC testing. (Refer to Table 6.5, page no. 94)

Note: Testing on "consecutive test days" means testing of QC strains each day tests are performed on isolates. It does not refer to calendar days.

Implementing weekly quality control testing

- Implement Weekly QC testing once satisfactory performance with daily QC testing has been documented.

- Along with weekly testing, QC shall be performed whenever reagent components of the test are changed.
- If any weekly test result is out of range, take corrective action.

Out-of-range results with quality control strains and corrective action

- Out-of-range QC results can be categorized into 1) random, 2) identifiable, or 3) system related.
- A single repeat of the QC test can usually resolve out-of-range results with QC strains due to random or identifiable errors.
- Out-of-range QC tests are often the result of contamination or the use of an incorrect QC strain; corrective action should first include repeating the test with a pure culture of a freshly sub-cultured QC strain.
- Out-of-range QC results due to a problem with the test system usually do not correct when the QC test is repeated and may indicate a severe problem that can adversely affect patient results. Every out-of-range QC result must be investigated.

Note: See disk diffusion Troubleshooting Guide in M100, 32nd edition Table 4D for troubleshooting and corrective action for out-of-range results with QC strains

Daily or weekly QC testing: out-of-range result due to identifiable error

- Identify the reason for an out-of-range result, correct the problem, document the reason, and retest the QC strain on the day the error is observed. No further corrective action is required if the repeated result is within range.
- Identifiable reasons for the out-of-range results may include, but are not limited to:

QC strain

- Use of the wrong QC strain
- Improper storage
- Inadequate maintenance (e.g., use of the same F2 subculture for > 1 month)
- Non-viability
- Changes in the organism (e.g., mutation, loss of plasmid)

Testing supplies

- Improper storage or shipping conditions
- Contamination
- Use of a defective agar plate (e.g., too thick or too thin)
- Use of damaged (e.g., cracked) plates / expired materials (disks)
- Inoculum suspensions incorrectly prepared or adjusted

- Inoculum prepared from a plate that has been incubated for the incorrect length of time
- Inoculum prepared from differential or selective media containing antimicrobial agents or other growth-inhibiting compounds
- Use of wrong incubation temperature or conditions
- Use of wrong disk, ancillary supplies
- Improper disk placement (e.g., inadequate contact with the agar) or disk falls off agar
- Incorrect reading or interpretation of test results
- Transcription error

Equipment

- Not functioning correctly or out of calibration (e.g., pipettes)

Weekly QC testing – out-of-range result not due to identifiable error

- If the reason for the out-of-range result with the QC strain cannot be identified, perform corrective action to determine if the error is random. Test out-of-range antimicrobial agent/organism combination on the day error is observed or when the QC strain's F2 or F3 subculture is available.
- If the repeat results are in range, evaluate all QC results available for the antimicrobial agent/organism combination when using the same lot numbers of materials used when the out-of-range QC result was observed. If five acceptable QC results are available, no additional days of QC testing are needed.
- Different scenarios of inconsistent QC results and action to be taken are given below for better guidance

Scenario #6.1

Ampicillin *E. coli* ATCC® 25922; acceptable range: 15 to 22 mm Week	Day	Lot Number (Disks)	Lot Number (MHA)	Result	Action
1	1	3564	16481	18	
2	1	3564	16481	19	
3	1	3564	16481	18	
4	1	3564	16481	19	
5	1	3564	16481	14	Out of range. Repeat QC same day.

Ampicillin *E. coli* ATCC® 25922; acceptable range: 15 to 22 mm Week	Day	Lot Number (Disks)	Lot Number (MHA)	Result	Action
6	2	3564	16481	17	In range. Five acceptable in-range QC tests for *E. coli* ATCC® 25922 with ampicillin disks lot 3564 and MHA lot 16481. Resume weekly QC testing.

For more trouble shooting / suggestive action refer to CLSI guideline M100-32nd Ed. Table 4D, Pg.192-.195

Scenario #6.2

Ampicillin *E. coli* ATCC® 25922; acceptable range: 15 to 22 mm Week	Day	Lot Number (Disks)	Lot Number (MHA)	Result	Action
1	1	9661	16785	18	
2	1	9661	16785	19	
3	1	9661	16785	14	Out of range. Repeat QC same day.
3	2	9661	16785	18	In range. Three acceptable in-range QC tests for *E. coli* ATCC® 25922 with ampicillin disks lot 9661 and MHA lot 16785. Repeat QC 2 more consecutive days.
3	3	9661	16785	18	In range.
3	4	9661	16785	17	In range. Five acceptable in-range QC tests for *E. coli* ATCC® 25922 with ampicillin disks lot 9661 and MHA lot 16785. Resume weekly QC testing.

Scenario#6.3

Ampicillin *E. coli* ATCC® 25922; acceptable range: 15 to 22 mm Week	Day	Lot Number (Disks)	Lot Number (MHA)	Result	Action
1	1	8541	16922	17	
2	1	8541	16922	21	
3	1	8541	16922	28	Out of range. Repeat QC same day.
3	2	8541	16922	21	In range. Three acceptable in-range QC tests for *E. coli* ATCC® 25922 with ampicillin disks lot 8541 and MHA lot 16922. Repeat QC 2 more consecutive days.
3	3	8541	16922	30	Out of range. Repeat QC same day.
3	4	8541	16922	28	Out of range. Perform corrective action if results for a QC strain/antimicrobial agent combination are out of range and the error is not identifiable and on two consecutive days of testing or if more than three results for a QC strain/antimicrobial agent combination are out of range during 30 consecutive days of testing. Daily QC tests must be continued until final resolution of the problem is achieved (described below).

Additional Corrective Action- Checklist

Additional corrective action is required if repeat results with QC strains are still out of range. It is possible that the problem is due to a system error rather than a random error. If repeat QC errors are observed then

- Continue Daily QC tests until final resolution of the problem is achieved.
- If necessary, obtain a new QC strain (either from stock cultures or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers.
- If the problem appears to be related to a manufacturer, contact and provide the manufacturer with the test results and lot numbers of materials used.
- It may be helpful to exchange QC strains and materials with another laboratory (may be Reference Lab) to determine the root cause of out-of-range QC results where the reason is not identifiable

As part of the corrective actions, make certain that you check all of the following:

1.	Zone diameters were measured correctly	☐ Yes ☐ No
2.	Turbidity standard is homogeneous and free of clumps	☐ Yes ☐ No
3.	Inoculum suspension was prepared properly	☐ Yes ☐ No
4.	Test materials were stored properly	☐ Yes ☐ No
5.	Test materials have not reached or passed their expiration dates	☐ Yes ☐ No
6.	Incubator temperature and atmosphere are within the specified range and equipment is properly maintained	☐ Yes ☐ No
7.	QC strain used for testing is acceptable	☐ Yes ☐ No
8.	QC strain maintained as per recommendations	☐ Yes ☐ No
9.	The person performing testing has been certified as competent to perform the test	☐ Yes ☐ No
10.	The pH of the medium is in the established range	☐ Yes ☐ No
11.	The depth of the agar in the plates is 4 mm.	☐ Yes ☐ No

Reporting patient results when out-of-range QC results are observed

When an out-of-range result occurs for the QC strains or corrective action is necessary, each patient test result must be carefully examined to determine if it can be reliably reported. Factors to consider may include, but are not limited to:

- Size and direction of QC strain error (e.g., slightly increased OR significantly increased OR decreased zone size)
- Actual patient result and its proximity to the interpretive breakpoint
- Results with other QC organisms
- Results with other antimicrobial agents
- The usefulness of the particular QC strain/antimicrobial agent as an indicator for a procedural or storage issue (e.g., inoculum dependent, heat-labile)

Options to consider for patient results include:

- Withhold the results for an individual antimicrobial agent
- Review individual patient or cumulative data for unusual patterns
- Use an alternative test method or a reference laboratory until the problem is resolved.

Accuracy checklist before reporting the patient AST results

To ensure that antimicrobial susceptibility test results on patients' isolates are accurate, review all results, and make certain:

1.	Results with QC strains are within the acceptable range.	☐ Yes ☐ No
2.	Growth is satisfactory.	☐ Yes ☐ No
3.	The test is not contaminated (mixed)	☐ Yes ☐ No
4.	The overall susceptibility profile or antibiograms are consistent with expected results for agents tested and the identification of the isolate; check results for all antimicrobial agents tested and not just those that will be reported.	☐ Yes ☐ No
5.	The results from individual antimicrobial agents within a specific drug class follow the established hierarchy of activity rules (e.g., third-generation cephalosporins are more active than first- or second-generation cephalosporins against Enterobacteriaceae).	☐ Yes ☐ No
6.	The isolate is susceptible to those antimicrobial agents for which resistance has not been documented (e.g., vancomycin and *Streptococcus* spp.) and for which only "susceptible" interpretive criteria exist in CLSI M100 Ed32, 2022	☐ Yes ☐ No
7.	Atypical resistance, if present, is confirmed.	☐ Yes ☐ No

While testing patient isolates, confirm the above checklist before reporting the results

NB: Multiple test parameters are monitored by following the QC recommendations. However, acceptable results derived from testing QC strains do not guarantee accurate results when testing patient isolates.

Further confirm unusual or inconsistent results by checking for

- Previous results on the patient (e.g. did the patient previously have the same isolate with an unusual antibiogram?)
- Previous QC performance (e.g., is there a similar trend or observation with recent QC testing?)
- Problems with the testing supplies, process, or equipment
- If a reason for the unusual or inconsistent result for the patient's isolate cannot be ascertained, a repeat of the susceptibility test or the identification, or both, may be needed.
- Use an alternative test method or a reference laboratory until the problem is resolved

Note: Refer CLSI M100-32nd edition, Appendix A- page 240-246 "Suggestions for Confirming Antimicrobial Susceptibility Test Results and Organism Identification

Each laboratory must develop its own policy for confirmation of unusual or inconsistent AST results. This policy should emphasize those results that may significantly impact patient care.

End-point interpretation control

Monitor end-point interpretation periodically to minimize variation in the interpretation of zone sizes among observers.

- All laboratory personnel who perform these tests should independently read a selected set of tests.
- Record the results and compare to the results obtained by an experienced reader; or, when using QC strains, compare to the expected results from CLSI M100 32nd edition Tables 4 A1-2.
- Generally, zone measurement readings from several individuals should not vary more than ± 2 mm.

References

1. CLSI. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Thirteenth Edition. CLSI document M02, 13th ed.. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
2. CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Thirty Second edition. CLSI document M100-A32 edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.

Table 6.1: Disk Diffusion QC ranges for ATCC QC strains

Antimicrobial Agent	Disk Content	Disk Diffusion QC Ranges (mm)		
		E. coli ATCC 25922	*Staphylococcus aureus* ATCC 25923	*Pseudomonas aeruginosa* ATCC 27853
Amikacin	30 µg	19–26	20–26	18–26
Ampicillin	10 µg	15–22	27–35	–
Azithromycin	15 µg	-	21-26	-
Aztreonam	30 µg	28-36	-	23-29
Cefazolin	30 µg	21-27	29-35	-
Cefepime	30 µg	31-37	23-29	25-31
Cefotaxime	30 µg	29-35	25-31	18-22
Cefoxitin	30 µg	23-29	23-29	-
Ceftaroline	30 µg	26-34	26-35	-
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	-
Ciprofloxacin	5 µg	29-37	22-30	25-33
Clindamycin	2 µg	-	24-30	-
Doxycycline	30 µg	18-24	23-29	-
Ertapenem	10 µg	29-36	24-31	13-21
Erythromycin	15 µg	-	22-30	-
Fosfomycin [b]	200 µg	22-30	25-33	-
Gentamicin [c]	10 µg	19-26	19-27	17-23
Imipenem	10 µg	26-32	-	20-28
Linezolid	30 µg	-	25-32	-
Meropenem	10 µg	28-35	29-37	27-33
Minocycline	30 µg	19-25	25-30	-
Nalidixic acid	30 µg	22-28	-	-
Nitrofurantoin	300 µg	20-25	18-22	-
Penicillin	10 units	-	26-37	-
Teicoplanin	30 µg	-	15-21	-
Tetracycline	30 µg	18-25	24-30	-
Tobramycin	10 µg	18-26	19-29	20-26
TMP/SXT	1.25/23.75 µg	23-29	24-32	-
Vancomycin	30 µg	-	17-21	-

a. Refer to M100-S32 Tables 4A1-2 Pgs 150-157 for the complete list.
When a Commercial test system is used for AST, refer to the manufacturer's instructions for QC test recommendations, and QC ranges

b. The 200-µg fosfomycin disk should contain 50 µg of glucose-6-phosphate.

c. For control ranges of gentamicin 120-µg and streptomycin 300-µg disk, use E.faecalis ATCC 29212(gentamicin:16–23 mm; streptomycin: 14–20 mm).

Table 6.2: Disk diffusion QC ranges for non-fastidious QC strains and β-lactam combination agents. (Un supplemented Mueller-Hinton medium)

Antimicrobial Agent	Disk Content	*E. coli* ATCC 25922 β-lactamase negative	*P. aeruginosa* ATCC 27853 Inducible AmpC	*S. aureus* ATCC25923 β-lactamase negative, mecA negative	*E. coli* ATCC 35218 [a,b] TEM-1
		MIC QC ranges, mm			
Amoxicillin-clavulanate (2:1)	20/10 µg	18–24	–	28–36	17–22
Piperacillin	100 µg	24–30	25–33	–	12–18
Piperacillin-tazobactam	100/10 µg	24–30	25–33	27–36	24–30

a. Careful attention to organism maintenance (e.g. minimal subcultures) and storage (e.g. −60°C or below) is especially important for the QC strain *E.coli* ATCC 35218 because spontaneous loss of the plasmid encoding the β-lactamase has been documented. If stored at temperatures above −60°C or if repeatedly sub cultured, the strain may lose its resistance characteristics and QC results may be outside the acceptable ranges.

b. To confirm the integrity of the QC strain (*E. coli* ATCC 35218), test the β-lactam agent highlighted in orange by disk diffusion when the strain is first sub cultured from a frozen stock culture. In-range results for the single agent indicate the QC strain is reliable for QC of β-lactam combination agents. It is not necessary to check the QC strain again with a single agent until a new frozen stock culture is put into use, providing recommendations for handling QC strain are followed.

Table 6.3: Characteristics of routine QC strains for antimicrobial susceptibility tests

QC Strains	Organism Characteristics	Disk Diffusion Tests	MIC Tests	Other Tests	Comments
E. faecalis ATCC 29212			Nonfastidious gram- positive bacteria	• Vancomycin agar • HLAR tests • High-level mupirocin resistance MIC test	Assess suitability of medium for sulfonamide or trimethoprim disk diffusion tests[a]
Escherichia coli ATCC 25922	• β-lactamase negative	• Nonfastidious gram-negative bacteria	• Nonfastidious gram- negative bacteria		
E. coli ATCC 35218	• TEM-1	• β -lactam combination agents	• β -lactam combination agents		
Pseudomonas aeruginosa ATCC 27853[b]	• Inducible AmpC β-lactamase	• Nonfastidious gram-negative bacteria	• Nonfastidious gram- negative bacteria		• Assess suitability of cation content in each batch/lot of CAMHB.
Staphylococcus aureus ATCC 25923	• β-lactamase negative •mecA negative • mupA negative	• Nonfastidious gram-positive bacteria		• High-level mupirocin resistance disk diffusion test • Inducible clindamycin resistance disk diffusion test (D-zone test)	• Little value in MIC testing due to its extreme susceptibility to most drugs
S. aureus ATCC 29213	• Weak β -lactamase–producing strain • mecA negative • mupA negative		• Nonfastidious gram- positive bacteria	• Oxacillin salt agar • High-level mupirocin resistance MIC test • Inducible clindamycin resistance MIC test • Penicillin zone-edg1e test	• Assess suitability of cation content in each batch/ lot of MHB for Daptomycin broth microdilution

a. If the medium has acceptable levels of thymidine, disk diffusion and MIC end points should be easy to read as 80% or greater reduction in growth.

b. May develop resistance to β -lactam antimicrobial agents after repeated subcultures.

Table 6.4: Conditions for disk diffusion antimicrobial susceptibility test for non-fastidious organisms (including routine QC strains)

Organism/ Organism Group	CLSI M100 Table	Medium	0.5 McFarland	Incubation	Incubation Time	Minimal QC Required
Enterobacteriaceae	2A	MHA	Direct colony suspension in broth or saline, or growth method	35°C± 2°C; ambient air	16–18 hours	*Escherichia coli* ATCC 25922 *P. aeruginosa* ATCC 27853 for carbapenems *E. coli* ATCC 35218 (for β-lactam/β-lactamase inhibitor combinations
Pseudomonas aeruginosa	2B-1	MHA	Direct colony suspension in broth or saline,	35°C± 2°C; ambient air	16–18 hours	*P. aeruginosa* ATCC 27853 *E. coli* ATCC 35218 (for β -lactam/ β -lactamase inhibitor combinations)
Acinetobacter spp.	2B-2	MHA	Direct colony suspension in broth or saline, or growth method	35°C± 2°C; ambient air	20–24 hours	*P. aeruginosa* ATCC 27853 *E. coli* ATCC 25922 for tetracyclines andtrimethoprim-sulfamethoxazole *E. coli* ATCC® 35218 (for β -lactam/ β -lactamase inhibitor combinations
**Staphylococcus* spp.	2C	MHA	Direct colony suspension in broth or saline	35°C ± 2°C; ambient air (Testing at temperatures above 35°C may not detect MRS)	16–18 hours 24 hours for cefoxitin with CoNS	*Staphylococcus aureus* ATCC 25923
***Enterococcus* spp.	2D	MHA	Direct colony suspension in broth or saline, or growth method	35°C± 2°C; ambient air	16–18 hours 24 hours for vancomycin	*Staphylococcus aureus* ATCC 25923

* Examine linezolid zones carefully with transmitted light; Vancomycin disk diffusion test is not recommended for S. aureus and CoNS

** Examine vancomycin zones carefully with transmitted light for small colonies or haze inside the zones of inhibition; any growth =resistance

Table 6.5: Plan 15-replicate (3×5 Day): Acceptance criteria and recommended action*

Number Out of Range With Initial Testing (Based on 15 Replicates)	Conclusion From Initial Testing (Based on 15 Replicates)	Number Out of Range After Repeat Testing (Based on All 30 Replicates)	Conclusion After Repeat Testing
0–1	Plan is successful. Convert to weekly QC testing	N/A	N/A
2–3	Test another 3 replicates for 5 days	2–3	Plan is successful. Convert to weekly QC testing
≥ 4	Plan fails. Investigate and take corrective action as appropriate. Continue QC each test day	≥ 4	Plan fails. Investigate and take corrective action as appropriate. Continue QC each test day

*Assess each QC strain/antimicrobial agent combination separately.

Table 6.6: IQC for monitoring AST by disk diffusion log sheet

Antimicrobial agent	Disk content	Lot no.	Disk diffusion QC ranges (in mm)							
			E. coli ATCC 25922		*S. aureus* ATCC 25923		*P. seud aeruginosa* ATCC 27853		*E. coli* ATCC 35218	
Amikacin	30 µg		19–26		20–26		18–26			
Ampicillin	10 µg		15–22		27–35					
Azithromycin	15 µg				21-26					
Aztreonam	30 µg		28-36				23-29			
Cefazolin	30 µg		21-27		29-35					
Cefepime	30 µg		31-37		23-29		25-31			
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					
Ciprofloxacin	5 µg		29-37		22-30		25-33			
Clindamycin	2 µg				24-30					
Co-amoxiclav	20/10 µg		18–24				28–36		17–22	
Cotrimoxazole	1.25/23.75 µg		23-29		24-32					
Doxycycline	30 µg		18-24		23-29					
Ertapenem	10 µg		29-36		24-31		13-21			
Erythromycin	15 µg				22-30					
Fosfomycin1	200 µg		22-30		25-33					
Gentamicin2	10 µg		19-26		19-27		17-23			
Imipenem	10 µg		26-32				20-28			
Linezolid	30 µg				25-32					
Meropenem	10 µg		28-35		29-37		27-33			
Nitrofurantoin	300 µg		20-25		18-22					
Norfloxacin	10 µg		28-35		17-28		22-29			
Penicillin	10 units				26-37					
Piperacillin	100 µg		24–30		25–33				12–18	
Piperacillin-Tazobactam	100/10 µg		24–30		25–33		27–36		24–30	
Teicoplanin	30 µg				15-21					
Tetracycline	30 µg		18-25		24-30					
Tobramycin	10 µg		18-26		19-29		20-26			
Vancomycin	30 µg				17-21					

1 Fosfomycin 200-µg disk should contain 50 µg of glucose-6-phosphate

2 For Gentamicin HLAR use 120-µg disk (& to monitor MHA agar quality) use *E. faecalis* ATCC 29212; QC range: 16–23 mm

Guidance on Media and Disks

Mueller Hinton Agar (MHA)

- Prepare MHA from a commercially available dehydrated base according to the manufacturer's instructions.
- Immediately after autoclaving, allow the agar to cool in a 45 to 50°C water bath.
- Pour freshly prepared medium into glass or plastic flat-bottomed Petriplates on a level, horizontal surface to give a uniform depth of approximately 4 mm (60 to 70 mL of medium for plates with a diameter of 150 mm; 25 to 30 mL for plates with a diameter of 100 mm and 20-24 mL for plates with a diameter of 90 mm).
- Fresh plates should be used the same day or stored in a refrigerator (2- 8°C).
- Examine a representative sample of each batch of plates for sterility by incubating at 35°C ± 2°C for 24 hours or longer
- Plates are stable for seven days but could have a longer shelf life if precautions are taken to prevent drying (wrapped in plastic to minimize evaporation), and QC is within range at the testing time.
- Just before use, if excess moisture is visible on the surface, plates should be placed in an incubator (35°C) or, with lids ajar, in a laminar-flow hood at room temperature until the moisture evaporates (usually 10 to 30 minutes).
- Check the pH of each batch of MHA and record in the Media QC reporting format below.
- The pH of MHA should be between 7.2 and 7.4 at room temperature (refer to CLSI guidelines M02 and manufacturer instructions for further details).
- If the pH is less than 7.2, drugs may certain ose potency (e.g., aminoglycosides, macrolides), whereas other antimicrobial agents may appear to have excessive activity (e.g., tetracycline). If the pH exceeds 7.4, the opposite effects can be expected.

For each batch of media prepared, in addition to sterility check, perform following quality control:

Quality Control	Tested for	Expected results
Escherichia coli ATCC® 25922	Nonfastidious gram-negative bacteria Nonfastidious gram-negative bacteria Assess the suitability of cation content in each batch/lot of CAMHB	Good growth
Pseudomonas aeruginosa ATCC® 27853		Good growth
Staphylococcus aureus ATCC® 25923	Nonfastidious gram-positive bacteria	Good growth
Negative control	**Un-Inoculated medium**	**No growth**

Media QC recording format

Media QC record									
Name of the medium									
ATCC strains used	Expected reactions								
Strain 1									
Strain 2									
Strain 3									
Acceptable	Expected reactions obtained								
Unacceptable	Expected reactions not obtained								
Date of preparation	Lot number	Expiry Date	Autoclave		PH	Physical examination	Sterility check	Growth check with ATCC strains	Reviewed by
			Hold time	Temperature					

Antimicrobial disks & quality specifications

Purchase Disks from a reliable commercial vendor. Check for the following at the time of receiving:

The disks should be accompanied, at minimum, with

- Certificate of analysis (CoA)stating the content of the disks
- Lot number
- Expiration date

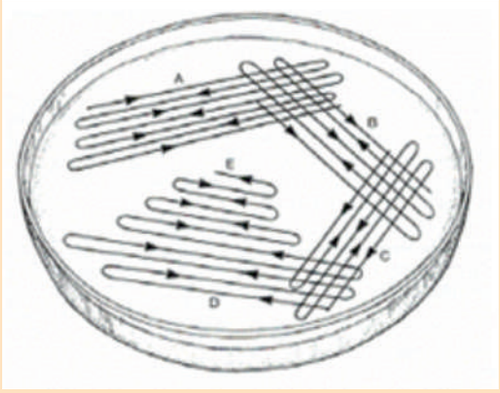
Storage of antibiotic disks

- Refrigerate the cartridges at 8°C or below in a refrigerator or below in a non-frost-free freezer until needed.
- Sealed packages of disks containing drugs from the ß-lactam class should be stored frozen (-20°C), except for a small working supply, which may be refrigerated for at least one week. Some labile agents (e.g., imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.
- Remove the disks cartridges from the refrigerator or freezer 1–2 hours before use to bring it to room temperature before use.
- Once a cartridge of disks has been removed from its sealed package, please place it in a tightly sealed, desiccated container for storage.

Batch or lot quality ontrol

- Check each batch or new lot of MHA/antibiotic discs with the appropriate QC strains before or with their first use for testing patient isolates. Performance tests with each lot of MHA must conform to the control limits of disk QC ranges for organisms and antibiotics.
- If zone diameters do not fall within acceptable range, proceed with corrective action.
- From each batch of prepared media, keep MHA plates and other agar media not containing antimicrobials overnight at 37°C for sterility checking before inoculation.
- Maintain records, at a minimum, of the lot numbers, and expiry dates of all materials and reagents used in performing susceptibility tests.

Chapter 7 - Guidance for Submission of AMR Surveillance Isolate for External Quality Assessment and Reporting Emerging AMR Alerts



National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India

Expectations from AMR surveillance laboratories

- To ensure quality results under NCDC EQAS, all participating laboratories are expected to submit one sensitive isolate every quarter and five isolates of each resistant priority bacterial pathogen for testing as per AMR surveillance program directive (Refer to Table 7.1 on page 103 and Table 7.2 on page 105).
- All participating laboratories are expected to submit EQAS isolates to NCDC every QUARTER for confirmatory testing as per below mentioned timeline:
  - Quarter 1 isolates (January 1 to March 31) are to be submitted by APRIL 15
  - Quarter 2 isolates (April 1 to June 30) to be submitted by JULY 15
  - Quarter 3 isolates (July 1 to September 30) to be submitted by OCTOBER 15
  - Quarter 4 isolates (October 1 to December 31) to be submitted by JANUARY 15 of next year
- Each surveillance network laboratory must enroll in Microbiology/Bacteriology EQAS conducted by the Indian Association of Medical Microbiology (IAMM), currently available from Sir Ganga Ram Hospital, New Delhi (http://www.iammeqasdelhi.com/) or Christian Medical College, Vellore, Tamil Nadu (http://www.microbiology.iammeqascmc.org/).
- Sites are expected to share the EQAS scores obtained from IAMM EQAS panels with NCDC. For details, please refer to page 106)
- Sites are expected to maintain a score of at least 80% in all the cycles of EQAS for bacterial identification/susceptibility.
- All the participating network sites are mandated to preserve the bacterial pathogens with crucial resistant types for future needs as agreed in the Memorandum of Understanding (MOU) between NCDC and the institution part of NARS-Net.
- The bacterial isolates should be sent to NCDC in nutrient agar media stabs in 1.5 ml/2 ml sterile plastic vials, and a duplicate stock should be preserved at the reporting lab.
- All AMR alert pathogens must be shipped to NCDC, New Delhi, for confirmation and further guidance.
  - o The strain identity should be confirmed before sending it to NCDC
  - o The confirmed isolate has to be reported immediately
  - o Alert isolate should reach NCDC within one week of confirmation
- If an Alert isolate sent is found to be contaminated or not revived at NCDC, the reporting lab should send the isolate again from the duplicate preserved stock at their lab after rechecking them.

Preparation of nutrient agar stabs

- Prepare and autoclave nutrient agar media. Ensure the media contains 1.5% agar in it.
- Dispense 1ml of Nutrient agar media in autoclaved 1.5 mL/2.0 mL plastic vials (approx. 2/3 full). (Autoclavable airtight cryovials are ideal for this, if available)
- Stab inoculate the media with pure colonies from fresh culture using a sterile straight wire and incubate overnight at 37°C.
- Next day, seal the plastic vials tightly and cover the edges of the lid using parafilm.
- Label the isolates with Lab ID or Sample ID/Lab code/Month/Year.
- Example: Sample ID is18004325; Lab code is GMC; the organism was submitted in October 2022 (October/2022); then the label on the strain should be 18004325/GMC/OCT/2022.

AST results reporting

- Report AST results only in zone diameters/MIC values and not as interpretation (RIS).
- Send the details of isolates with the packet containing isolates in the prescribed excel format (Refer to Page 107).
- Also, email this excel sheet to the AMR surveillance secretariat at amrsurveillance@gmail.com
- For AMR Alert strains, send a duly filled hard copy of the alert form and the isolates (Refer to Pages 108-109).
- The soft copy (filled and signed PDF/ word file) must be shared with amrsurveillance@gmail.com.
- For each isolate sent to NCDC as EQAS or ALERT strain, ensure that AST is performed for ALL the listed antibiotics in the surveillance AST panel.
- All labs should report MIC values based on broth microdilution AST for
  - Colistin for all gram-negative priority pathogens (*E. coli, Klebsiella* species, *Acinetobacter baumanii/Acinetobacter calcoaceticus* complex, and *Pseudomonas aeruginosa*)
  - Vancomycin for *Staphylococcus aureus*
  - Linezolid for *Staphylococcus aureus* and *Enterococcus* species

Standard precautions

The persons performing the culture and AST should follow "standard precautions" and wear appropriate PPEs while handling the isolates for packaging.

Table 7.1: List of strains to be shipped to NCDC for External Quality Assessment (EQAS)/ Alert confirmation

Pathogen	AMR Alerts (All)	Resistant strain (5 isolates)	Intermediate Resistant strain (All)	Sensitive strain (1 isolate)
Enterococcus species	Linezolid Resistant (All)	Vancomycin		
Staphylococcus aureus	Vancomycin (All); Linezolid (All)		Vancomycin (All)	
Escherichia coli	Colistin (All)	Imipenem		
Klebsiella species	Colistin (All)	Imipenem		
Acinetobacter baumanii/ Acinetobacter calcoaceticus complex	Colistin (All)	Imipenem		
		Piperacillin-Tazobactam		
Pseudomonas aeruginosa	Colistin (All)	Imipenem		
		Piperacillin-Tazobactam		
Salmonella enterica sero. Typhi	All isolates to be submitted, All Resistant as alerts (Ceftriaxone; Azithromycin; Imipenem)			
Salmonella enterica sero. Paratyphi	All isolates to be submitted, All Resistant as alerts (Ceftriaxone; Azithromycin; Imipenem)			
Shigella species	All isolates to be submitted			
Vibrio cholerae	All isolates to be submitted			

Isolate packaging and transportation to NCDC

- The isolates or specimens with priority AMR pathogens are covered by UN 3373 shipment procedures and according to International Air Transport Association's Dangerous Goods Regulations (IATA-DGR) packing instruction 650.
- Packaging must be good quality and strong enough to withstand the shocks and loadings usually encountered during transport. Packaging must be constructed and closed to prevent any leakage or loss of contents during transport.
- The packaging must consist of three components: (Refer to Figure 7.1)

A primary container: This can be a screw-capped container or leakproof cryovials. If multiple primary containers are to be sent, they should be individually wrapped or separated to prevent contact. Adequate absorbent material must be packed around the primary receptacle/s to absorb any fluid leakage from the primary receptacle/s.

a) **A leak-proof secondary container/packaging:** The primary container/s and absorbent material are put into a leak-proof secondary container. Sealed plastic bags (zip lock bag) is a good alternative for the recommended secondary container. An itemized list of contents must be enclosed in a separate sealed bag (zip lock bag). Pre-frozen ice packs should be packed around the secondary container.

b) **A rigid outer packaging/box [Tertiary Container]:** The secondary container containing the primary container must be put into a shipping container with adequate cushioning material. At least one surface of the outer packaging must have a minimum dimension of 100 mm x 100 mm. The outer packaging must be marked with **UN 3373 and the 'Biological substances, Category B'** label adjacent to the diamond-shaped mark.

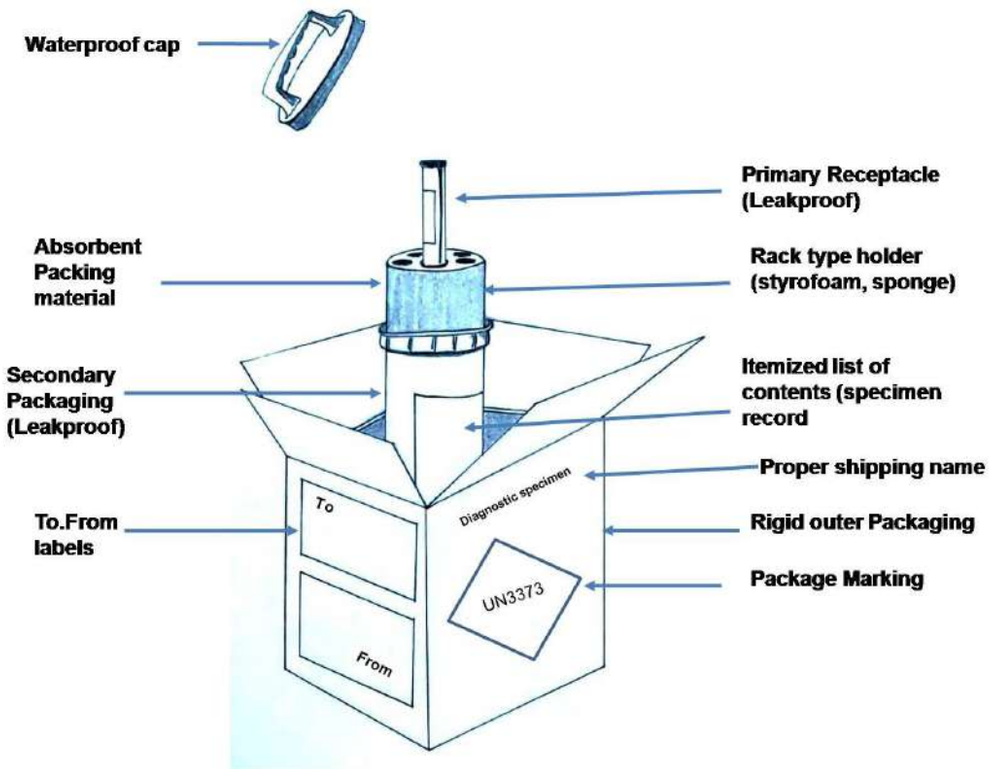


Figure 7.1: Triple packaging recommended by IATA

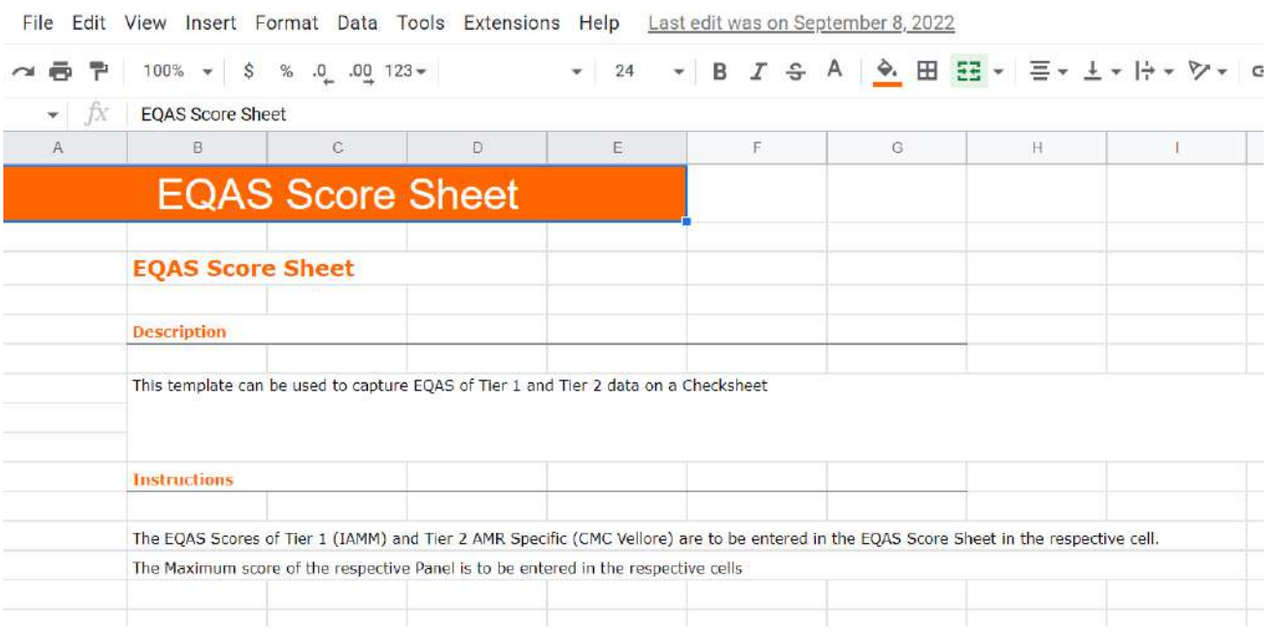
Figure 7.2: Zip-lock bags: Alternative for the secondary container used commonly in resource-limited settings

Figure 7.3: The UN labels for the Tertiary container for specimen/isolate transport/referral

Table: 7.2 Checklist for sending EQAS or ALERT strains to NCDC

Sl No	Things to do before shipping isolates and alert strains to NCDC	Please tick
1.	Observe the growth of culture after overnight incubation inside the vial	☐Yes ☐No
2.	Vials sealed tightly	☐Yes ☐No
3.	Vial lid edges covered with Parafilm	☐Yes ☐No
4.	Labelled neatly and correctly as per the enclosed list	☐Yes ☐No
5.	Hardcopy of list of isolates for EQAS with the AST results in zone diameters/ MIC enclosed as per the format provided by NCDC	☐Yes ☐No
6.	Isolate vial labels & isolate list enclosed in the pack is cross-checked	☐Yes ☐No
7.	Triple-layer packaging done as recommended by IATA	☐Yes ☐No
8.	AMR alert reporting form is filled and duly signed by the AMR nodal officer	☐Yes ☐No
9.	Package labeled with legibly written "From and To address."	☐Yes ☐No
10.	Scan copy of the list of isolates and their AST details was sent to amrsurveillance@gmail.com	☐Yes ☐No

EQAS score sheets and instructions



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EQAS Score Sheet

EQAS Score Sheet

EQAS Score Sheet

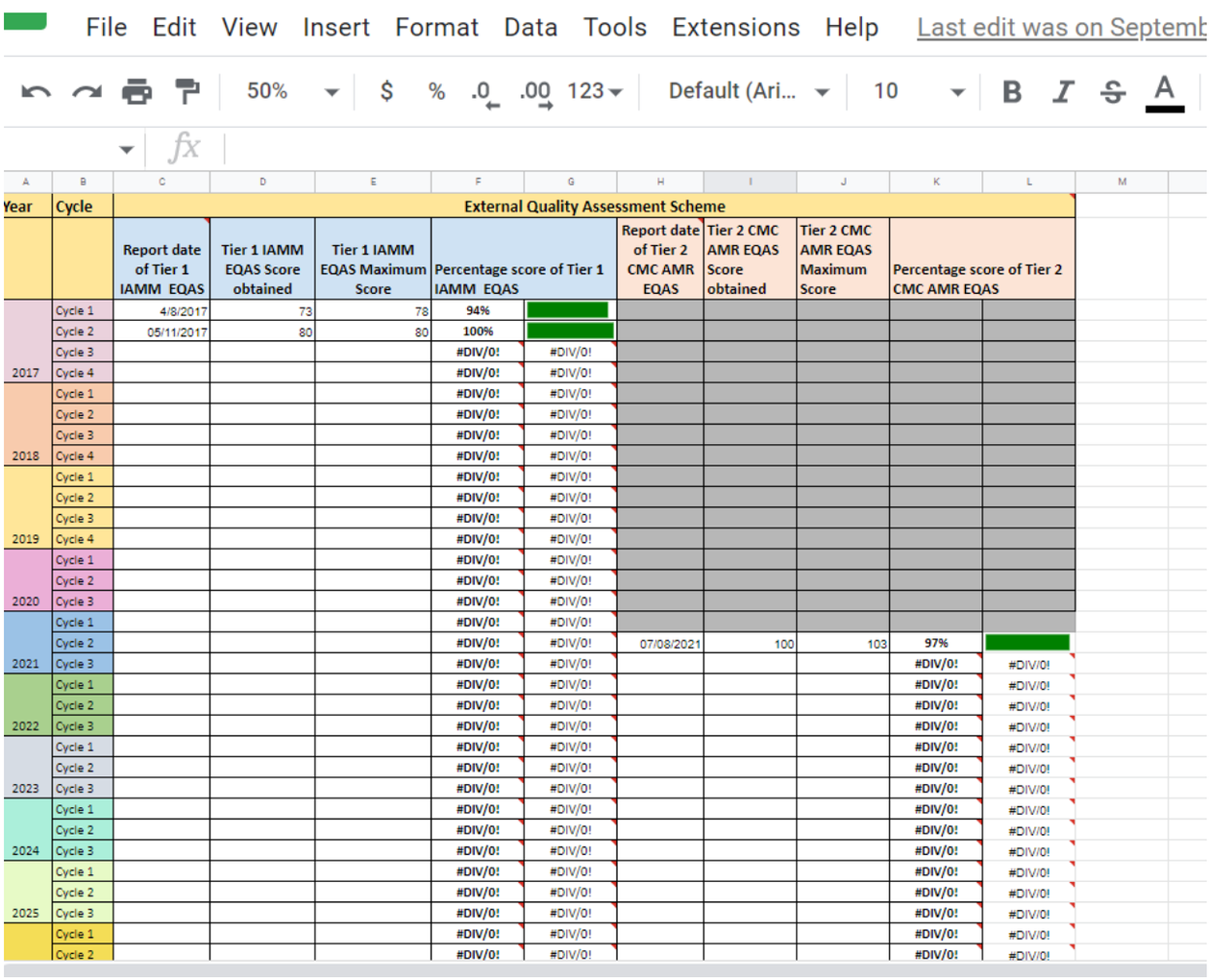
Description

This template can be used to capture EQAS of Tier 1 and Tier 2 data on a Checksheet

Instructions

The EQAS Scores of Tier 1 (IAMM) and Tier 2 AMR Specific (CMC Vellore) are to be entered in the EQAS Score Sheet in the respective cell.

The Maximum score of the respective Panel is to be entered in the respective cells



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Year	Cycle	External Quality Assessment Scheme										
		Report date of Tier 1 IAMM EQAS	Tier 1 IAMM EQAS Score obtained	Tier 1 IAMM EQAS Maximum Score	Percentage score of Tier 1 IAMM EQAS		Report date of Tier 2 CMC AMR EQAS	Tier 2 CMC AMR EQAS Score obtained	Tier 2 CMC AMR EQAS Maximum Score	Percentage score of Tier 2 CMC AMR EQAS	
	Cycle 1	4/8/2017	73	78	94%						
	Cycle 2	05/11/2017	80	80	100%						
	Cycle 3				#DIV/0!	#DIV/0!					
2017	Cycle 4				#DIV/0!	#DIV/0!					
	Cycle 1				#DIV/0!	#DIV/0!					
	Cycle 2				#DIV/0!	#DIV/0!					
	Cycle 3				#DIV/0!	#DIV/0!					
2018	Cycle 4				#DIV/0!	#DIV/0!					
	Cycle 1				#DIV/0!	#DIV/0!					
	Cycle 2				#DIV/0!	#DIV/0!					
	Cycle 3				#DIV/0!	#DIV/0!					
2019	Cycle 4				#DIV/0!	#DIV/0!					
	Cycle 1				#DIV/0!	#DIV/0!					
	Cycle 2				#DIV/0!	#DIV/0!					
2020	Cycle 3				#DIV/0!	#DIV/0!					
	Cycle 1				#DIV/0!	#DIV/0!					
	Cycle 2				#DIV/0!	#DIV/0!	07/08/2021	100	103	97%	
2021	Cycle 3				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 1				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 2				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
2022	Cycle 3				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 1				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 2				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
2023	Cycle 3				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 1				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 2				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
2024	Cycle 3				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 1				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 2				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
2025	Cycle 3				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 1				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!
	Cycle 2				#DIV/0!	#DIV/0!				#DIV/0!	#DIV/0!

Antibiotics to be tested for priority bacterial pathogens before shipping the isolates to NCDC

Organism	Specimen	Antibiotics																																				
		Amikacin 30 µg	Ampicillin 10 µg	Ampicillin-Sulbactam 10 ug/ 10 ug	Amox-Clav 20/10 µg	Azithromycin 15 µg	Aztreonam 30 ug	Benzylpenicillin (MIC)	Cefepime 30 µg	Cefixime 30 ug	Cefotaxime 30 µg	Cefoxitin 30 µg	Ceftazidime 30 µg	Ceftriaxone 30 µg	Cefuroxime 30 µg	Chloramphenicol 30 µg	Ciprofloxacin 5 µg	Clindamycin 2 µg	Doxycycline 30 µg	Erythromycin 15µg	Ertapenem 10 µg	Fosfomycin 200 µg	Gentamicin 10 µg	Gentamicin High 120 µg	Imipenem 10 µg	Linezolid 30 µg	Meropenem 10 µg	Minocycline 30 µg	Netilmicin 30 µg	Nitrofurantoin 300 µg	Pefloxacin 5 µg	Piperacillin/Tazobactam 100/10 µg	Tetracycline 30µg (MIC)	Teicoplanin (MIC)	TMP/SMX*	Vancomycin 30µg	Vancomycin screen Agar	Colistin MIC	Vancomycin MIC	Comments
Enterococcus species	B P O**		✓					✓											✓	✓				✓		✓							✓	✓		✓				
Enterococcus species	U		✓					✓									✓					✓		✓		✓				✓			✓	✓		✓				
Staphylococcus aureus	B, P,O											✓					✓	✓	✓	✓			✓			✓							✓		✓		✓		✓	
Escherichia coli	B, P, O	✓	✓		✓				✓		✓			✓	✓		✓				✓		✓		✓		✓								✓			✓		
Escherichia coli	U	✓	✓		✓				✓		✓			✓	✓		✓				✓	✓	✓		✓		✓			✓					✓			✓		
Klebsiella species	B, U, P, O	✓			✓				✓		✓			✓	✓		✓				✓		✓		✓		✓					✓			✓			✓		
Acinetobacter baumannii/ Acinetobacter calcoaceticus complex	B, U, P, O	✓		✓									✓				✓						✓		✓		✓	✓				✓	✓		✓			✓		
Pseudomonas aeruginosa	B, U, P, O	✓					✓						✓				✓						✓		✓		✓		✓			✓						✓		
Salmonella enterica serotype Typhi	B		✓			✓	i			✓				✓		✓	✓								✓						✓				✓					
Salmonella enterica serotype Typhi	S		✓							✓				✓		✓	✓								✓						✓				✓					
Salmonella enterica serotype Paratyphi	B		✓							✓				✓		✓	✓								✓						✓				✓					
Shigella species	S		✓			✓								✓		✓	✓																		✓					
Vibrio cholerae	S		✓			✓										✓			✓														✓		✓					

***TMP/SMX- Trimethoprim Sulfamethoxazole 1.25/23.75 µg; **B – Blood, U- Urine, O – Other sterile body fluids, S- Stool.** All tick marked (✓) antibiotics to be mandatorily tested before shipping to NCDC

Alert form for reporting pathogens with Emerging Antibiotic Resistance (EAR)

A. Laboratory Information	
1. Name of the reporting laboratory:	2.Name and Contact Information of Microbiologist:
B. Patient Demographic Information	
3.Patient ID:	4. Specimen ID:
5. Completed Age (in years/months/weeks/days):	6. Sex (Tick one box): Male ☐ Female ☐ Other ☐
7.District:	8.Village (rural) / Locality (urban):
C. Admission Information	
9. Date of Hospital Admission D D M M Y Y Y Y	10. Location of patient at the time of sample collection (*Tick one box*) ICU☐ IPD ☐ OPD☐ Other ☐
D. Specimen Type and Pathogen Isolated	
11. Specimen Collection Date Click here to enter a D D M M Y Y Y Y	
12. Type of Specimen *(Tick one box)* a. Blood ☐ b. Urine ☐ c. Stool☐ d. Pleural Fluid ☐ e. CSF☐ f. Pus Aspirate☐(specify location:_____) g. Other Sterile Body Fluid ☐ (specify:_____)	13. Isolated Pathogen *(Tick one box)* 1.*Staphylococcus aureus* ☐ 2.*Escherichia coli* ☐ 3.*Klebsiella* species ☐*specify species if known:* 4.*Acinetobacter baumannii/Acinetobacter calcoaceticus* complex ☐*specify species if known* 5.*Pseudomonas aeruginosa* ☐*specify species if know* 6.*Enterococcus* species ☐ *specify species if known:* 7.*Salmonella* Typhi/Paratyphi ☐*specify serotype if known*

E. Detected/ Suspected Resistance Pattern	**B. Method of Detection*(Tick all that apply)***	**C. AST details**
1.Suspected VISA *(Vancomycin Intermediate S. aureus)*	☐Growth on Vancomycin Screen Agar ☐MIC 4-8 µg/ml by automated AST ☐MIC 4-8 µg/ml by broth microdilution	
2. Suspected VRSA *(Vancomycin Resistant S. aureus*	☐Growth on Vancomycin Screen Agar ☐MIC ≥ 16 µg/ml by automated AST ☐MIC ≥ 16 µg/ml by broth microdilution	
4. Suspected Colistin resistance *(Enterobacteriaceae & Non fermenters)*	☐MIC ≥ 4 µg/ml by BMD for Enterobacteriaceae and *Acinetobacter baumannii/ Acinetobacter calcoaceticus* complex ☐MIC ≥ 8 µg/ml by BMD for *P. aeruginosa*	
5. Suspected Linezolid resistance *(in Enterococci and S. aureus)*	☐ Zone diameter ≤ 20 mm by disc diffusion ☐MIC ≥ 8 µg/ml by automated AST ☐MIC ≥ 8 µg/ml by broth microdilution	

6. Suspected Ceftriaxone resistance in *Salmonella enterica* sero. Typhi	☐ Zone diameter ≤ 19 mm by disc diffusion ☐MIC ≥ 4µg/ml by automated AST ☐MIC ≥ 4µg/ml by broth microdilution	
7. Suspected ceftriaxone intermediate sensitive in *Salmonella enterica* sero. Typhi/ Paratyphi	☐ Zone diameter 20-22 mm disc diffusion	
8. Suspected Azithromycin resistance in *Salmonella enterica* sero. Typhi/Paratyphi	☐ Zone diameter ≤ 12 mm by disc diffusion ☐ MIC ≥ 32 µg/ml by automated AST ☐ MIC ≥ 32 µg/ml by broth microdilution	
9. Suspected Imipenem or Meropenem resistant *Salmonella enterica* sero. Typhi / Paratyphi	☐ Zone diameter <19 mm by disc diffusion ☐ MIC ≥ 4µg/ml by automated AST ☐ MIC ≥ 4µg/ml by broth microdilution	
10. Other significant resistance Pathogen: (If other than listed in E. 8) Drug 1:_____ Drug 2._____ Drug 3: _____ Drug 4: _____	Drug 1: ☐ Zone diameter ____ by disc diffusion ☐MIC _____ µg/ml by automated AST ☐MIC _____ µg/ml by broth microdilution Drug 2: ☐ Zone diameter ____ by disc diffusion ☐MIC _____ µg/ml by automated AST ☐MIC _____ µg/ml by broth microdilution Drug 3: ☐ Zone diameter ____ by disc diffusion ☐MIC _____ µg/ml by automated AST ☐MIC _____ µg/ml by broth microdilution Drug 4: ☐ Zone diameter ____ by disc diffusion ☐MIC _____ µg/ml by automated AST ☐MIC _____ µg/ml by broth microdilution	

H. Clinical Notes

Date of Reporting:

Reported by:

(Name, signature & seal)

Chapter 8 - Standard Operating Procedure for Preservation of Bacterial Isolates/ Control Strains

National Programme on Antimicrobial Resistance Containment
National Centre for Disease Control, India

Objectives and Scope

This SOP aims to describe the method to preserve the bacterial pathogens of significance.

Preparation of nutrient agar stabs (long-term storage 1 year)\

Preparation of 15% glycerol stocks for long-term storage

For long-term storage of bacterial isolates, we need 15% glycerol stocks. For the preparation of 15% glycerol stocks of isolates to be preserved, first prepare 30% glycerol using the following procedure:

Preparation of 30% Glycerol to prepare 15% glycerol stocks (100ml)

- Mix 30 ml of sterilized glycerol and 70 ml of sterilized saline to make 30% glycerol
- Aliquot 0.5ml of 30% glycerol in autoclavable 2 ml cryotubes and slightly loosened the screw caps.
- Sterilize the aliquoted cryotubes by autoclaving.
- Once cool, tighten the screw caps and store the cryotubes in the refrigerator.

Preparation of 15% glycerol stocks of control strains/bacterial isolates

- Ideally, for each isolate, prepare a minimum of 2 stocks, one to be stored at -20°C and the other at -80°C (if available).
- From a pure overnight growth of the isolate on an appropriate non-selective medium, inoculate 4-5 colonies in 5ml of suitable broth medium like TSB and incubate at 37°C.(Figure 8.1)
- After 4-5 hrs of incubation, when the growth is in the mid-log phase, take 0.5ml of broth containing inoculum and inoculate cryotubes containing 0.5ml of 30% glycerol (prepared as above)
- Vortex the tubes to make the suspension homogenous
- Label properly with isolate Id, name of the pathogen, date of stock preparation, etc.
- Store the cryotubes in a deep freezer.

Retrieval of Stocked strains

- Remove a portion of the frozen bacterial suspension with a loop and inoculate appropriate liquid broth or solid media. Return the cryotube immediately to the freezer.
- Alternatively, remove cryotubes and thaw contents rapidly in warm water. Once thawed, do not refreeze the tube. Use contents to subculture non-selective agar, for example, tryptic soya or nutrient agar to isolate pure bacterial colonies.

Preparation of nutrient agar stabs (short-term storage < 1 year)

Stab cultures at room temperature (used for non-fastidious organisms like (*Staphylococcus* and Enterobacteriaceae)

Media Preparation

- Prepare carbohydrate-free agar media like nutrient agar or tryptic soya agar as per the manufacturer's instructions and autoclave.
- After autoclaving, pour 1 ml of media in autoclaved/sterile screw capped 2 ml tubes and let them set as agar deeps (higher volumes may be poured depending on the capacity of storage tubes, tubes may be filled till 2/3rd of capacity).
- Date the batch of tubes and store in a cool place.

Preparation of Agar Media Stabs

- Label each tube with isolate inventory ID, lab or Sample ID/Lab code/month/Year, and date of inoculation. E.g. sample ID is18004325; the Lab code is GMC; the organism was submitted in October 2022 (October/2022); then the label on the strain should be 18004325/GMC/OCT/201.
- Using a sterile straight wire, stab inoculate the media at several places with overnight organism growth on plate media. (Figure 8.1)
- Incubate overnight at 37°C with slightly loose screw caps.
- After incubation, check the surface of the agar stab for growth, tighten screw caps and seal with Para film or molten paraffin wax.
- Store at room temperature or 4°C for up to 6 months.

Revival of Stock Strains

- Remove a portion of the visible growth using a sterile inoculating loop or straight wire and inoculate into liquid culture medium. Incubate at the optimum growth temperature and streak onto fresh culture plates

For transport of bacterial isolates for EQAS or AMR alerts to National Reference Laboratory at NCDC:

- The agar stabs must be prepared as above
- For transport refer to the document on Guidance for submission of EQAS and Alert Strains
- An inventory register may be maintained with details of isolates stored, site where stored etc.
- Sample format for inventory register is at Page 106.

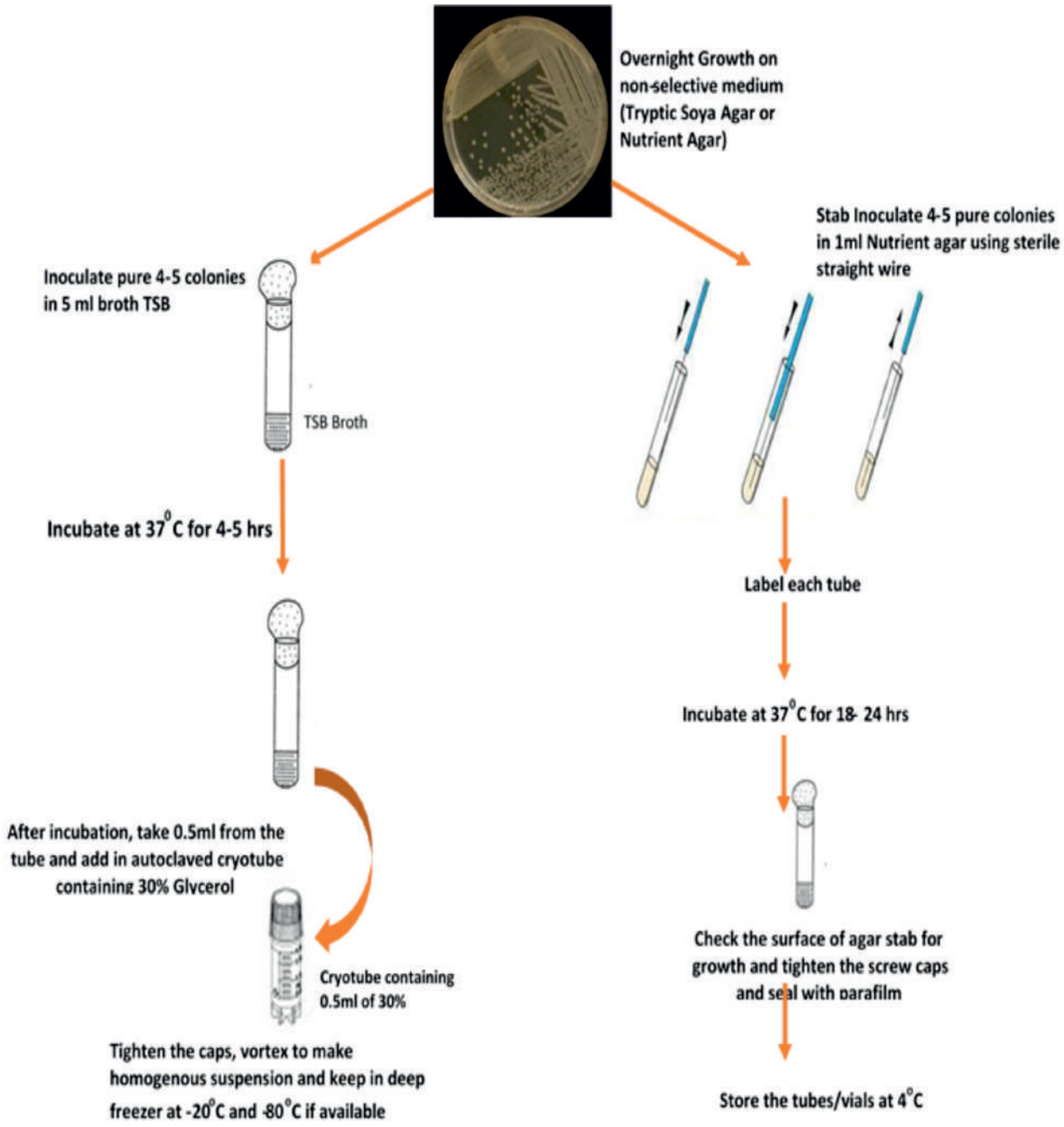
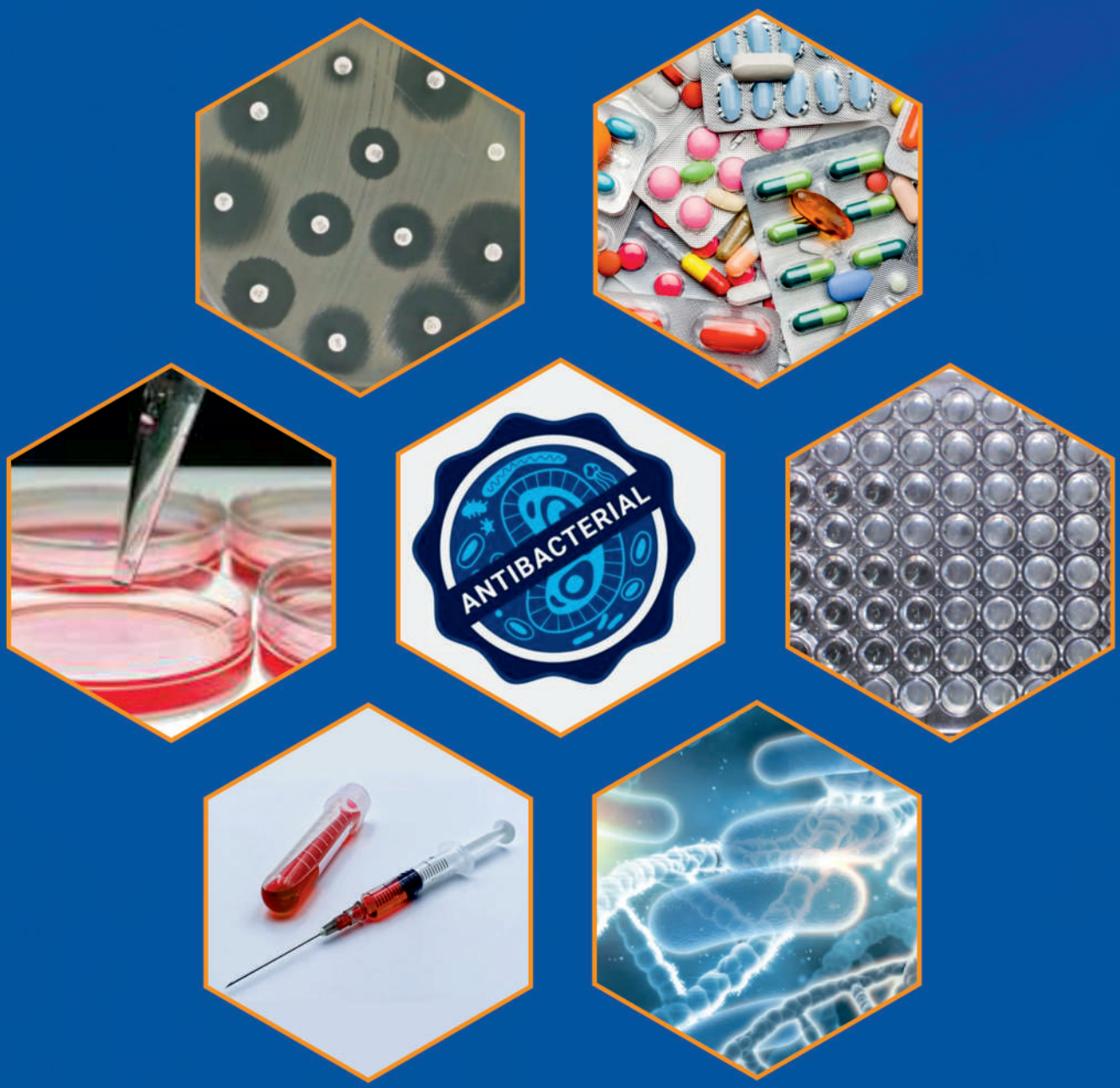


Figure 8.1: Preparation of 15% TSB Glycerol and Nutrient Agar Stabs to preserve isolates.

Format for the inventory of preserved isolates

Strain inventory Id	Date Stock Created	Name of the strain	Isolate Details (like serotype	Specimen Type	Location			Date Discarded
					Facility (Room and/or Floor	Storage Device (Freezer)	Storage Details (box, shelf)	



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